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Crystal Structure Analysis of Cytochrome C₅₅₃

from

***Desulfovibrio vulgaris* Miyazaki F**

An Application of the Multi-wavelength

Anomalous Dispersion Method

1989

Atsushi Nakagawa



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--- An Application of the Multi-wavelength

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by

Atsushi Nakagawa

A Doctoral Thesis submitted to

Faculty of Science

Osaka University

1989

ABSTRACTS

Multi-wavelength anomalous dispersion phasing technique has been applied to the determination of three-dimensional structure of an electron transfer protein cytochrome c₅₅₃ isolated from sulfate-reducing bacterium, *Desulfovibrio vulgaris* Miyazaki F. This novel method makes use of crystallographic phases determined from measurements made at several wavelengths. In contrast with all of the conventional methods of solving overall structures of biological macromolecular crystals, which require either multiple (or single) isomorphous derivatives or atomic coordinates of a similar structure for molecular replacement, this method does not need any other informations than diffraction intensities from native biological macromolecular crystals and allows direct solution of classical "phase problem". This result has proved successful through the use of intense and polychromatic synchrotron radiation at Photon Factory, National Laboratory for High Energy Physics, with accurate and high-speed data collection from the combination of the Weissenberg camera for macromolecular crystallography and the imaging plate, which is a flexible plate coated with photostimulable phosphor crystal and its sensitivity is several tens times higher than the conventional high sensitivity X-ray film. And local scaling procedure has succeeded to reduce systematic errors of data.

The model building of the structure has been carried out by the interpretation of the electron density map which was calculated by multiple anomalous dispersion technique using four native data sets taken at different wavelength below 2.2Å resolution. The structure has been refined by Hendrickson-Konnert's restrained parameter least-squares refinement proce-

ture to an R value of 0.226 at 6.0-1.6Å resolution including 63 water molecules.

The structure of cytochrome c553 has four α -helices, and about a half of all amino acid residues have α -helical conformations. The c-type heme group is attached covalently to the amino acid chain by thioether linkage from Cys10 and Cys13 to the heme vinyl groups at positions 2 and 4, respectively. Coordination bonds are also formed from the epsilon nitrogen of His14 and the sulfur of Met57 to the fifth and sixth coordination sites of the heme iron. The relative locations of two helices at both N-terminus and C-terminus and style of bonding and coordination to the heme group is similar to that of other cytochromes c. But other parts of the structure are quite different from that of other cytochromes c, and its function and physical properties, for example redox potential and so on, are also quite different from those of the others. In this sense, the folding pattern of cytochrome c553 shows "cytochrome c folding" like other cytochromes c superfamily but cytochrome c553 is not classified into the same class of the other small type cytochromes c, for example cytochrome c551 from *Pseudomonas aeruginosa* and so on. Its three-dimensional structure provides a novel insight into the physical and chemical properties and evolution of cytochromes c superfamily.

CONTENTS

ABSTRACT	...	iii
CONTENTS	...	v
ACKNOWLEDGEMENT	...	viii
ABBREVIATIONS	...	x
1. INTRODUCTION		
1-1. Protein Crystallography	...	1
1-2. Utilization of Synchrotron radiation for Protein Crystallography	...	3
1-3. Structure Study of Cytochrome c553	...	8
2. PRELIMINARY STAGE OF STRUCTURE ANALYSIS		
2-1. Crystallization		
2-1-1. Purification and Crystallization	...	16
2-1-2. Crystallographic Study of Cytochrome c553	...	18
2-2. Single Isomorphous Replacement Method		
2-2-1. Theoretical Background	...	21
2-2-2. Heavy Atom Reagents Survey	...	31
2-2-3. Selection of Space Group Enantiomorph	...	33
2-2-4. Native Anomalous Scattering Effect for Phase Calculation	...	33
3. MULTI-WAVELENGTH PHASE DETERMINATION STAGE OF STRUCTURE ANALYSIS		
3-1. Data Collection		
3-1-1. Apparatus and Equipment		
3-1-1a. X-ray Source	...	37
3-1-1b. Beamline Optics at BL-6A2	...	38

3-1-1c. The Weissenberg Camera for Macromolecular Crystallography	...	40
3-1-1d. Imaging Plate	...	43
3-1-2. Experiments		
3-1-2a. Choice of Wavelengths	...	44
3-1-2b. Condition of Data Collection	...	48
3-2. Data Handling		
3-2-1. Scale - Merge		
3-2-1a. Data Reduction -- Indexing --	...	50
3-2-1b. Absorption Correction and Local Scaling	...	54
3-2-2. Native Anomalous Difference Patterson Maps	...	64
3-2-3. Absolute Scale	...	64
3-3. Phase Determination by Multi-wavelength Anomalous Dispersion Method		
3-3-1. Theoretical Background	...	72
3-3-2. Refinement of Atomic Parameters	...	81
3-3-3. Phasing	...	82
3-4. Model Building	...	82
4. REFINEMENT STAGE OF STRUCTURE ANALYSIS		
4-1. Refinement		
4-1-1. Theoretical Background	...	89
4-1-2. Refinement survey	...	92
5. RESULT AND DISCUSSION		
5-1. Refined structure		
5-1-1. Molecular packing	...	97
5-1-2. Secondary structure, Main chain folding and Heme packing	...	101

5-1-3. Heme environment	... 114
5-1-4. Hydrogen Bonds	... 117
5-1-5. Water Structure	... 117
5-2. Comparison of the Structure of Cytochrome <i>c</i> families	
5-2-1. Folding Pattern and Charge Distribution	... 121
5-2-2. Interactions of Cytochrome <i>c</i> 553	... 132
5-2-3. Structure and Redox Potential	... 135
5-2-4. Alignment of Amino Acid Sequence	... 140
5-2-5. Evolution of Cytochrome <i>c</i> Superfamily	... 146
5-3. Usage of Multi-wavelength Anomalous Phase determination for Protein Crystallography	... 148
CONCLUDING REMARKS	... 152
REFERENCES	... 154
APPENDIX	
A. Imaging Plate System at Photon Factory and its software system	... 158
B. Atomic Parameters of Cytochrome <i>c</i> 553	... 167
LIST OF PUBLICATIONS	... 181

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ABBREVIATIONS

Ac	acetate
A.S.	ammonium sulfate
CNDP	4-carboxyl-2,6-dinitrophenol
DvH	<i>Desulfovibrio vulgaris</i> Hildenborough
DvM	<i>Desulfovibrio vulgaris</i> Miyazaki
EXAFS	extended X-ray absorption fine structure
IP	imaging plate
MAD	multi-wavelength anomalous dispersion
MIR	multiple isomorphous replacement
Mersalyl	[3-[[2-(carboxymethoxy)benzoyl]amino]-2-methoxypropyl]-hydroxymercury monosodium salt
PCMB	<i>p</i> -chloromercuribenzoic acid
PHMB	<i>p</i> -hydroxymercuribenzoic acid
PHMBS	<i>p</i> -hydroxymercuribenzenesulfonic acid
PMA	phenylmercuric acetate
PTTC	platinum(II)(2,2',6',2''-terpyridine)chloride dihydrate
SIR	single isomorphous replacement
SIRA	single isomorphous replacement with anomalous dispersion effect
SR	synchrotron radiation
TFA	trifluoroacetate
TFC	trifluoromethylphenyl carbamate
oxi.	oxidized form (ferri form)
pI	isoelectric point
red.	reduced form (ferro form)

r.m.s. root-mean-square

Ala	A	alanine	Arg	R	arginine
Asn	N	asparagine	Asp	D	aspartic acid
Cys	C	cystein	Gln	Q	glutamine
Glu	E	glutamic acid	Gly	G	glycine
His	H	histidine	Ile	I	isoleucine
Leu	L	leucine	Lys	K	lysine
Met	M	methionine	Phe	F	phenylalanine
Pro	P	proline	Ser	S	serine
Thr	T	threonine	Trp	W	tryptophan
Tyr	Y	tyrosine	Val	V	valine

1 Å = 0.1 nm

1. INTRODUCTION

1-1. Protein Crystallography

Protein crystallography is the application of X-ray diffraction technique to crystals of proteins and it can determine three-dimensional structures of proteins, one of the most important kind of molecules in living system. It is advantageous to know how the atoms of major molecules of life - not only proteins but also nucleic acids - are disposed in space if the way they perform their vital function is to be understood in detail. Knowledge of three-dimensional structure will usually also be useful elucidating its function at the atomic level and to make the intelligent design of proteins with modification. At present, such structural information is most effectively obtained by analysis of diffraction from crystals of macromolecules. Even though the time-averaged structure might be determined by monochromatized X-ray diffraction technique and the environment around a proteins molecule may be a little different from that in solution, the structure information determined by X-ray diffraction method is very useful to study its function and mechanism. Protein crystals are highly hydrated - usually about half of the volume is due to solvent of crystallization - and for this reason molecular conformation in crystals are, for the most part, closely related to the situation in solution. And large part of the lattice interactions are mediated by water molecules. For this reason also, the environment of a protein molecule in crystal is quite similar to that in the solution in a living system.

Recent advances of in gene synthesis and genetic engineering have made it possible, in principle, to construct any desired amino acid sequences. To make more useful, more stable and more available proteins is one of the most important theme of protein engineering. So it is necessary to know relationship between structure and function of proteins in the native form as the first step. Although there are too many proteins to count in the nature, only less than 300 of coordinate sets from over 150 distinctive macromolecules have been deposited in the Protein Data Bank (Bernstein *et al.*, 1977).

Three-dimensional structures of proteins are restricted not only by its amino acid sequence but also by solvent-protein and protein-protein interactions. And it is very hard to predict its three dimensional structure only from the information about its amino acid sequence of the protein of which three-dimensional structure has been known. To determine three dimensional structures of proteins and to know relationship between structure and function of proteins are very important not only for protein engineering but also for molecular biology.

Recently because of the progress of mathematical procedure, computers and diffraction intensity measurement technique, the structure determination of proteins by X-ray diffraction technique became a very useful and powerful technique. If the crystal is nearly isomorphous with a known crystal structure of a similar protein, the difference Fourier method can be used with phases calculated from the known structure to show up the changes in structure. If the crystal is a novel one but is composed of molecules which are similar to those of known structure, the molecular replacement method following rotation and translation search can often be used to build a model of

properly positioned components from which phases can be computed by the structure factor equation. If the molecule is a novel one, the crystallographic phase problem must be solved *ab initio*. The method of isomorphous replacement has played a central role in the diffraction analysis of nearly all truly new protein structures. But many protein crystals do not give any good isomorphous derivatives to determine crystallographic phases by which unambiguous electron density map to elucidate three-dimensional structure can be calculated.

The most serious limitation for X-ray crystal structure analysis is the uncertainty of the crystallization of molecules. And the other serious problem for protein crystallography is to determine crystallographic phases of diffraction data, even if a large and well ordered native protein crystal is obtained.

1-2. Utilization of Synchrotron Radiation for Protein Crystallography

In 1912, M. Laue *et al.* discovered the X-ray diffraction phenomenon by crystals (Friedrich *et al.*, 1912). X-ray beam is scattered according to the three dimensional periodicity in a crystal. This phenomenon means that diffraction pattern of a crystal conveys information about an internal structure of a crystal. In other words, analysis of X-ray diffraction pattern should give an internal structure of a crystal. At the present time, X-ray crystal structure analysis is one of the most powerful techniques to determine crystal and molecular structure, not only of small molecules but also especially of biological macromolecules, such as proteins, viruses, DNA and

so on.

The most serious limitation for X-ray crystal structure analysis is the difficulty of obtaining good crystals. Success in crystallization of protein crystals depends on experience and fortune. It is not only necessary to get a crystal but also the crystal must be grown to the size of 0.5 mm in all dimensions to get a good high resolution data. Even if we can get a good crystal, there are another problem on the structure analysis.

Suppose that there are some atoms in a crystal; number of atoms (N), coordinate of j -th atom (x_j, y_j, z_j); then the diffraction intensity for index hkl ($I(hkl)$) can be written as

$$I(hkl) = F(hkl) \cdot F^*(hkl) \quad (1-1)$$

$$F(hkl) = \sum_j^N f_j \exp(-B(\sin\theta/\lambda)^2) \cdot \exp(2\pi i(hx_j + ky_j + lz_j)) \quad (1-2)$$

We can observe the only intensities, $|I(hkl)|$, by X-ray diffraction technique, not the structure factors $F(hkl)$. In other words, phase angles of structure factors, $\alpha(hkl)$, are lost on intensity measurement. The most difficult problem to determine structure by the X-ray diffraction method is how we can determine crystallographic phases for the Fourier series calculation. Crystallographers call this problem the "PHASE PROBLEM".

To determine a structure of small molecular crystal, the direct method is very useful and very powerful technique. By this method, phase angles are calculated *ab initio* using only the intensity relationship between reflections. Recently, it has become possible to determine a crystal structure of small molecule fully automatically by the progress of computer technology and mathematical techniques. But to determine a macromolecule crys-

tal structure, it is very difficult to determine phases only by direct method, although many crystallographers have been trying to solve the phase problem and have also been trying to determine the structure using only native crystal data by the direct method. So some empirical methods must always be employed for phase determination in biological macromolecular crystallography. Isomorphous replacement technique is the most useful method to determine crystallographic phases of diffraction intensities from macromolecular crystal. In isomorphous replacement technique, phases are determined from the intensity difference between the native and the derivative(s). This technique is very useful when good isomorphous derivatives can be prepared. Another technique, molecular replacement method, may be the most useful when three-dimensional structure of a similar protein has been solved because it is then not necessary to prepare any isomorphous derivative. Molecular replacement may be very useful for protein engineering.

When the structure of a similar protein is not understood and it is very hard or it is not able to prepare isomorphous derivative, it should be very difficult to determine the structure of macromolecule by X-ray diffraction technique.

Recently a new type of light source called synchrotron radiation (SR) has been widely used for many fields in which photons were used.

When electronic particle, such as electron or positron, is accelerated close to the light speed and changed its direction by a magnetic field, very strong electromagnetic wave is observed toward its tangent direction. This electromagnetic wave is called "Synchrotron Radiation". This synchrotron radiation has some special characters compared with X-rays from a conven-

tional X-ray generator. These characteristics include

1. Very high brightness
2. Polychromatic beam
3. Small divergency
4. Pulse beam
5. Polarized light

The synchrotron radiation beam is also very useful for protein crystallography. With regard to the first advantage, the high power X-ray beam, high resolution data can be obtained and even if the size of crystal is small, for example 0.2 mm in all directions, it is possible to get high resolution data. Although protein crystals are very easy to get radiation damage by X-ray beam, a shorter exposure time helps make radiation damage to a smaller extent, even though the intensity of the beam may be higher. With regard to the second advantage, polychromatic X-rays, the Laue method, which is the technique to get many reflections at the same time using white X-rays, may become the best technique for time-resolved macromolecule crystal structure analysis. Another usage of the second advantage is that we can choose any wavelength of the X-rays. Tunability of wavelength is very useful for anomalous dispersion technique. Even for isomorphous replacement method, anomalous dispersion effect is very useful for crystallographic phase determination. For protein crystals which have heavy atom(s) in its native form, they have an ability to determine crystallographic phases without heavy atom derivatives and to determine three-dimensional structure. By a conventional X-ray generator, characteristic X-ray is strong but white X-ray is very weak. So it is impossible to get any wavelength of X-ray other than characteristic X-ray of target, $\text{CuK}\alpha$ is always used for protein

crystallography (1.54 Å), using a conventional X-ray generator. Because of the synchrotron radiation is a polychromatic beam, we can choose any wavelength of X-ray. For anomalous dispersion experiment, this wavelength tunability is very useful because any wavelength of X-ray can be chosen including that near absorption edge which is used in the anomalous dispersion experiments. The third advantage, small divergency, is that the beam divergency is very close to parallel and when it is monochromatized by a monochromator, the energy dispersion of beam will be small. In the energy region of near absorption edge, real part of the anomalous scattering factor ($\Delta f'$) varies dramatically. In other words, when energy resolution of X-ray beam is not very good, it is difficult to use real term of the anomalous difference efficiently. The fourth advantage, the pulse beam, also provides a possibility for time-resolved measurement, but averaged X-ray beam is always used for static structure determination of biological macromolecule crystals. The fifth advantage, polarization of X-ray beam does not give any information of the static protein structure. But recently, elliptically polarized synchrotron beam for X-ray region has been obtained using undulator constructed in the Accumulation Ring (AR) in KEK, National Laboratory for High Energy Physics, and it will be possible to give some informations of protein structure.

The crystal of cytochrome c553 diffracts data at quit high resolution, that means the quality of cytochrome c553 crystal is good. But it was very difficult to get good isomorphous derivatives and to determine the three-dimensional structure of cytochrome c553.

In this thesis, the three-dimensional structure of cytochrome c553 has been determined by the multi-wavelength anomalous dispersion method using

anomalous dispersion effect of the iron atom of the heme group in the native protein, and this method does not require to prepare any isomorphous derivative. The multi-wavelength anomalous dispersion method provides a possibility to determine the three-dimensional structure of any kind of metallo-proteins which contain heavy atoms, of which atomic number ranges from ^{29}Cu to ^{47}Ag and from ^{50}Sn to ^{92}U , in the native form.

1-3. Structure study of cytochrome c553

The sulfate-reducing bacteria were discovered by Beijerinck in 1895 (Beijerinck, 1895). Sulfate-reducing bacteria are classified as a group of microbes which conduct dissimilatory sulfate reduction. In the metabolism of a sulfate-reducing bacterium, the sulfate ion acts as a terminal electron acceptor, as oxygen acts in conventional respiration, and therefore it has been called "sulfate respiration", analogous to "nitrate respiration" found among nitrate-reducing bacteria. The sulfate-reducing bacteria have been considered to consist of a small group of highly specialized anaerobic bacteria with similar phylogenetical and bioenergetic systems. As oxygen always inhibits their growth, they always live in the anaerobic environment such as a marshy place and so on.

Today at least seven genera of sulfate-reducing bacteria are discovered and confirmed. The two genera of sulfate-reducing bacteria, *Desulfovibrio* and *Desulfotomaculum*, are well established, and these two species seem to be closed in the phylogenetic tree. *Desulfotomaculum* involves spore forming. *Desulfovibrio* contains methophilic and halophilic bacteria and

they do not form spores. *Desulfovibrio* is the best known, because it is easy to isolate and purify. The cytochrome *c*₅₅₃ of which three-dimensional structure is discussed in this thesis comes from *Desulfovibrio vulgaris* Miyazaki F (IAM no. 12604, Kobayashi and Skyring, 1982). This strain was initially isolated from a paddy field in Miyazaki Prefecture, Japan (Ishimoto and Koyama, 1953), and its enzymes have been investigated by various biochemical methods.

These bacteria are Gram-negative and tend to have single polar flagella with an average size of $0.4 \times 10 \mu\text{m}$.

Electron transfer system of *Desulfovibrio vulgaris* is not well known, but Figure (1-1) shows a tentative scheme for it, Aerobes reduce oxygen to water, whereas the sulfate-reducing bacteria reduce sulfate to water and sulfide.

Desulfovibrio have many *c*-type cytochromes although they are anaerobic bacteria. It was the first anaerobic bacterium which involved cytochromes *c* in its metabolic path (Ishimoto *et al.*, 1954, Postgate, 1954). At least three kinds of *c*-type cytochromes, having the heme *c* in a molecule, are found in *Desulfovibrio vulgaris* Miyazaki F. They are cytochrome *c*₅₅₃, cytochrome *c*₃ and high molecular weight cytochrome *c*, and their properties are summarized in Table (1-1).

Cytochrome *c*₃ acts as an electron transfer protein to hydrogenase, and its electrochemical, physicochemical and other properties are well studied from many facets (Ishimoto *et al.*, 1954, Postgate, 1954, Yagi and Maruyama, 1971). The three-dimensional structure of cytochrome *c*₃ from *Desulfovibrio vulgaris* Miyazaki (Higuchi *et al.*, 1981, Higuchi *et al.*, 1981, Higuchi *et al.*, 1984) and *Desulfovibrio denitrofricans* Norway (Haser *et al.*, 1979,

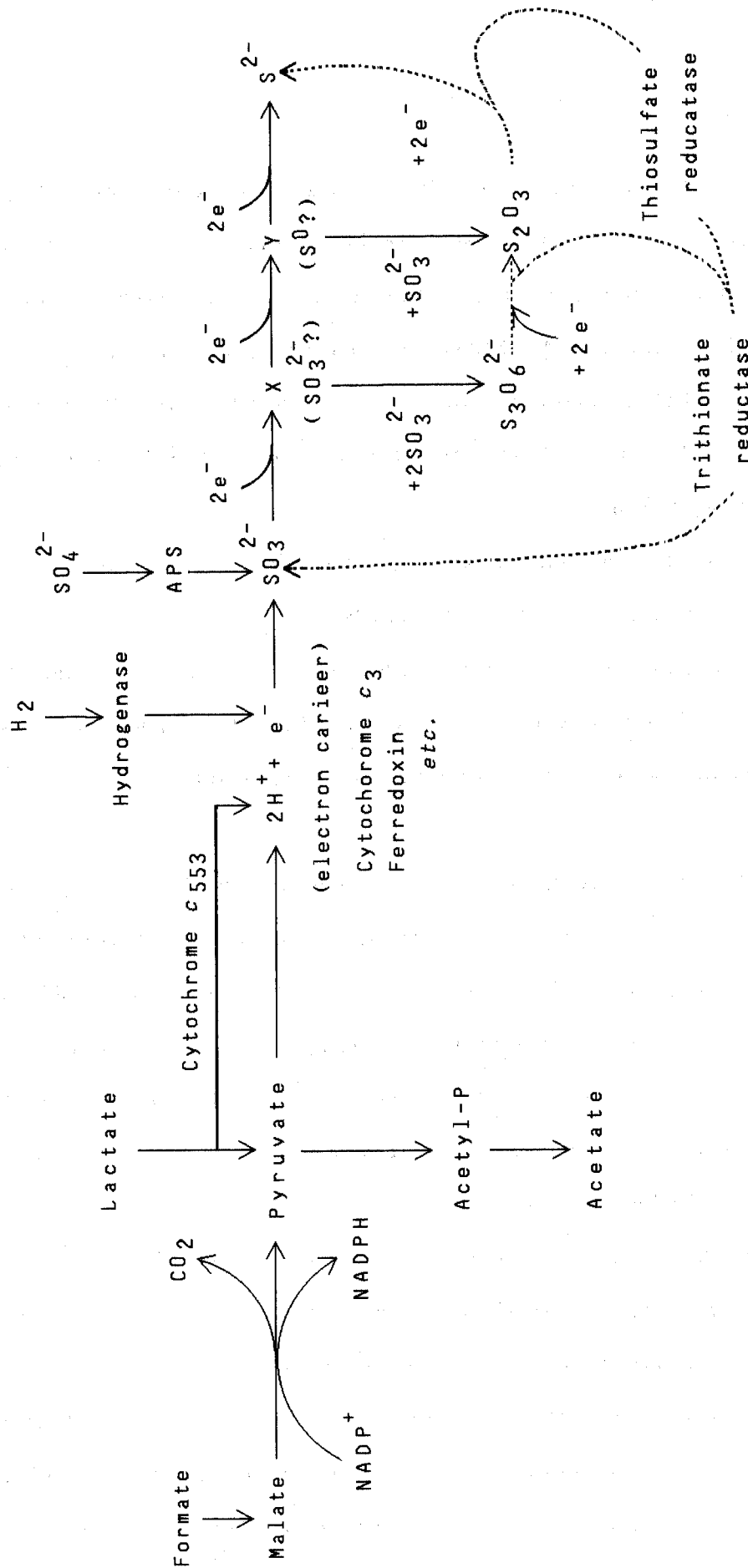


Figure 1-1

The tentative scheme for electron transfer system
of cytochrome c553

	M.W.	No. of residues	Heme contents
Cytochrome c ₅₅₃	9,000	79	1
Cytochrome c ₃	14,000	107	4
High molecular weight cytochrome c	70,000	?	12?

Table 1-1

Cytochromes in *Desulfovibrio vulgaris* Miyazaki

Pierrot *et al.*) has already been determined and the structure of cytochrome *c*₃ from *Desulfovibrio vulgaris* Miyazaki has been refined to 1.8Å resolution (Higuchi *et al.*, 1984).

High molecular weight cytochrome *c*, which was at first separated from extracts of *Desulfovibrio vulgaris* Miyazaki F by Yagi (Yagi, 1969), is composed of two subunits and has a molecular weight of about 70000. Its purification and crystallization has been successful (Higuchi *et al.*, 1987).

Cytochrome *c*₅₅₃ (Le Gall and Bruschi-Heriland, 1968; Bruschi *et al.*; Yagi, 1969; Yagi, 1979; Bruschi and Le Gall, 1972; Peck and Le Gall, 1982) is reported to act as an electron carrier protein for formate dehydrogenase (Yagi, 1979) and lactate dehydrogenase (Ogata *et al.*, 1981) at the starting point of carbon and energy metabolism. It accepts electrons from lactate and transfers them to cytochrome *c*₃ and the other enzymes involved in the inorganic sulfur metabolism. Cytochrome *c*₅₅₃ from *Desulfovibrio vulgaris* Miyazaki has a single polypeptide chain containing 79 amino acids residues and one heme group (Table (1-2)). And it is the smallest cytochrome *c* that has been isolated so far (Nakano *et al.*, 1983). This suggests that cytochrome *c*₅₅₃ is one of the most primitive *c*-type cytochrome of the known members of cytochromes *c* super family.

The amino acid sequence of cytochrome *c*₅₅₃ from *Desulfovibrio vulgaris* Hildenborough strain was determined by Bruschi and Le Gall (Bruschi and Le Gall, 1972). Dickerson proposed the alignment of amino acid sequence of cytochrome *c*₅₅₃ and other *c*-type cytochromes and, on the basis of that, the location of sulfate-reducing bacteria in the phylogenetic tree (Dickerson, 1980). However a comparison of amino acid sequences of cytochrome *c*₅₅₃ from *Desulfovibrio vulgaris* Miyazaki and *Desulfovibrio vul-*

Molecular weight	9000 dalton	
Number of residues	79 amino acids	
Heme content	1	
pI	10.5	
Absorption spectra	(red.) 553nm	(23.9)
(molar absorbance)	(red.) 524nm	(16.2)
	(red.) 417nm	(142.0)
	(red.) 317nm	(32.1)
	(oxi.) 525-526nm	(10.6)
	(oxi.) 410 nm	(109.0)
	(oxi.) 360 nm	(27.8)
	(oxi.) 227-280nm	(193.0)
Redox potential	0 mV	
(pH 7.0)		

Table 1-2

Physicochemical Properties of
cytochrome c553
from *Desulfovibrio vulgaris* Miyazaki

garis Hildenborough does not show any similarity except for several residues at the N- and C-terminal although amino acid composition of both species is quite similar to each other (Table (1-3)). Yagi and his colleague has suggested a new alignment of the primary structures of cytochrome c₅₅₃ from *Desulfovibrio vulgaris* Miyazaki and other small type cytochromes c (Nakano *et al.*, 1983). Comparison of tertiary structures will give a new insight to the alignment of cytochromes c superfamily.

The redox potential of cytochrome c₅₅₃ from *Desulfovibrio vulgaris* Miyazaki was measured by Niki (Niki, *unpublished result*) and it is about 0mV, whereas other cytochromes c have values about 200-400mV. This unusually low redox potential must depend on its structure. It is also necessary to know the three-dimensional structure for understanding of the relationship between structure and redox potential.

	DvMF	DvH
Lys	12	12
His	2	1
Arg	1	1
Asp + Asn	6 + 1	5 + 1
Thr	1	1
Ser	5	6
Glu + Gln	4 + 2	5 + 1
Pro	0	1
Gly	11	12
Ala	11	14
Cys	2	2
Val	5	2
Met	5	6
Ile	0	1
Leu	5	5
Tyr	5	6
Phe	1	0
Trp	0	0

Table 1-3

Amino acid Composition of cytochrome c553
from two strains,
Desulfovibrio vulgaris Miyazaki F (DvMF)
and
Desulfovibrio vulgaris Hildenborough (DvH)

2. PRELIMINARY STAGE OF STRUCTURE ANALYSIS

2-1. Crystallization

2-1-1. Purification and Crystallization

The purification of cytochrome c553 was carried out according to the method reported by Yagi (Yagi, 1979). The procedure for purification is described in Figure (2-1). Just before crystallization, the solution of cytochrome c553 was purified by gel-filtration with Sephacryl S-200 gel. The fractions having the value of the purity index¹ greater than 1.2 were collected and then condensed by ultrafiltration.

As cytochrome c553 is soluble in Tris-HCl buffer saturated with ammonium sulfate at the neutral pH, it could be crystallized by the combined method of the salting out by ammonium sulfate and the vapor diffusion of ammonia to make the pH of the protein solution close to the isoelectric point (10.5) of the protein. A solution of purified cytochrome c553 (10%(W/V)) was used for crystallization. Ammonium sulfate powder was added stepwise to make the solution saturated on cooling with crushed ice. This solution was then centrifuged (15000rpm, 30min.) to remove insoluble ammonium sulfate powder. This purified protein solution was put in the microdialysis cell

1. The purity index is defined as:

Absorbance at 553 nm (red.)/Absorbance at 280 nm (oxi.)

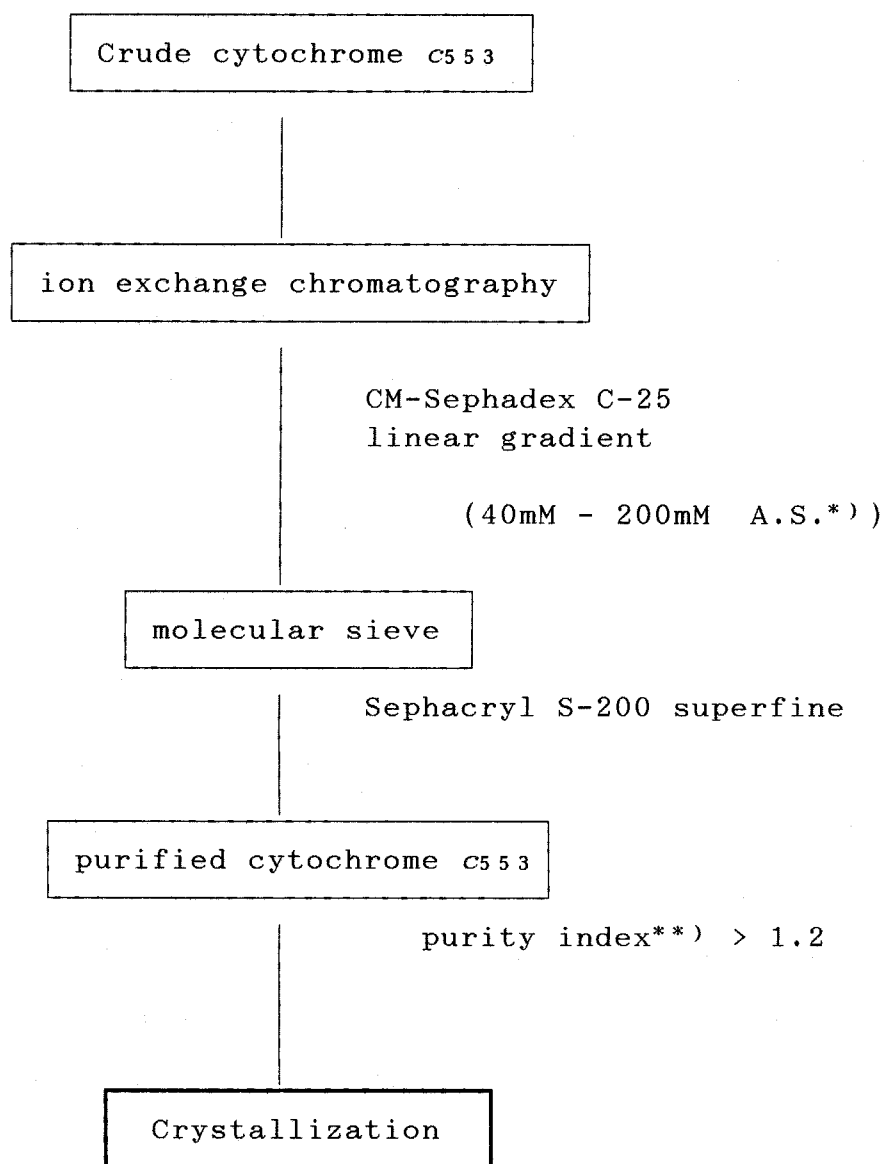


Figure 2-1

Flow chart of the purification
of cytochrome c553

*) Ammonium sulfate

**) Absorbance at 553nm(red.) / Absorbance at 280nm(oxi.)

(crystallization button) and was dialyzed against 50 mM of the ammonium sulfate-saturated Tris-HCl buffer solution (pH 8.0) and placed in an air-tight box with another 50 mM of the ammonium sulfate-saturated Tris-HCl buffer solution (pH 9.0) containing ammonia (Figure (2-2)). After about 10 days at 15 °C, as the pH of the protein solution increased and approached its isoelectric point, some crystals suitable for X-ray analysis were grown. The crystals were red tetragonal bipyramid, and their typical dimensions were approximately 0.8x0.8x1.0mm³ (Figure (2-3)).

2-1-2. Crystallographic Study of Cytochrome c553

The crystal data were determined with Ni-filtered CuK α radiation on a four-circle diffractometer. The X-ray generator was a rotating-anode type RIGAKU RU-200 (40 kV, 200 mA), and the diffractometer was the Rigaku AFC-5 system. The tentative cell parameters were determined by using the vector minimum method from 15 reflections collected by the diffractometer using the peak search system. Then preliminary measurement of intensities of Bijvoet pairs was carried out at 5 Å resolution. From the inspection of the symmetry of diffraction intensities and systematic absences of diffraction, the space group was determined as $P4_32_12$ (or its enantiomorph $P4_12_12$). Unit cell parameters were reduced from Bragg angles of twenty reflections carefully measured on the diffractometer; $a=b=42.7$ Å, $c=103.4$ Å. The correct enantiomorph, $P4_32_12$, was determined by the inspection of two Fourier maps with SIR phases (see Chapter 2-2-3); one of them was calculated for the space group $P4_32_12$ and the other was calculated for the space group $P4_12_12$.

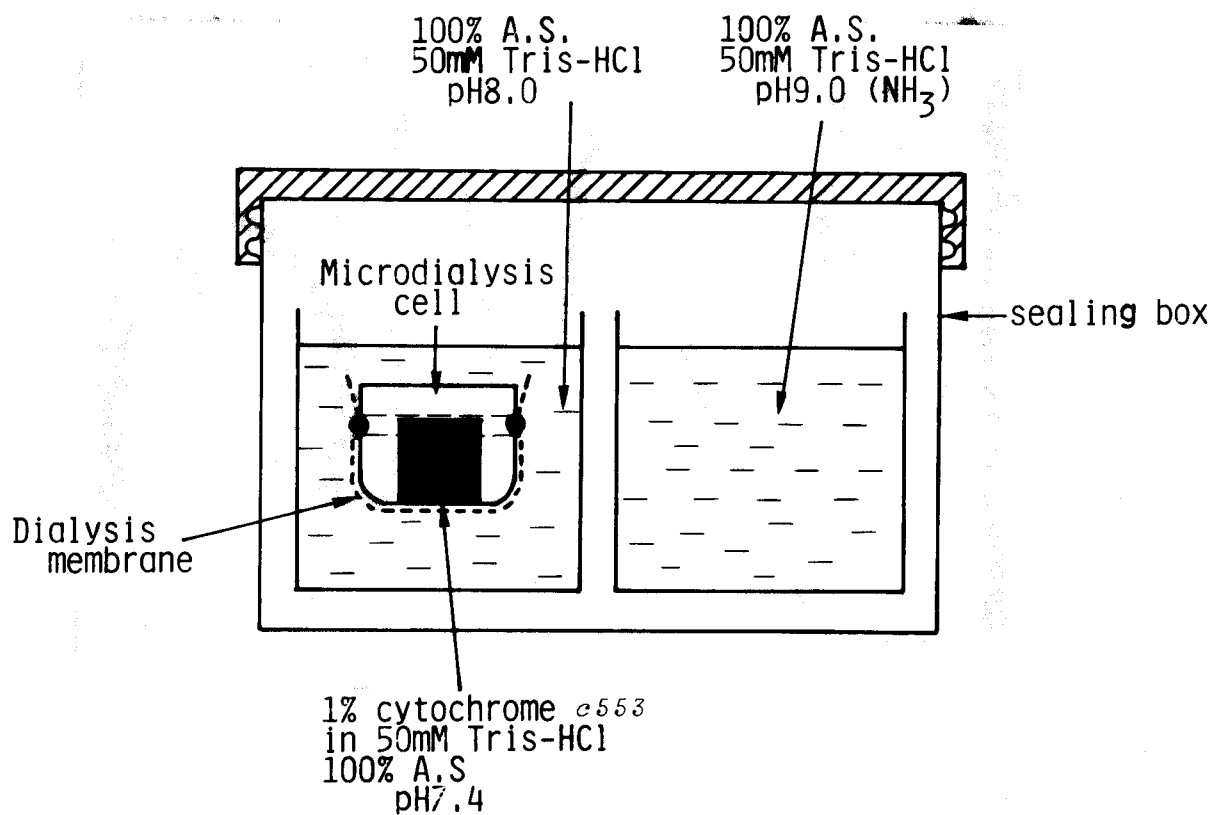
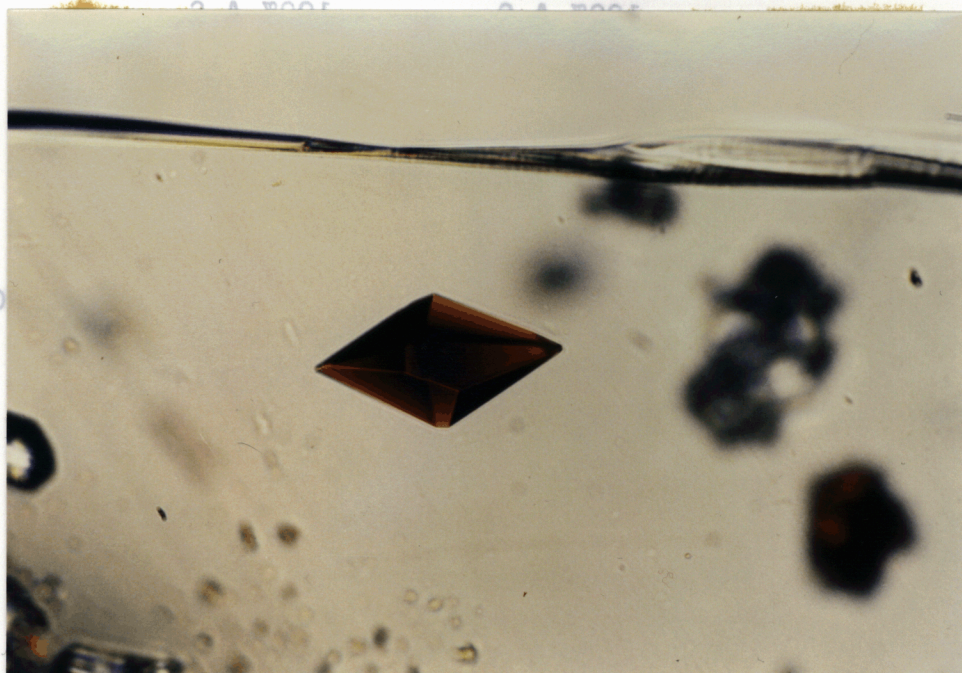


Figure 2-2

Crystallization of cytochrome *c553*
by the combined method of the salting-out and
the vapor diffusion technique



S-Figure 2-3

Crystal of cytochrome c553
Approximate crystal size is 0.4x0.4x0.7 mm³
the vapor diffusion technique

The consideration of anomalous scattering effect supported this selection of enantiomorph. If there is one molecule in an asymmetric unit, a V_M value of 2.61 Å³/dalton is obtained, which is a reasonable value for a commonly found protein crystal (Matthews, 1968). Table (2-1) summarizes the crystallographic data of a cytochrome c553 crystal.

2-2. Single Isomorphous Replacement Method

2-2-1. Theoretical Background

Suppose that there are N atoms in a unit cell of a crystal and the coordinate of j -th atom in a crystal is (x_j, y_j, z_j) in the fractional coordinates system, the structure factor of particular reflection which has a Miller index hkl is described as

$$F(hkl) = \sum_j^N f_j \cdot \exp 2\pi i(hx_j + ky_j + lz_j) \quad (2-1)$$

where f_j is the atomic scattering factor of j -th atom. Alternatively, the structure factor is given using phase, α , as follows,

$$F(hkl) = |F(hkl)| \cdot \exp i\alpha \quad (2-2)$$

An inversed Fourier transformation of equation (2-1) is written as

$$\rho(x, y, z) = \frac{1}{V} \sum_h \sum_k \sum_l F(hkl) \cdot \exp -2\pi i(hx + ky + lz) \quad (2-3)$$

Crystal system	Tetragonal
Space group	P4 ₃ 2 ₁ 2
Cell Constants	$a = b = 42.7 \text{ \AA}$ $c = 103.4 \text{ \AA}$
Volume	$V = 188 \times 10^3 \text{ \AA}^3$
Number of molecule in one unit cell (1 molecule per asymmetric unit)	$Z = 8$
Volume per 1 dalton	$V_M = 2.61$
Volume of solvent	$V_{\text{solv.}} = 0.53$

Table 2-1

Crystallographic data of cytochrome c553 crystal

Equation (2-3) shows that if the structure factors $F(hkl)$ are known, then electron density at point (x,y,z) can be calculated.

By X-ray diffraction method, only an intensity $I(hkl)$ can be observed. The intensity $I(hkl)$ is given by

$$I(hkl) = F(hkl) \cdot F^*(hkl) \quad (2-4)$$

where $F^*(hkl)$ denotes the conjugate complex of a structure factor $F(hkl)$.

Equation (2-2) shows that structure factor has both amplitude term and phase term. As the intensity $I(hkl)$ can be described by equation (2-4), the phase term of a structure factor vanishes in the diffraction intensity measurement. Thus it is necessary to determine phases of each reflections for structure determination.

For structure analysis of a protein crystal, the isomorphous replacement method is mainly used for phase determination.

Assume that F_P , F_{PH} , f_H are structure factors of a protein, a heavy atom derivative and a heavy atom itself, respectively. Here these three terms are related as follows,

$$F_{PH} = F_P + f_H \quad (2-5)$$

If the positions of heavy atoms are reduced from the difference Patterson maps, the structure factor of heavy atom can be calculated by

$$f_H = \sum_j G_j \cdot T_j \cdot f_j \cdot \exp(2\pi i(hx_j + ky_j + lz_j)) \quad (2-6)$$

here,

$$T_j = \exp(-B_j (\sin\theta/\lambda)^2) \quad (2-7)$$

where $G_j, B_j, f_j, (x_j, y_j, z_j)$ are the occupancy, the isotropic temperature factor, the atomic scattering factor and the fractional coordinates of j -th atom of each heavy atom, respectively.

As shown in Figure (2-4), if modulus of structure factor of the native protein crystal and both modulus and phase of the heavy atom derivative are known and the structure factor of the heavy atom is also known, the phase angle of structure factor of protein can be calculated. But in this method, there is an ambiguity of selecting the correct phase from two possible phases, P and Q in Figure (2-5).

To resolve phase ambiguity inherent in the single isomorphous replacement method (SIR), either of the two methods is usually employed. One is the multiple isomorphous replacement method (MIR) and the other is the single isomorphous replacement method with anomalous scattering effect (SIRA).

Figure (2-6) shows a Harker diagram of MIR case and Figure (2-7) shows a Harker diagram of SIRA case. Combination of MIR and anomalous scattering effect are always used to get a better result than MIR only.

In an actual experiment, many kinds of errors are included in data. Therefore all circles do not cross at the one unique phase on the Harker's diagram, and the correct phases are hard to determine uniquely and phases can only be determined with assessed probability. The lack of closure error can be expressed as the difference between the observed and the calculated

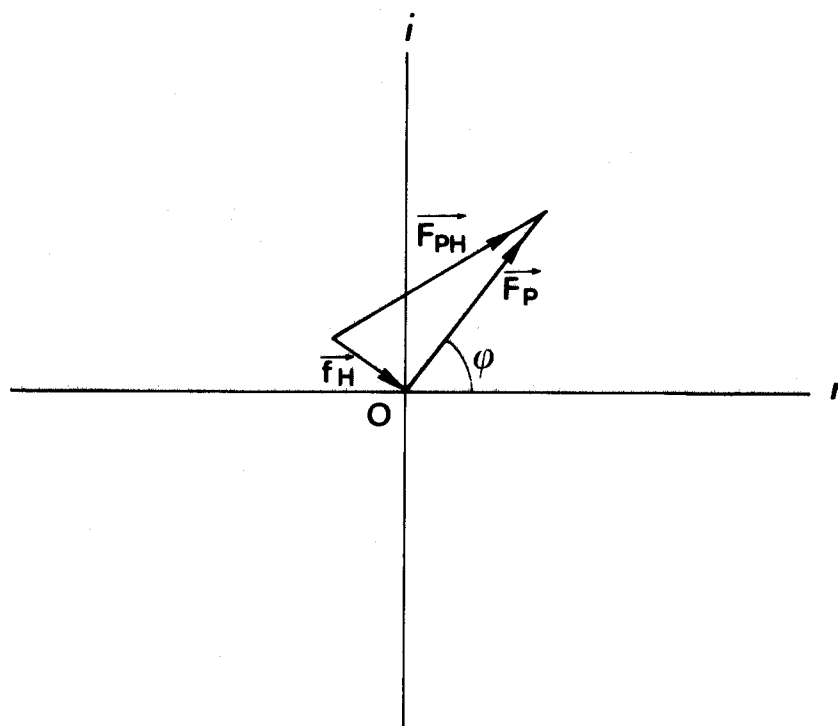


Figure 2-4

Vector diagram
showing single isomorphous replacement case

F_P : Structure factor of protein
 F_{PH} : Structure factor of isomorphous derivative
 f_H : Structure factor of heavy atom(s)

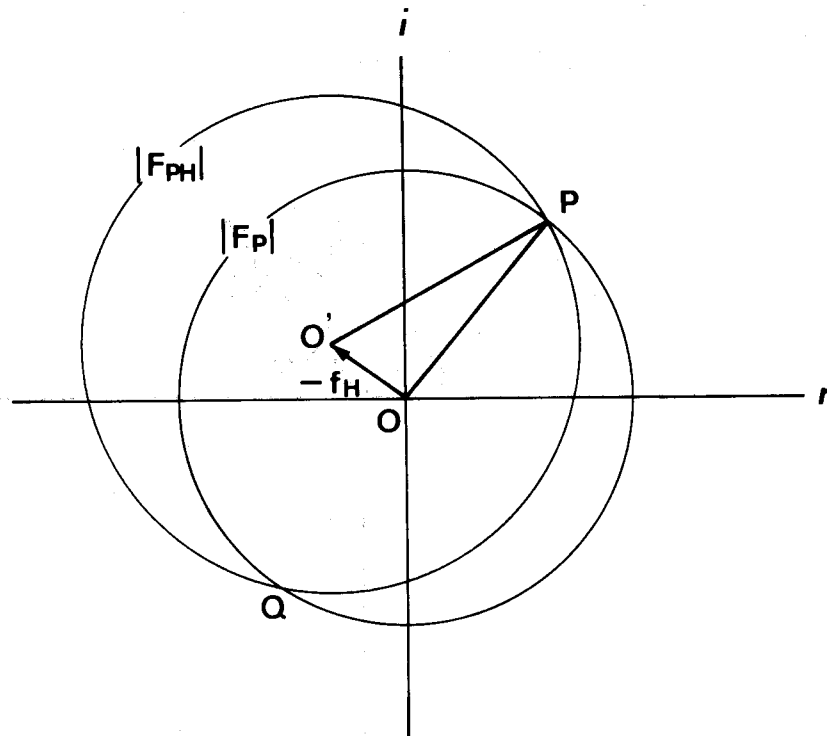


Figure 2-5

Harker diagram showing SIR case

Two possible phases (P and Q) are obtained and it is not possible to select the correct phase

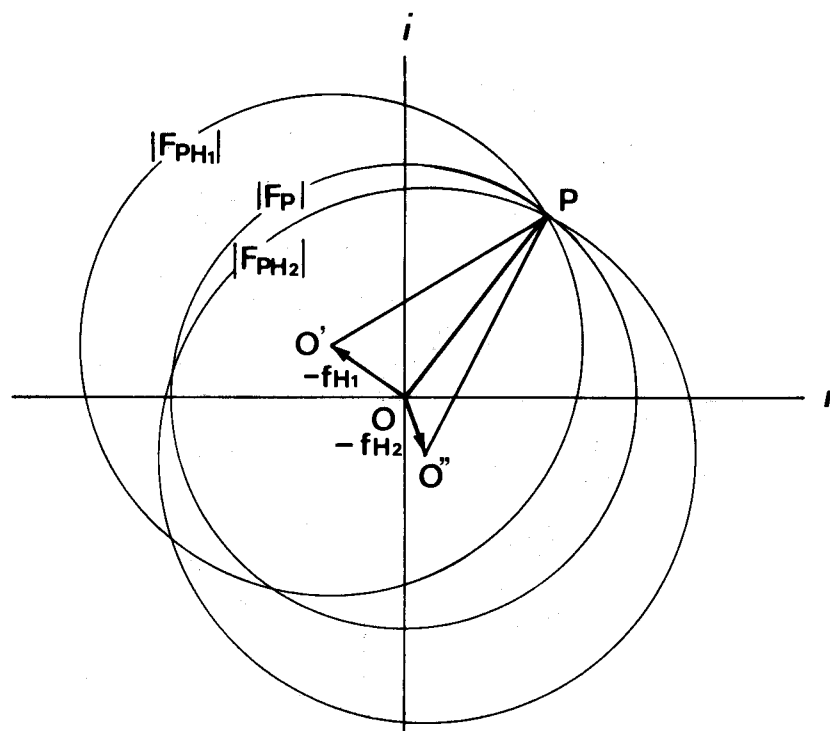


Figure 2-6

Harker diagram showing MIR case

In this case, correct phase can be selected
without ambiguity.

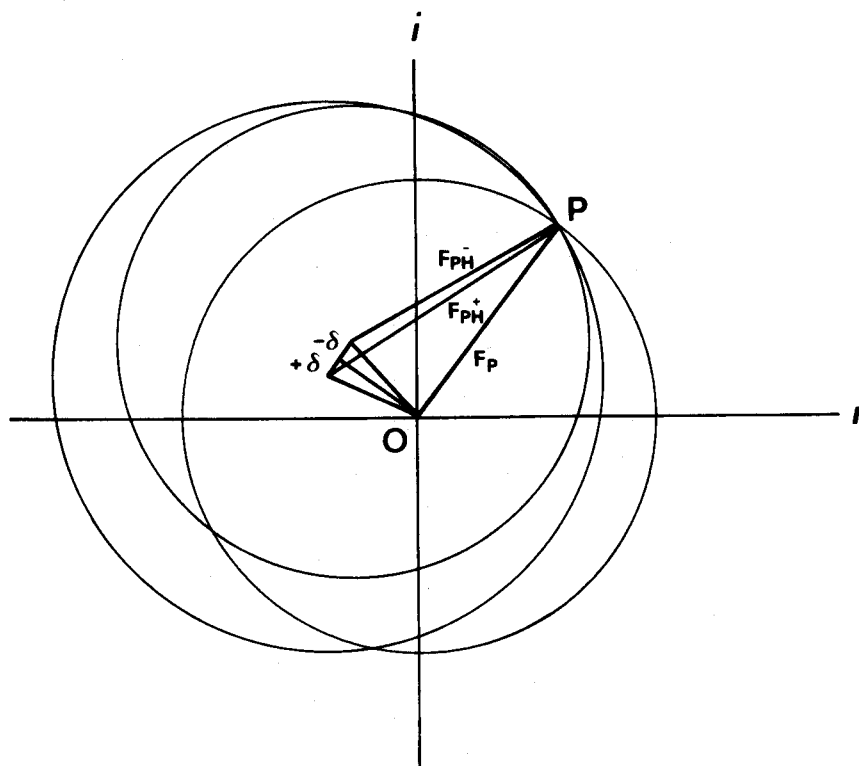


Figure 2-7

Harker diagram showing SIRA case

F_P : Structure factor of protein

F_{PH} : Structure factor of isomorphous derivative

δ : Imaginary part of anomalous term of heavy atom

value of the structure factor as follows.

$$\varepsilon = |F_{obs}| - |F_{calc}| \quad (2-8)$$

For the isomorphous replacement with anomalous difference phase calculation case, the lack of closure error is defined as follows (Matthews, 1966),

$$\begin{aligned} \varepsilon_+^2 + \varepsilon_-^2 &= \{(\varepsilon_+ + \varepsilon_-)^2 + (\varepsilon_+ - \varepsilon_-)^2\} / 2 \\ &= (\chi_{iso}^2 + \chi_{ano}^2) \end{aligned} \quad (2-9)$$

Assume a Gaussian distribution in the lack of closure errors, the probability of a phase angle for the protein structure factor, α , is given by

$$P_j(\alpha) = \exp[-\varepsilon_j(\alpha)^2 / 2E_j^2] \quad (2-10)$$

for the j -th heavy atom derivative.

When several heavy atom derivatives are used for phase calculation, the overall probability of a phase angle is given by the product of individual probabilities

$$P(\alpha) = \prod_j P_j(\alpha) = \exp[-\sum_j \varepsilon_j(\alpha)^2 / 2E_j^2] \quad (2-11)$$

Blow and Crick had shown that the centroid of the phase distribution is a better choice to get a good result (Blow and Crick, 1959). Suppose the

true structure factor F_t , that can be written as

$$F_t = |F_P| \cdot \exp(i\alpha) \quad (2-12)$$

where $|F_P|$ is an amplitude of protein structure factor and α is a phase angle. Then the mean squares error of electron density over the unit cell from this reflection is

$$\langle \Delta\rho^2 \rangle = \frac{1}{V} (|F_s| - |F_t|) \quad (2-13)$$

where F_s is the value of the coefficient used in the synthesis. To minimize the error of electron density, $\langle \Delta\rho^2 \rangle$, F_s should be expressed as,

$$\begin{aligned} F_s(\text{best}) &= \frac{|F_P| \cdot \int \exp(i\alpha) \cdot P(\alpha) d\alpha}{\int P(\alpha) d\alpha} \\ &= m \cdot |F_P| \cdot \exp(i\alpha_{\text{best}}) \end{aligned} \quad (2-14)$$

Here, α_{best} is known as the "best phase". This equation (2-14) means that the centroid of the probability distribution can be expressed as polar coordinate $(m \cdot |F_P|, \alpha)$. The m value is known as the "figure of merit", and has a value between 0 and 1 and $m=1$ means there are no errors in the phase angle.

The mean square error of the electron density can be expressed using figure of merit

$$\langle \Delta\rho^2 \rangle = \frac{1}{V} \sum F_h^2 \cdot (1-m) \quad (2-15)$$

Refinement of heavy atom parameters of each derivative uses minimiza-

tion of the lack of closure error for each derivatives.

Following parameters should be refined,

1. Relative scale factor and temperature factor to native data : $K, \Delta B$
2. Coordinates of each heavy atom sites : (x_j, y_j, z_j)
3. Temperature factor of each heavy atom : B_j
4. Occupancy of each heavy atom : G_j

Two refinement techniques are always used for the refinement of heavy atom parameters. One is FHLE refinement, or centric refinement as its modification, and the other is phase refinement.

2-2-2. Heavy Atom Reagents Survey

At first, in the structure determination of cytochrome c553, the author has been tried to determine phases by the isomorphous replacement technique as it is the best procedure in many cases.

As described in Chapter 2-2-1, cytochrome c553 was crystallized in ammonium sulfate saturated Tris-HCl(50mM) buffer at pH 9.0. Few heavy atom reagents are dissolved in basic buffer solution and many heavy atom ions form complexes with sulfate ion. Thus it was very hard to get good isomorphous derivatives. More than twenty heavy atom reagents were surveyed (Table (2-2)). Only mersalyl derivative could be successfully used for phase determination (Nakagawa *et al.*, 1979).

At first, diffraction data sets of native and mersalyl derivative were collected on a four-circle diffractometer (RIGAKU AFC-5) with rotating anode X-ray generator (RIGAKU RU300; $\text{CuK}\alpha$).

Reagent	Conc. (mM)	Time (day)	PH
K ₃ UO ₂ F ₅	sat.	30	9.0
UO ₂ Ac ₂	sat.	10	9.0
UO ₂ (NO) ₂	sat.	10	9.0
HgAc ₂	2.0	9	9.0
Mersaly1	4.0	12	9.0
PCMB	2.0	10	9.0
PHMB	sat.	30	9.0
PHMBS	sat.	30	9.0
PMA	sat.	14	9.0
K ₂ HgBr ₄	sat.	20	2.0
Mercurochrome	sat.	30	9.0
K ₂ PtCl ₄	sat.	6	8.8
K ₂ PtCl ₆	sat.	9	9.1
PTTC	4.0	3	9.0
LaCl ₃	sat.	12	9.0
SmCl ₃	sat.	7	9.0
NaAuCl ₆	2.0	9	9.0
PdCl ₂	2.0	11	9.0
OsCl ₃	2.0	8	9.0
CsCl	10.0	12	9.0
AgNO ₃	5.0	12	9.1
Th(NO ₃) ₄	sat.	11	9.0
WO ₃	2.0	13	9.0
K ₃ IrCl ₆	4.0	11	9.0

Table 2-2

Soaking Conditions for Heavy Atom Derivatives

Only mersaly1 derivative can be used
for phase determination process

Figure (2-8(a)) and Figure (2-8(b)) show difference Patterson maps of modulus $|F_{PH}-F_P|^2$ and $|F_{PH^+}-F_{PH^-}|^2$, respectively.

2-2-3. Selection of Space Group Enantiomorph

From the interpretation of the difference Patterson map using the mersalyl derivative, the author found one reasonable peak for a heavy atom site. After the refinement of the atomic parameter by phase refinement method (program : LS3DA; Matsuura, 1976, *private communication*), native Fourier maps with SIRA phases were calculated both in the space group $P4_12_12$ and $P4_32_12$. Only the map that was calculated in the space group $P4_32_12$ shows molecular boundary at 6Å resolution map.

2-2-4. Native Anomalous Scattering Effect for Phase Calculation

If we could get one good isomorphous derivative, with large anomalous dispersion effect, we would be able to calculate a good electron density map which leads to the interpretation of molecular folding. However mersalyl derivative of cytochrome c553 crystal was not good enough to get an electron density map by which the molecular folding can be visualized. So it was necessary to get phase information from other sources.

Though the imaginary term of the anomalous scattering effect ($\Delta f''$) of heme iron of these data was not so large (3.2 electrons at 1.54 Å), native anomalous difference Patterson map, modulus $(F_{P^+}-F_{P^-})^2$, showed an Fe-Fe self

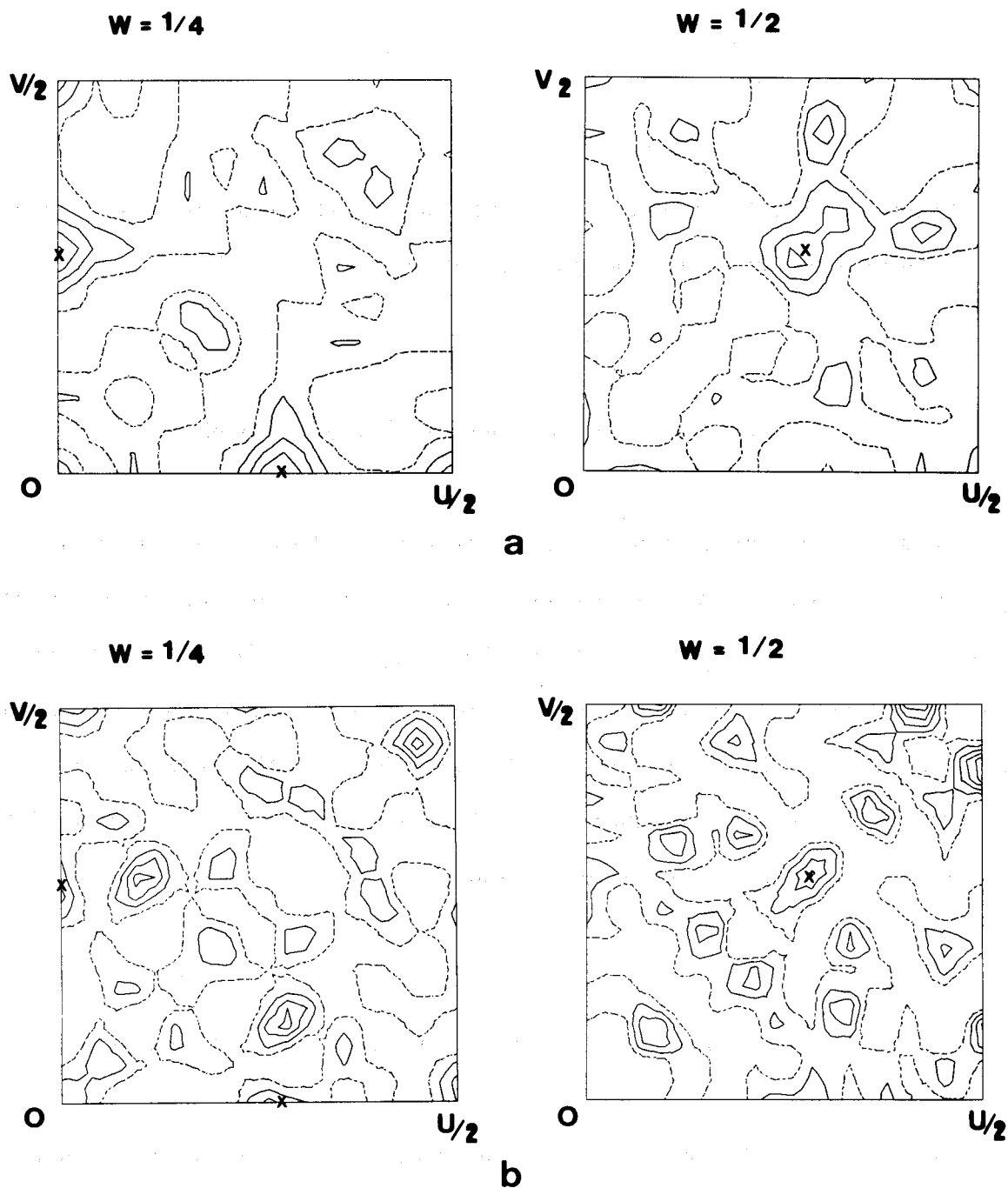


Figure 2-8

Difference Patterson maps of mersalyl derivative
showing Harker sections

- (a) Isomorphous difference with coefficient of $|F_{PH} - F_P|^2$
- (b) Anomalous difference with coefficient of $|F_{PH^+} - F_{PH^-}|^2$

Dashed line shows zero level and these maps are contouring
of equal intervals in arbitrary unit

vector on the Harker section (Figure (2-9)). To use the phase information from the native anomalous scatterer, the author coded a program for phase refinement which can accept native anomalous scattering effect (program : LS3DN, Nakagawa, 1984, *unpublished result*).

The lack of closure error of the native anomalous case is defined as follows (Argos and Mathews, 1973),

$$\varepsilon = (|F^+_c| - |F^-_c|) - (|F^+_o| - |F^-_o|) \quad (2-16)$$

Total phase probability distribution is calculated by the product of individual probabilities of isomorphous and anomalous difference of mersalyl derivative and native anomalous difference.

After heavy atom parameters were refined, native Fourier map was calculated.

To build a molecular model from the interpretation of electron density map, the author used the random stroke type monochrome three-dimensional graphic display (VG/3) and model building program PTNFITG (Iga *et al.*, *private communication*).

But electron density map calculated by crystallographic phases using SIRA of mersalyl derivative and native anomalous dispersion effect was not good enough to trace the main chain folding.

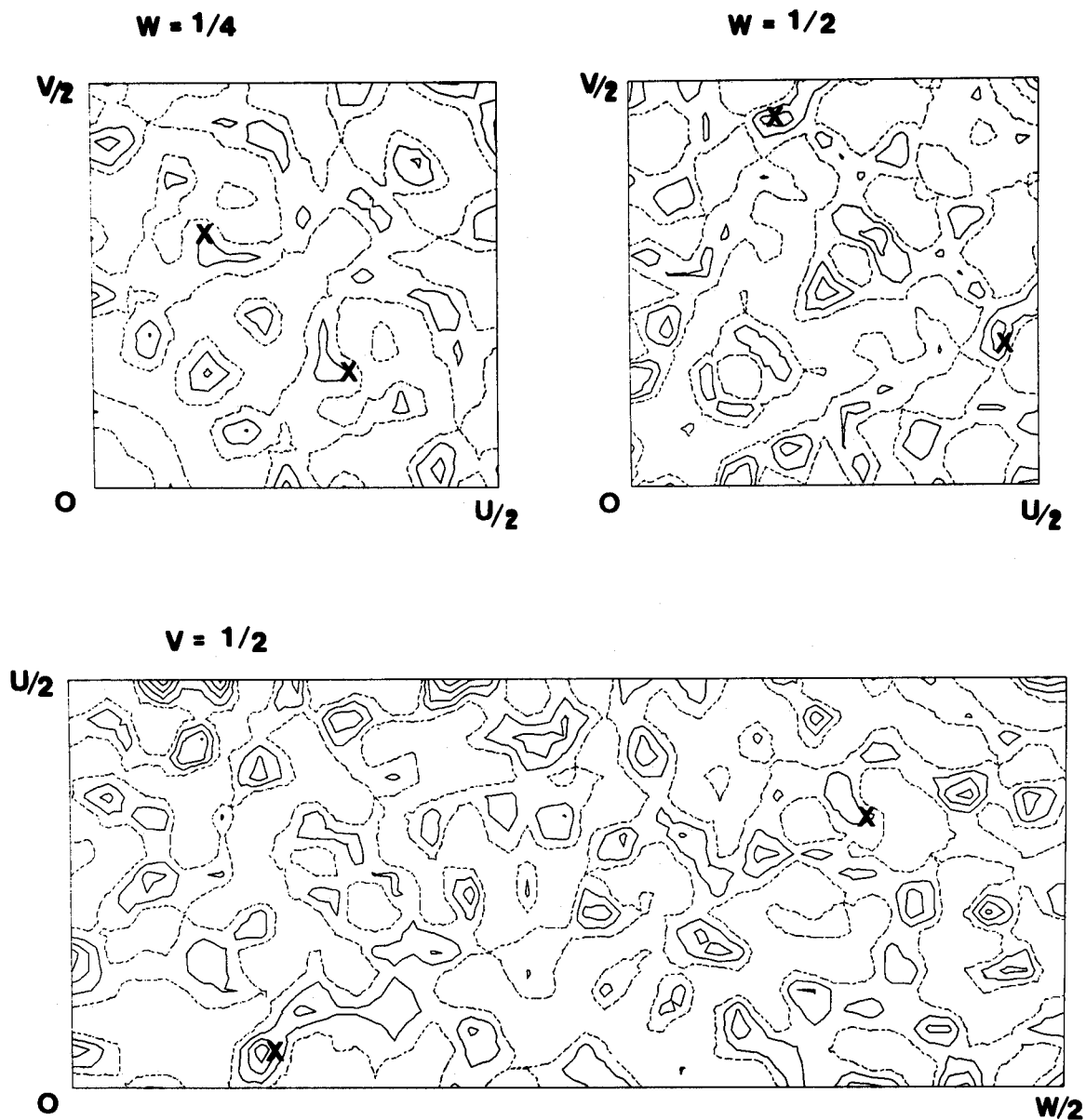


Figure 2-9

Harker sections of native anomalous difference
 Patterson map with coefficient of $|F_p^+ - F_p^-|^2$
 using data collected by a four-circle diffractometer

Dashed line shows zero level and these maps are contouring
 of equal intervals in arbitrary unit

3. MULTI-WAVELENGTH PHASE DETERMINATION STAGE OF STRUCTURE ANALYSIS

3-1. Data Collection

3-1-1. Apparatus and Equipment

3-1-1a. X-ray Source

All diffraction data for multi-wavelength anomalous dispersion method were collected at Photon Factory using synchrotron radiation source.

There are two main accelerators in Photon Factory, one is a linear accelerator and the other is a storage ring. The linear accelerator is called Linac and is 200m long. Electron or positron beam is accelerated to 2.5GeV and is injected to storage ring. Electron or positron beam is stored in PF ring at 2.5GeV. Electron beam was used to get synchrotron radiation at the time when all data for this study were collected. Since July, 1988, positron beam has been stored. Positron beam is more stable than electron beam because of lack of ion trapping effect. But when all diffraction data were collected using synchrotron radiation, PF ring was operated using electron beam.

Normal bending magnets and some insertion devices, such as undulator, vertical wiggler and multi-pole wiggler, are used as light sources in Photon Factory. All diffraction data were collected at branch beamline BL-6A2, and the light source of BL-6A2 is produced by a normal bending magnet.

As synchrotron radiation is polychromatic beam, any wavelength of X-

ray beam can be chosen.

3-1-1b. Beamline Optics at BL-6A2

The branch beam line BL-6A2 is mainly used for macromolecular crystallography using camera method. The light source of this beamline is a normal bending magnet. At this station, continuous X-ray beam can be used for various experiments. The hutch is placed 15m far from the light source point. At 11.8m from the light source point, the fused quartz mirror is placed to focus X-ray beam and to reduce high order X-ray. This mirror is 100cm long and two types of mirror, one is a flat type and the other is a cylindrical type, can be used (Satow *et al.*, 1989). To collect diffraction data from macromolecular crystals, the flat type mirror with bending to focus vertical direction is always used (Figure (3-1)).

The optical bench with a single plate monochromator is placed in this hutch to collect diffraction data by the Weissenberg method. When diffraction data will be collected by Laue method, the monochromator should be removed to get white X-ray radiation.

The monochromator is a triangle-shaped Si(111) plane. It is an asymmetrical cut type with small bending, $\alpha=7.8^\circ$, to focus horizontal direction of X-ray.

The vertical divergence of beam is focused by the mirror and the horizontal direction is focused by the asymmetrical cut bending monochromator.

Air in the slit and monochromator chamber are replaced by helium gas

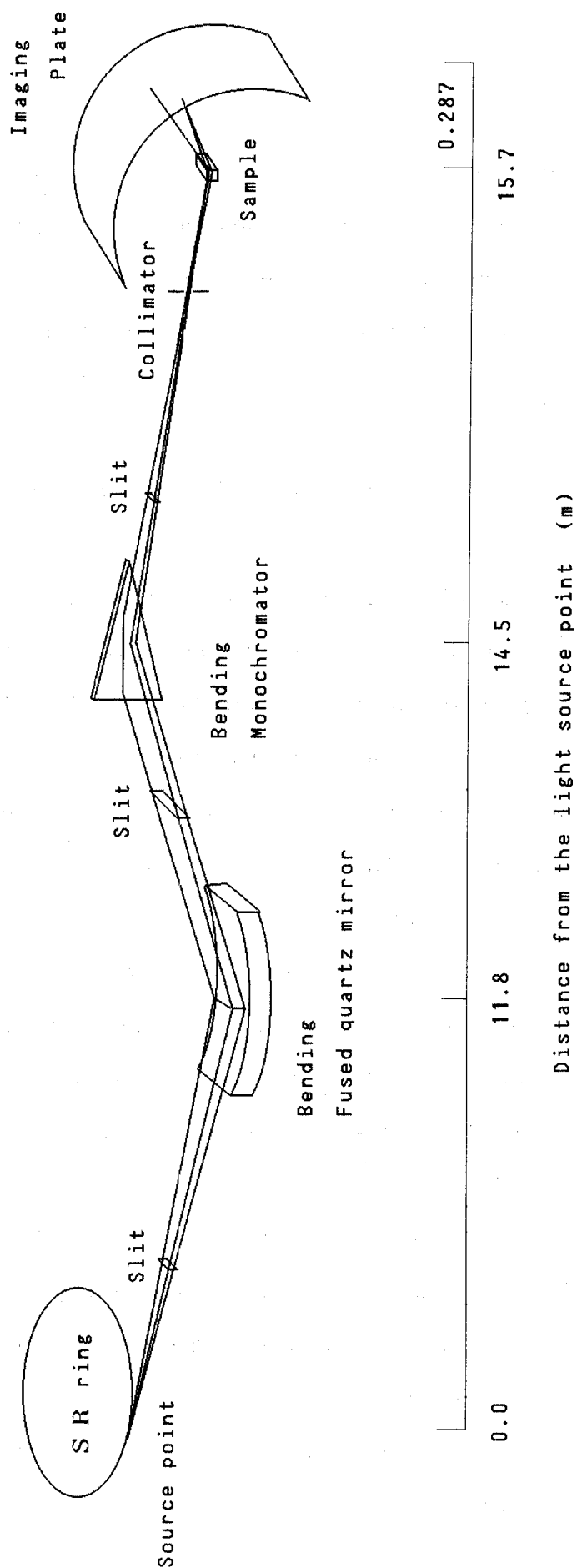


Figure 3-1

Beamline components at BL-6A2 at Photon Factory

Vertical direction is focused by the bending mirror and horizontal direction is focused by the asymmetrical cut bending monochromator

to reduce absorption and scattering by air.

The slit and the optical bench, those were designed by Kamiya and constructed by KEK workshop, are controlled by the personal computer NEC PC-9801E placed outside of the hutch with a program coded by the author.

3-1-1c. The Weissenberg Camera for Macromolecular Crystallography

The weissenberg camera for macromolecular crystallography was used to collect diffraction data in this study. An advantage of the camera method is to allow us to collect many reflections at a same time. This Weissenberg type camera to adopt for data collection in macromolecular crystallography was designed by Sakabe (Sakabe, 1979), and the modified type-II is now working at BL-6A2 hutch at Photon Factory (Sakabe *et al.*, 1989).

A conventional Weissenberg camera for crystals of small molecules (Weissenberg, 1924) has a single layer-line screen to get only one layer data on one film and to spread these data two-dimensionally on a film. But this camera has no screen or multilayer-line screen, which is used to reduce background noise and not to select any layer-lines of diffraction. All layer data should be recorded on one film.

Ardnot-Wonacott type oscillation camera (Ardnot and Wonacott, 1977) is widely used to collect reflection data of biological macromolecular crystal in the world. This Weissenberg camera has many advantages compared with the Ardnot-Wonacott type oscillation camera.

Advantages of this type Weissenberg camera are,

- Wide oscillation range

- Bijvoet-paired data are recorded simultaneously, if symmetry is higher than monoclinic system.
- Easy to collect high angle (high resolution) data, on account of a cylindrical cassette
- Easy to replace to helium gas in the film cassette

Disadvantages of this camera are,

- Relatively high background, as oscillation range is wider than oscillation method
- Necessity of crystal alignment before data collection

The first disadvantage can be ignored by replacing to helium gas in the film cassette, which reduce scattering by air, and by large film cassette, because random scattering should be reduced according to $1/R^2$ (R : camera radius). Second disadvantage is related to the second advantage, simultaneous recording of Bijvoet-paired data. Practically, as crystal alignment is very easy and it usually takes less than one or two minutes for exposure, radiation damage during alignment is not a serious problem.

The type-II camera has six axes of motors to move

- | | | |
|--|---|----------|
| 1. Film cassette movement | : | Z |
| 2. Crystal rotation | : | ω |
| 3. Inclination angle of the camera | : | μ |
| 4. Crystal alignment axis of X-direction | : | X |
| 5. Crystal alignment axis of Y-direction | : | Y |
| 6. Crystal movement along rotation axis | : | Z' |

These six motors are controlled by the personal computer NEC PC-9801E (Clock : 8MHz) and recently this camera can also be controlled by a new personal computer, NEC PC-9801RX4 (CPU : 80286, Clock : 12MHz), using the modified

version of the control program (Version 3.1). The control program was originally coded by Sakabe in Photon Factory and then it was modified and improved by the author. Synchronized movement of ω and Z results Weissenberg movement.

Now, any of four type camera cassettes can be chosen among 143.0, 286.5, 430.0 and 573.0 mm camera radius. The cassette of which camera radius was 286.5 mm was used to collect diffraction data of cytochrome c553. When 200x400 mm² size the imaging plate (see chapter 3-1-1d) was mounted onto this cassette, the maximum resolution was above 1.5 Å with use of 1.38 Å X-ray beam. In the film cassette, air was replaced with helium gas to reduce background noise according to reducing scattering of X-ray by air.

Recorded image data on the imaging plate was read-out by the imaging plate system (BA-100, Fuji Photo Film Co.LTD.) and stored in magnetic tapes. These data were used to estimate intensity data. This process was done at the computer center at Photon Factory (FACOM M-360MP) using the program WEIS coded by Higashi (Higashi, 1989). This program at first refines the film setting matrix and the crystal setting matrix. Calculated and observed positions for any reflections should agree sufficiently to measure its intensity accurately. The initial film setting matrix is determined by positions of three fiducial marks, which are marked by very short-time exposure of the direct beam, which is attenuated by some metal sheets, with the detector appropriately translated in each case. The crystal and film setting matrices are refined from positions of reflections. Mis-indexing is avoided by monitoring the shape of the spot intensity profile, the percentage of reflections with intensities greater than background and the quality of agreement of intensities of symmetry-related reflections. Initially film

setting matrix is refined with low-resolution data. The mis-orientation matrices and the cell parameters are then included in the refinement. And the resolution limit of the data used increased in a stepwise manner after parameters are converged within each step.

Finally data up to 2.0 - 2.5 Å resolution were used for processing of intensities. Integrated intensities are calculated by the profile-fitting method (Rossmann, 1979), and the standard profiles are determined in nine different regions of the detector area.

3-1-1d. Imaging Plate

Normal X-ray film which uses silver compound have been usually used as the most useful detector of X-ray diffraction. But normal X-ray film has many disadvantages, such as low sensitivity, high background noise called chemical fog, narrow dynamic range, non-linearity of blacking of film by X-ray intensity, difficulty of uniform developing and so on. Since computer controlled four-circle diffractometer with scintillation counter was invented, it has been widely used in crystallography because all diffraction data are collected automatically with high precision. But scintillation counter has no spatial resolution and all reflections must be measured step by step. Recently, new type area detectors have been invented, such as multi-wire proportional counter (MWPC), TV detector, CCD and the imaging plate. The former three detectors can handle real-time data measurement but later one is similar to X-ray film in the sense of necessity of read-out system. But imaging plate has many advantages, such as high sensitivity, no

chemical fog, wide dynamic range, large detective area, linearity of response, no dead time and so on (Miyahara *et al.*, 1986).

The imaging plate is a flexible plate coated with fine photostimulable phosphor crystal, BaFBr:Er²⁺, combined in an organic binder. X-ray image is stored as a distribution of F-centers in the photostimulable phosphor crystal. The stored image is read out by measuring the intensity of fluorescence stimulated by He-Ne laser beam scanned over the surface of the screen. And the image stored in a plate is erasable by irradiation of visible light. Performance of the imaging plate is summarized in Table (3-1).

In this study, all data were collected using the imaging plate which size was 200x400 mm².

The read-out system of the imaging plate image data was a BA-100 manufactured by Fuji Photo Film Co.LTD. Image data were read out as two-dimensional intensity data of 0.1x0.1mm² pixels and 10bit A/D converter was used to get digital data. Total of 16MByte data were stored on the disk and then saved on the magnetic tape.

3-1-2. Experiments

3-1-2a. Choice of Wavelengths

To determine the crystallographic phases of cytochrome c553 by the multi-wavelength anomalous dispersion method, following four different wavelengths were chosen for data collection.

(1) Active Area Size	...	400 x 200 mm ² (max. size for BA-100)
(2) Pixel Size	...	0.1 x 0.1 mm ² (min. size for BA-100)
(3) Spacial Resolution	...	< 0.2 x 0.2 mm ²
(4) Linearity of Response	...	4 order of magnitude
(5) Dynamic Range	...	5 order of magnitude
(6) Detective Quantum Efficiency	...	86 % (for MoK α -ray)
(7) Background Level	...	< 3 photons/pixel
(8) Dead Time	...	Zero
(9) The plate can be used <u>REPEATEDLY</u>		

Table 3-1

Performance of imaging plate

- 1.040Å ($\Delta f' = 0.197e$, $\Delta f'' = 1.664e$) ... λ_1
- 1.380Å ($\Delta f' = -0.440e$, $\Delta f'' = 2.678e$) ... λ_2
- 1.743Å ($\Delta f' = -9.211e$, $\Delta f'' = 3.951e$) ... λ_3
- 1.746Å ($\Delta f' = -6.299e$, $\Delta f'' = 0.469e$) ... λ_4

Real and imaginary part of anomalous scattering terms are calculated by Cromer and Liberman's method (Sasaki, 1984). Gold foil was used to select 1.04 Å wavelength X-ray, copper foil was used to select 1.380 Å. Iron foil was used to select X-ray at absorption edge of iron, 1.7345 Å. 1.743 Å and 1.746 Å were selected by mechanical movement of monochromator from absorption edge of iron foil. As shown in Figure (3-2), λ_3 and λ_4 are near to the K absorption edge of iron atom, and both $\Delta f'$ at λ_3 and $\Delta f'$ at λ_4 were quite large. And $\Delta f''$ at λ_3 was large, although $\Delta f''$ at λ_4 was negligible. The data at λ_1 is far from absorption edge and both $\Delta f'$ and $\Delta f''$ were negligible. The data at λ_2 was at first collected to use as native data for refinement. $\Delta f'$ at λ_2 was small and $\Delta f''$ was not so large but native anomalous difference Patterson ($F_P^+ - F_P^-$)² shows a significant peak corresponds to Fe-Fe self vector (see Chapter 3-1-2b) as this data set was merged by five crystals and non-systematic errors should be reduced.

Following differences were used for the crystallographic phase determination.

- $F^+(\lambda_2) - F^-(\lambda_2)$... $\Delta f''(\lambda_2)$
- $F^+(\lambda_3) - F^-(\lambda_3)$... $\Delta f''(\lambda_3)$
- $F(\lambda_3) - F(\lambda_1)$... $\Delta f'(\lambda_3 - \lambda_1)$
- $F(\lambda_4) - F(\lambda_1)$... $\Delta f'(\lambda_4 - \lambda_1)$

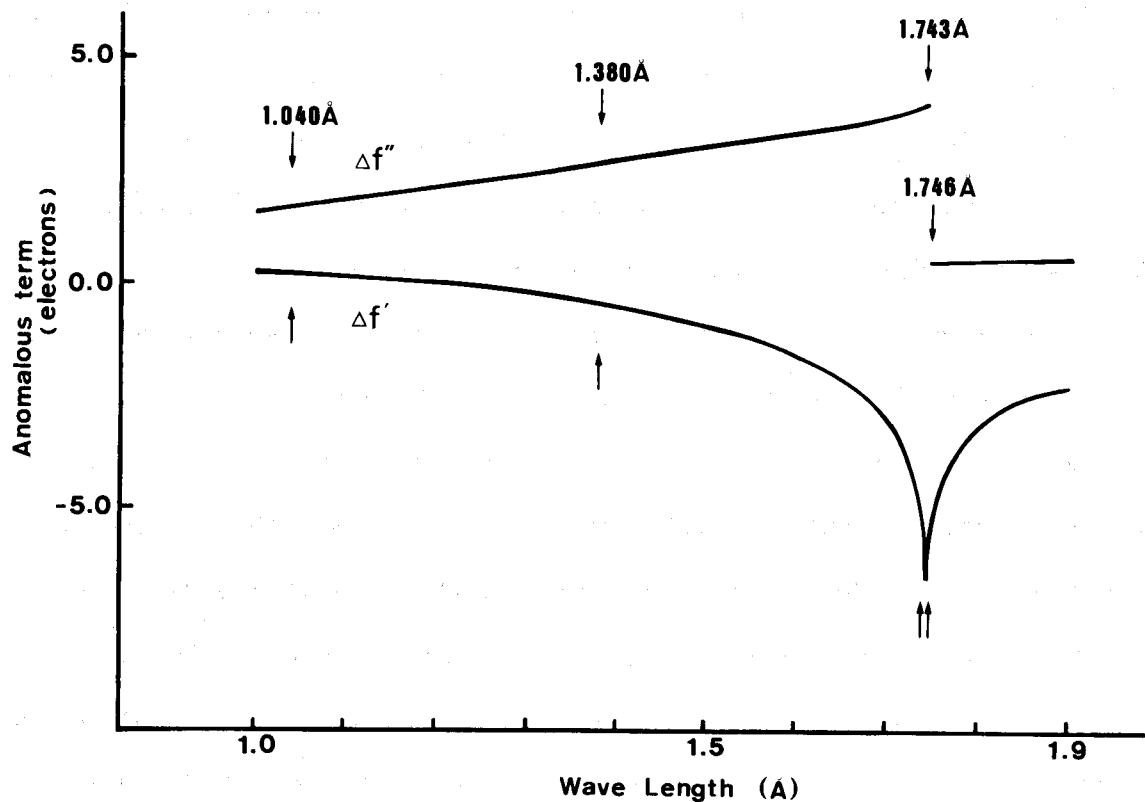


Figure 3-2

Wavelength dependence of anomalous terms
of atomic scattering factor of iron

Arrows show wavelengths at which
diffraction data was collected

These data are calculated by Cromer and
Liberman's method (Sasaki, 1984)

3-1-2b. Condition of Data Collection

The conditions of data collection were summarize in Table (3-2). Monochromatized X-ray beam was collimated by the $0.2 \times 0.2 \text{ mm}^2$ collimator just in front of the sample. Film cassette of which camera radius was 286.5mm was carried out for all data collection. The diffraction data sets at 1.380 Å were merged using data of five crystals both a - and c -axis rotation. The data near rotation axis which are taken using the Weissenberg geometry are not so good, because the shape of these data usually becomes worth than the other part of data. So usually it is neccesary to collect at least two data set rotating along two different directions. But the diffraction data of cytochrome *c553* has 4/mmm symmetry and it means that intensity of the reflection of which the Miller index is hkl is equal intensity of the reflection of khl , $I(hkl)=I(khl)$, thus it is enough for data collection of cytochrome *c553* to rotate along only one axis which is not the unique axis (c -axis). Three data sets except for data at 1.380 Å were collected using only one crystal for each data set.

As the author wanted to get higher resolution data at 1.380 Å as can as possible, total exposure time for these data sets were relatively longer than those for other data sets.

Crystal number	Wavelength(A)	Rotation axis	ϕ -range($^{\circ}$)	exposure time	irradiated power(mA·min.)*)
1	1.380	c	103.9	54'27"	9796
2	1.380	a	118.25	63'42"	12421
3	1.380	c	57.75	18'15"	3921
4	1.380	a	129.0	34'00"	9537
5	1.380	c	60.75	21'21"	4792
6	1.743	a	220.6	18'50"	4765
7	1.040	a	200.5	13'52"	2665
8	1.746	a	198.6	20'24"	3765

Table 3-2

Conditions of data collection for multi-wavelength anomalous dispersion method

All data are collected at BL-6A2 at Photon Factory
using the Weissenberg camera for
macromolecular crystallography and imaging plates

*) Irradiated power is defined as
Ring current(mA) x Exposure time(min.)

3-2. Data Handling

3-2-1a. Data Reduction -- Indexing --

The program system WEIS (Higashi, 1989) was used to get integrated intensity data of each Miller index from two dimensional image data of Weissenberg type diffraction pattern. Conditions of data reduction and their results were summarized on Table (3-3). Crystals those data were collected at 1.380 Å diffracted better than 1.5 Å resolution, that was limited by the size of an imaging plate, but diffraction data below 1.6 Å resolution were indexed and collected to avoid the data at the edge of a imaging plate.

Lorentz and polarization corrections for each reflections were applied at the same time when each reflections were indexed and integrated.

Polarization factor P is defined as

$$P = (I(h) - I(v)) / (I(h) + I(v)) \quad (3-1)$$

where $I(h)$ and $I(v)$ are intensities of horizontally and vertically polarized X-ray, respectively. As synchrotron radiation is highly linear polarized, polarization correction is effective for accuracy of data. It was used 0.92 for polarization correction from the measurement using powder diffraction (Sakabe, *private communication*). Relatively large effective mosaic spread of $\Delta=0.3$ were used, and this value was large enough to avoid true partial reflections being classified as full reflections even if the crystal setting matrix has a little error. Size of measurement box was changed according to the size of diffraction spot, and from 9x9 to 13x13 pixels of measurement

Wave length (Å)	IDENT	RSET ¹⁾	RINT ²⁾	DR ³⁾	DS ⁴⁾	No.of refln.	R_{merge} ⁵⁾
1.380	101	2.0	1.6	0.23	0.16	4886	6.2
	102			0.29	0.17	4940	7.5
	103			0.25	0.17	5814	5.7
	104			0.26	0.19	5004	6.0
	105			0.34	0.22	4972	5.8
	106			0.35	0.20	5460	5.8
	107			0.28	0.19	5294	5.7
	108			0.29	0.17	4888	6.0
	109			0.25	0.25	3902	7.7
	110			0.22	0.15	5114	5.3
	111			0.27	0.19	5098	5.9
	201	2.0	1.6	0.25	0.15	9030	6.9
	202			0.32	0.19	6066	6.0
	203			0.30	0.22	10018	6.0
	204			0.36	0.21	4992	5.6
	205			0.29	0.17	12582	5.8
	206			0.28	0.16	13134	5.4
	301	1.6	1.6	0.33	0.21	2726	6.9
	302			0.20	0.13	2840	7.2
	303			0.36	0.21	2772	6.3
	304			0.28	0.20	2840	6.6
	305			0.32	0.19	2544	8.4
	306			0.29	0.18	2728	6.6
	307			0.27	0.16	2936	6.1
	308			0.29	0.21	2480	7.7
	309			0.31	0.19	3012	6.3
	310			0.25	0.12	2768	6.9
	311	2.0	1.6	0.26	0.14	3026	5.8
	312	1.6	1.6	0.26	0.18	2516	9.6
	313			0.25	0.17	2902	5.7
	314			0.31	0.18	2666	6.1

Table 3-3

Data reduction of cytochrome c553 on each films

Wave length (Å)	IDENT	RSET ¹⁾	RINT ²⁾	DR ³⁾	DS ⁴⁾	No. of refln.	R _{merge} ⁵⁾
1.380	401	2.0	1.6	0.26	0.17	10828	6.1
	402			0.21	0.19	10374	5.7
	403			0.20	0.15	10680	6.4
	404			0.21	0.14	10646	6.8
	405			0.22	0.14	10334	6.6
	406			0.19	0.12	9236	7.1
	407			0.27	0.23	8538	7.3
	408			0.24	0.14	5188	6.1
	501	2.0	1.6	0.18	0.11	5078	6.8
	502			0.20	0.13	5258	5.9
	503			0.21	0.16	5284	4.9
	504			0.19	0.13	5126	5.7
	505			0.18	0.13	5150	5.1
	506			0.27	0.14	4600	6.9
	507			0.22	0.13	4694	6.8
	508			0.21	0.14	4390	7.1
1.743	601	2.2	2.0	0.45	0.29	6152	6.4
	602			0.37	0.27	6544	6.5
	603			0.34	0.18	6170	6.5
	604			0.34	0.22	5710	6.8
	605			0.44	0.25	4348	7.1
	606			0.32	0.17	6098	6.2
	607			0.41	0.26	5990	6.7
	608			0.48	0.21	6424	6.9
	609			0.30	0.18	6708	6.1
	610			0.51	0.27	6768	7.0

Table 3-3
(continued)

Wave length (Å)	IDENT	RSET ¹⁾	RINT ²⁾	DR ³⁾	DS ⁴⁾	No.of refln.	<i>R</i> _{merge} ⁵⁾
1.040	701	2.2	1.6	0.24	0.22	9516	6.4
	702			0.18	0.14	10148	5.7
	703			0.19	0.16	9992	5.5
	704			0.19	0.16	9982	5.8
	705			0.17	0.14	9222	5.5
	706			0.18	0.14	8758	5.8
	707			0.18	0.14	7974	5.5
	708			0.17	0.14	6846	5.3
	709			0.23	0.18	7190	6.5
	710			0.22	0.18	6328	6.1
	711			0.20	0.19	7454	6.3
	712			0.19	0.17	7780	6.5
	713			0.21	0.16	8136	5.7
	714			0.20	0.15	9004	6.1
	715			0.22	0.16	9102	5.9
	716			0.18	0.15	9436	6.1
1.746	801	2.2	2.0	0.24	0.20	6024	6.3
	802			0.33	0.22	5982	6.8
	803			0.24	0.16	6548	6.1
	804			0.41	0.27	6212	7.0
	805			0.42	0.33	5366	7.3
	806			0.23	0.12	5822	6.1
	807			0.29	0.18	4798	6.4
	808			0.38	0.24	4982	6.5
	809			0.22	0.18	5362	6.5

Table 3-3
(continued)

1) Resolution limit to determine setting matrix

2) Resolution limit to collect diffraction data

3) r.m.s. deviation along R-direction

4) r.m.s. deviation along S-direction

5) *R*_{merge} is defined as

$$R_{\text{merge}} = \frac{\sum \sum |I_{hj} - \langle I \rangle_h|}{\sum \sum \langle I \rangle_h}$$

box was used for integration of intensities.

3-2-1b. Absorption Correction and Local Scaling

Observed diffraction data should be included both systematic and non-systematic errors.

Non-systematic errors should be reduced by averaging of many symmetry-related reflections. But some correction technique should be necessary to reduce systematic errors. Absorption effect by crystal, mother liquor and glass capillary and difference of diffraction volume according to its rotation angle should be the most serious parts of systematic errors. The scaling program in the WEIS system, named SCALE, can reduce these errors using Katayama's method (Katayama *et al.*, 1972). This method needs some symmetry related reflections, but camera method is suitable for this purpose as many symmetry related reflections can be collected at a same time. This correction was applied to the data set at 1.380 Å using the data collected by diffractometer, which was applied absorption correction by described North & Phillips (North *et al.*, 1968), as reference data.

Matthews suggested that local scaling method was very useful to reduce systematic errors (Matthews and Czerwinski, 1975). Modified technique of this method was attempted to the data collected by this Weissenberg method. Small differences $\Delta f'$ and $\Delta f''$ are used for phase determination by the multi-wavelength anomalous dispersion method, it was necessary to reduce systematic errors as could as possible.

In this local scaling procedure, scaling factors applied to each shell

were determined by the following procedure,

1. Blocking reflection data according to their polar coordinates (ϕ, ψ) in the reciprocal space (Figure (3-3(a))).
2. For each shell, the scale factor k_j of j -th shell will be calculated as follows,

$$\left(\sum_h \sum_k \sum_l (F_{obs}(hkl)/F_{ref}(hkl)) \right) / N = 1/k_j \quad (3-2)$$

where N is number of reflections in the j -th shell

The reference data must be needed for this method. The reflection data set collected at 1.040 Å was used as reference data. Figure (3-3(b)) shows an example of distribution of polar angles of reflections on a film. Figure (3-4) shows an example of distribution of local scale factors according to ϕ angles.

To calculate local scale factors, the following equation were also tried to apply,

$$\left(\sum_h \sum_k \sum_l (F_{obs}(hkl) / \sum_h \sum_k \sum_l F_{ref}(hkl)) \right) / N = 1/k_j \quad (3-3)$$

but this scale factor did not show so much improvement. The equation (3-2) gave much weight to weak reflections. Anomalous effect is more effective to higher resolution data and higher resolution data is always weaker than lower resolution data. That was because the equation (3-2) gave better result in this study.

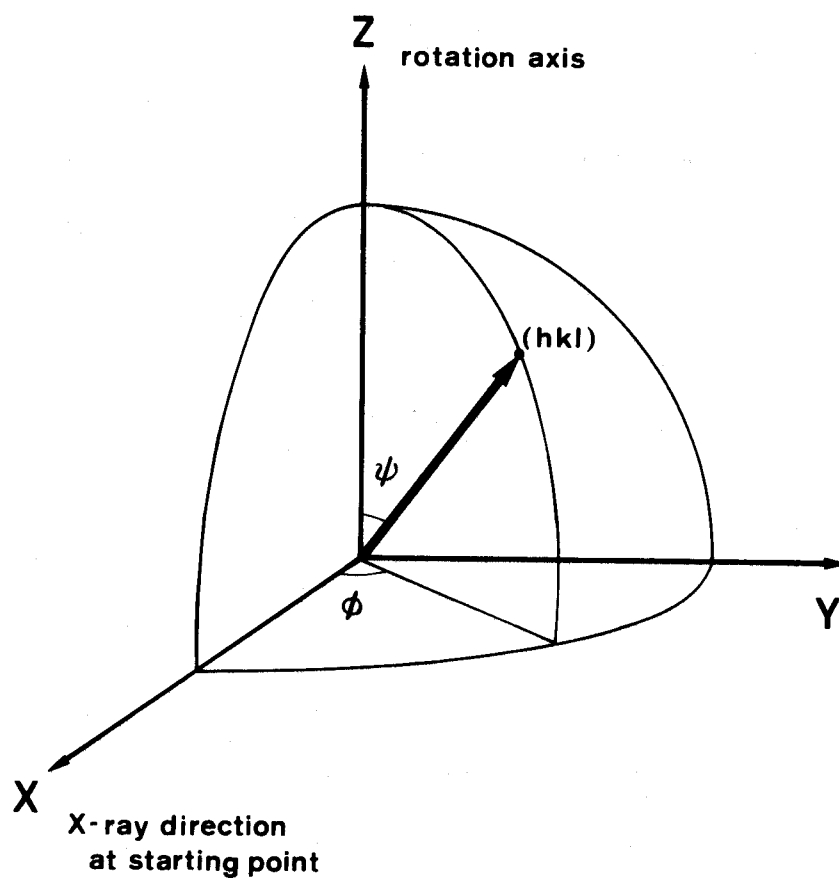


Figure 3-3

(a) Polar coordinates (ϕ, ψ) to determine diffraction point for Local scaling

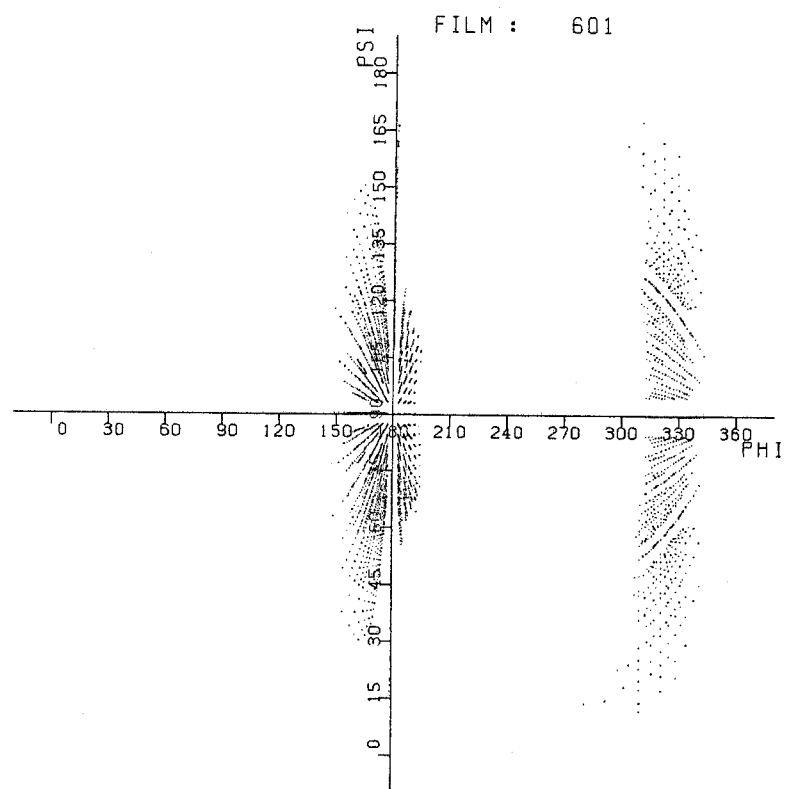


Figure 3-3
(continued)

- (b) An example of distribution of polar angles of diffractions on a film
- Each point shows a polar coordinate of a diffraction spot

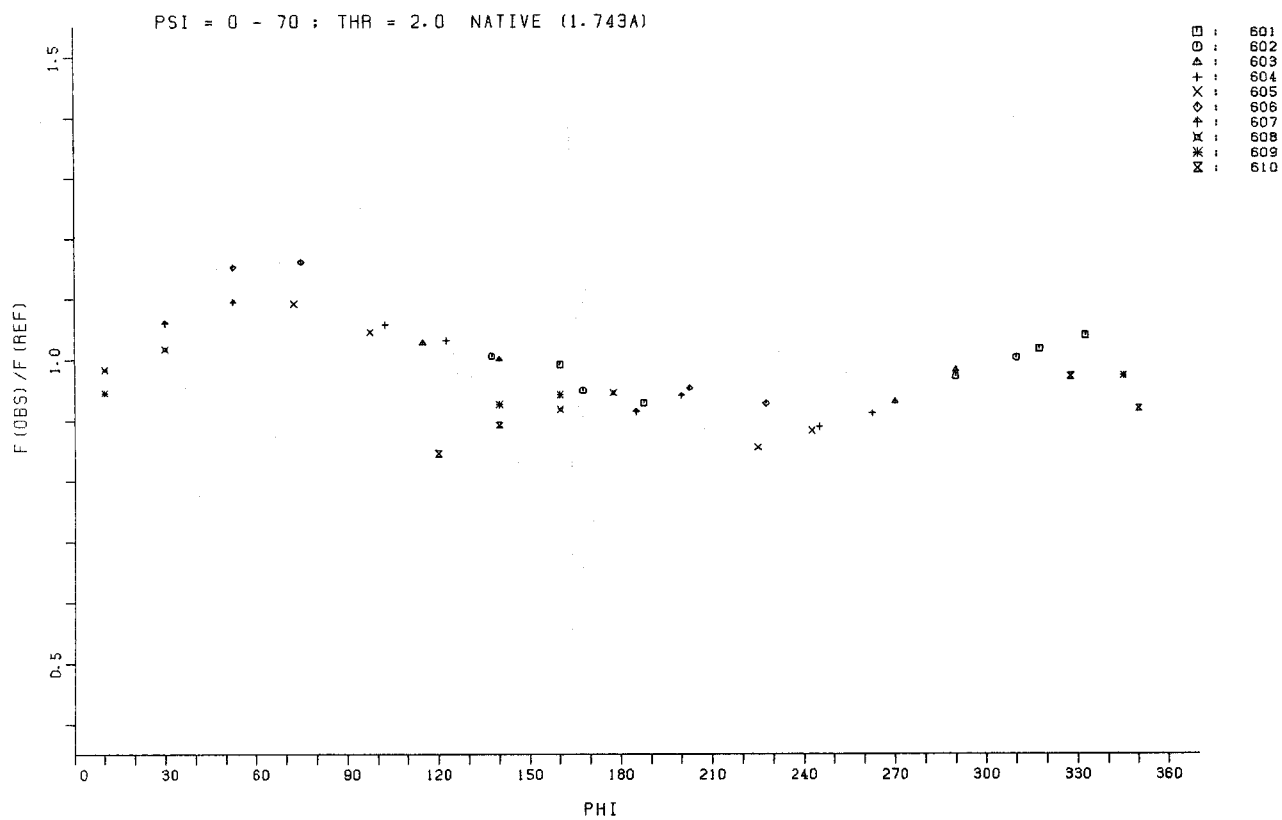


Figure 3-4

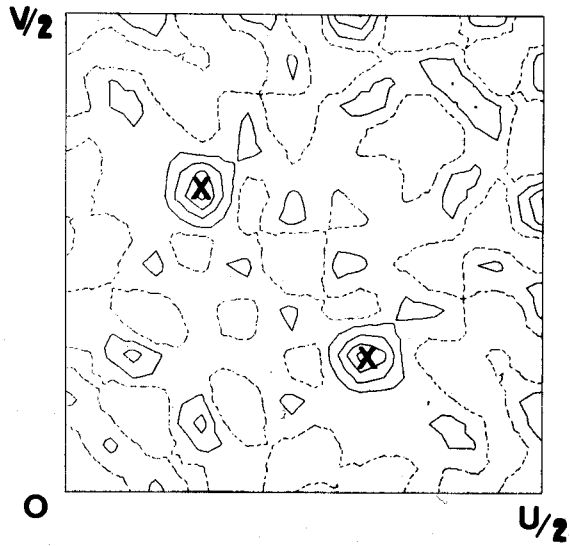
An example of angle distribution of Local scale factors

Figure (3-5(a)) and Figure (3-5(b)) showed anomalous difference Patterson maps of the data at 1.743 Å without the local scaling. The Patterson coefficient of former was corresponds to $\Delta f'$ term and that of later was corresponds to $\Delta f''$ term. The former Patterson map did not show clear Fe-Fe self vector on the Harker section, although the later showed clear peaks. The difference of qualities of these maps was due to film-to-film scaling problem. The later coefficient, $F_p^+ - F_p^-$, was used differences on the same film of only one crystal, although the former included scaling problem between two different crystals. To reduce this scaling error, the local scaling procedure was very effective for improvement of quality of map. Figure (3-6) showed Patterson maps with both before and after local scaling data. The coefficient of both two maps was $|F_p(1.743) - F_p(1.380)|^2$. Apparently, after local scaling data showed high signal-to-noise ratio peak at the position corresponding to Fe-Fe self-vector of heme iron. And Table (3-4) shows an example of changes of R_{merge} according to the local scaling procedure.

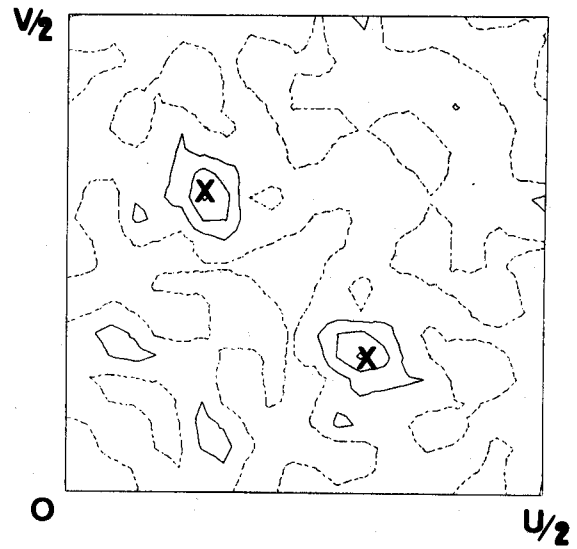
Since the intensities recorded on the imaging plate would correspond to the average intensities from the crystal during the exposure time, radiation damage correction was difficult to be applied. But as the exposure time to take intensities on one sheet of imaging plate was very short, radiation damage must be negligible on one sheet. Difference of diffraction patterns which were cause by radiation damage on the first and the last sheet could not recognized in the same crystal, and small radiation damage might be corrected by the film to film scaling based on equivalent reflections.

Table (3-5) shows final data statistics for multi-wavelength anomalous

$$W = 1/4$$

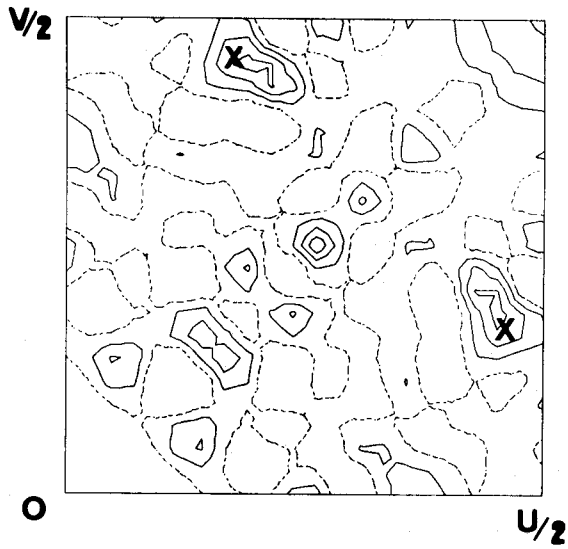


a

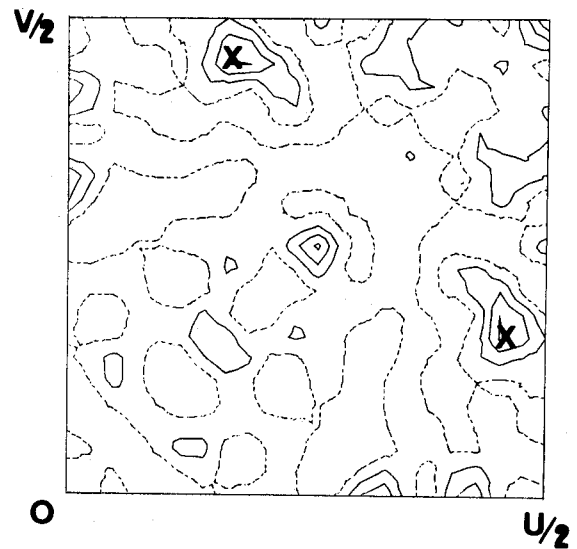


b

$$W = 1/2$$



a



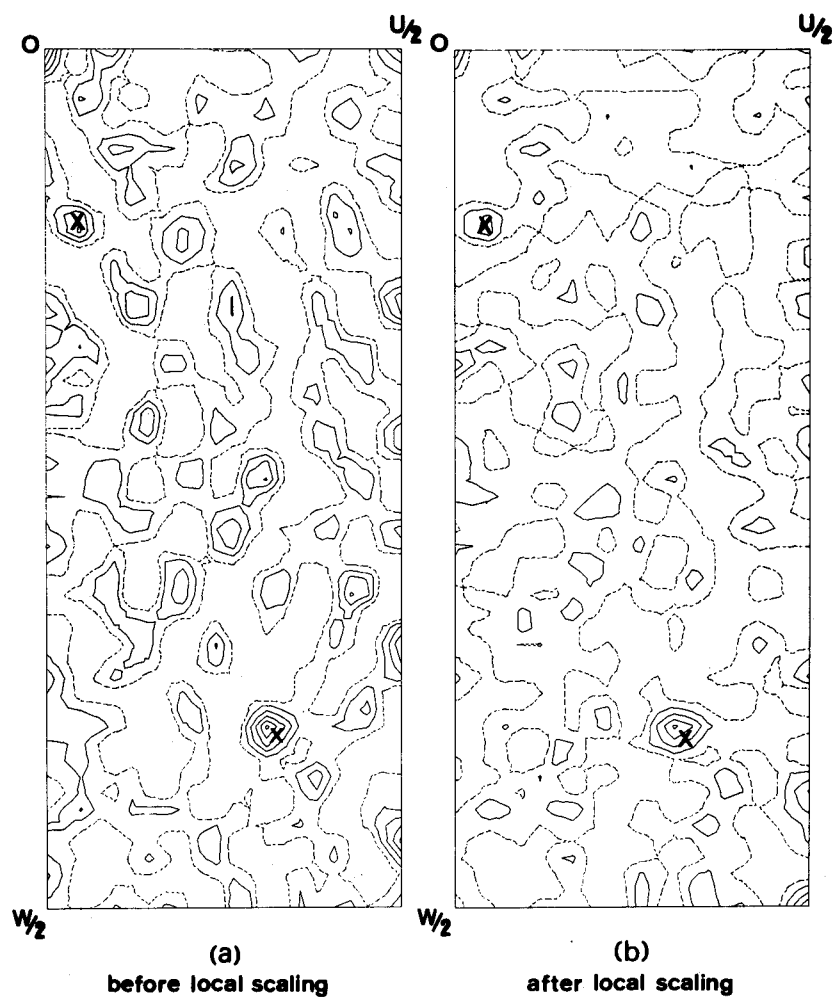
b

Figure 3-5

Native anomalous difference Patterson maps
using data without local scaling

- (a) coefficient of $|F_p(1.743) - F_p(1.38)|^2$, corresponding to $\Delta f'$
- (b) coefficient of $|F_p^+(1.743) - F_p^-(1.743)|^2$, corresponding to $\Delta f''$

Dashed line shows zero level and these maps are contouring
of equal intervals in arbitrary unit



$$\frac{(F_p^{\pm}(1.743) - F_p^{\pm}(1.380))^2}{\text{section} = \sqrt{2}}$$

Figure 3-6

Effect of local scaling procedure

Showing native anomalous difference Patterson maps
of which coefficient is $|F_p(1.743) - F_p(1.380)|^2$

(a) without local scaling, (b) after local scaling

Dashed line shows zero level and these maps are contouring
of equal intervals in arbitrary unit

Film IDENT ²⁾	$R_{\text{merge}}^{1)}$ (%)		No. of Reflection
	No Local Scaling	After Local Scaling ³⁾	
601	7.2	6.4	6152
602	7.7	6.5	6544
603	8.7	6.5	6170
604	9.8	6.8	5710
605	11.4	7.1	4348
606	11.7	6.2	6098
607	10.2	6.2	5990
608	8.2	6.9	6424
609	7.2	6.1	6708
610	7.5	7.0	6768
Total		6.57	30954 (4726) ⁴⁾

Table 3-4

Effect of local scaling of data of cytochrome c553

$$1) R_{\text{merge}} = \frac{\sum_h \sum_i |I_{hi} - \langle I \rangle_h|}{\sum_h \sum_i \langle I \rangle_h}$$

2) Fp(1.743Å) data

3) Local Scaling against Fp(1.040Å) data

4) No. of independent reflections

Wave Length(Å)		$R_{\text{merge}}^{1)}(\%)$	Resol.(Å)	No. of refln. ²⁾
λ_1	1.040	5.89	1.6	8101
λ_2	1.380	6.21	1.6	12378(1942) ³⁾
λ_3	1.743	6.57	2.0	4725(1549)
λ_4	1.746	6.50	1.8	4561

Table 3-5
Summary of F-data for each wavelength

$$1) R_{\text{merge}} = \frac{\sum_h \sum_j |I_{hj} - \langle I \rangle_h|}{\sum_h \sum_j \langle I \rangle_h}$$

2) Number of Bijvoet's pair-related reflections
count as one

3) Number of Bijvoet's pair data

phase determination.

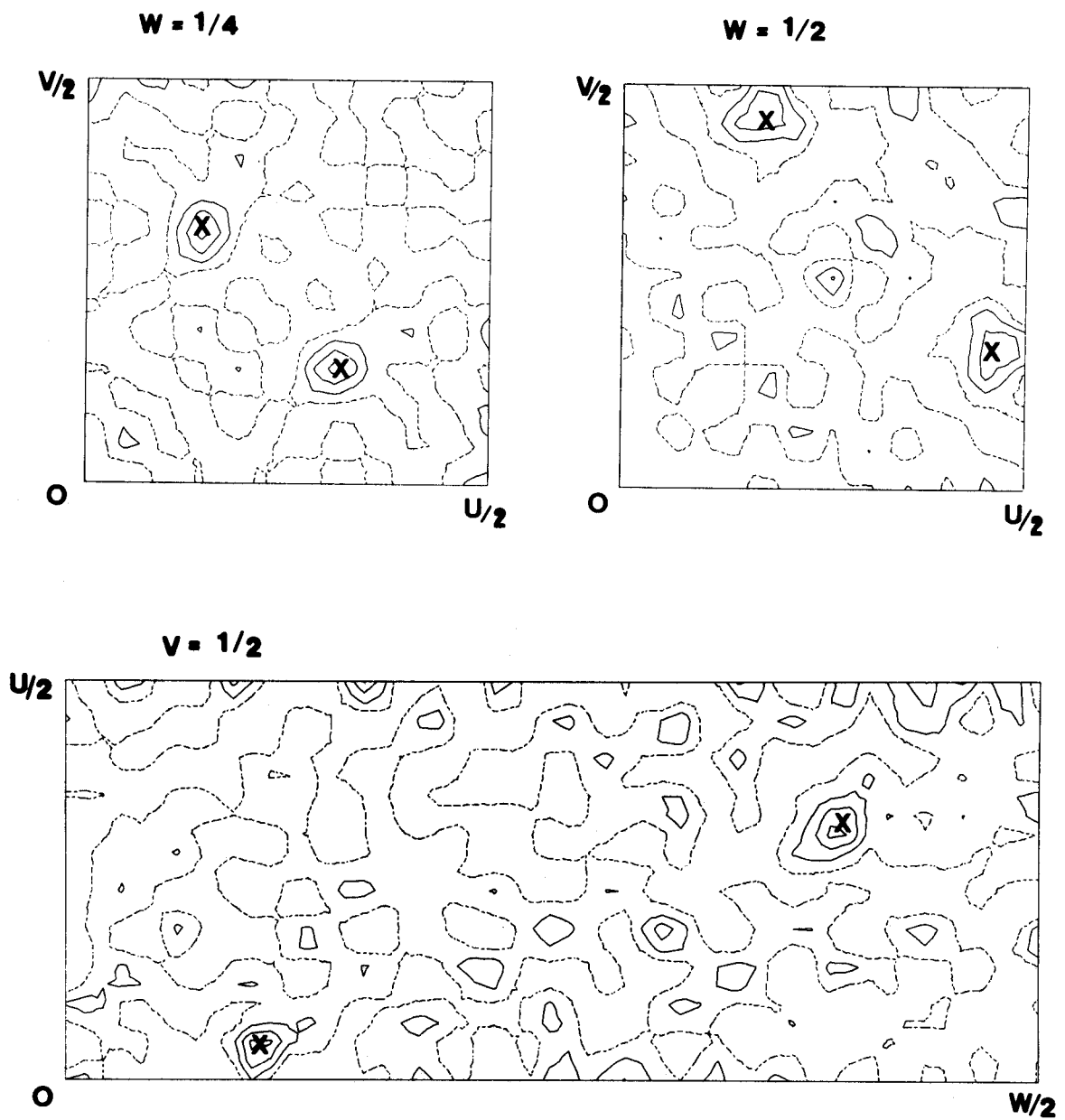
3-2-2. Native Anomalous Difference Patterson Maps

Figure (3-7(a)) to Figure (3-7(d)) showed native anomalous difference Patterson maps of both $\Delta f'$ and $\Delta f''$ term for phase determination. Figure (3-7(e)) did not show any significant peak, it indicated the wave length which was used for data collection of the data set at λ_4 was apparently longer than the wave length at K absorption edge of iron and $\Delta f''$ was negligible.

3-2-3. Absolute Scale

Figure (3-8) showed a Wilson's plot (Wilson, 1949) of data collected at 1.380 Å. Considering of this plot, scale factor and temperature factor were determined as 1.727 and 19.0, respectively.

Scale factors for other data sets were calculated from relative scale factors to the data at 1.380 Å and summarized in Table (3-6).

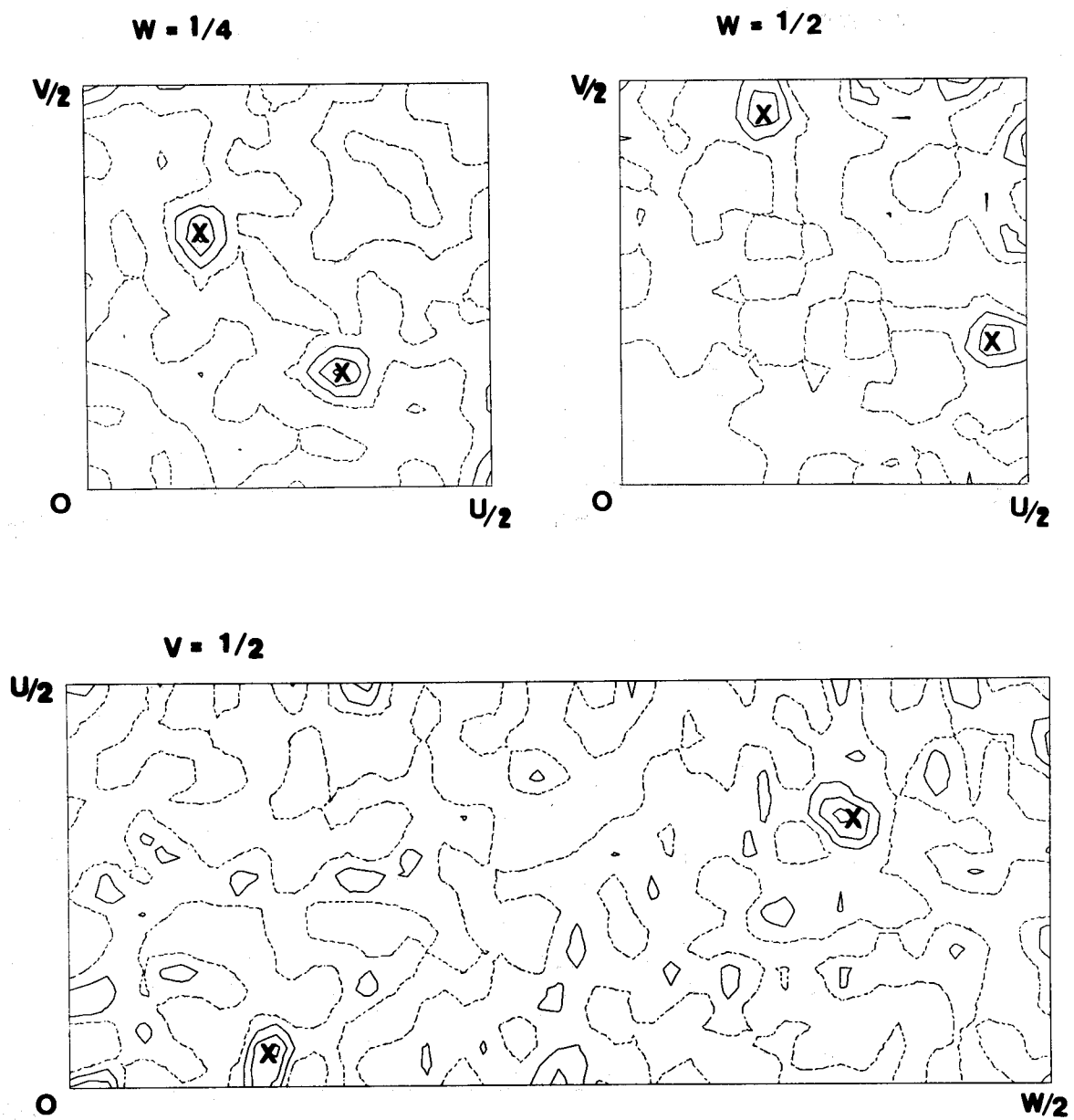


(a) $|F_P(1.743) - F_P(1.040)|^2$

Figure 3-7

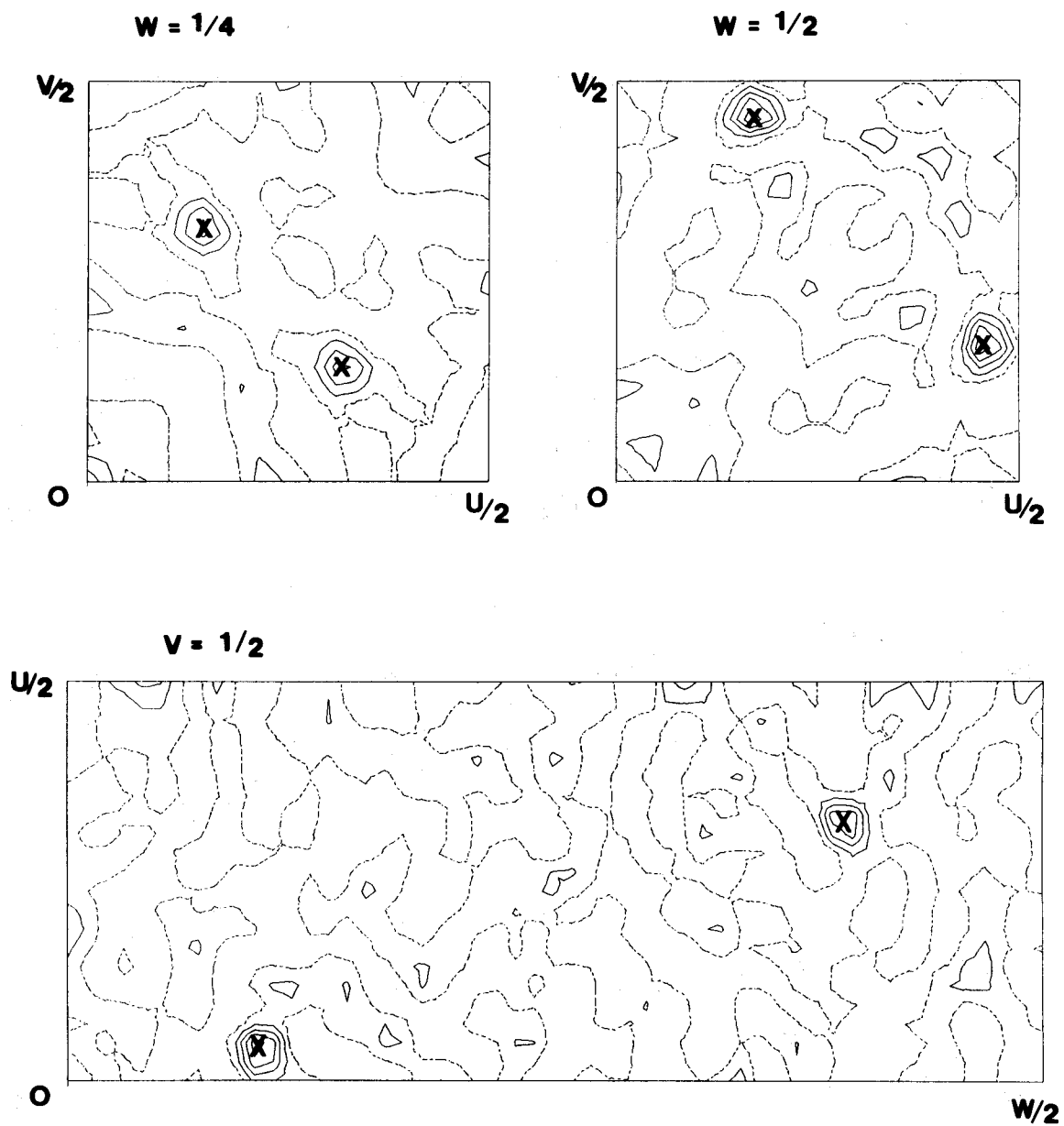
Native anomalous difference Patterson maps

Dashed line shows zero level and these maps are contouring
of equal intervals in arbitrary unit



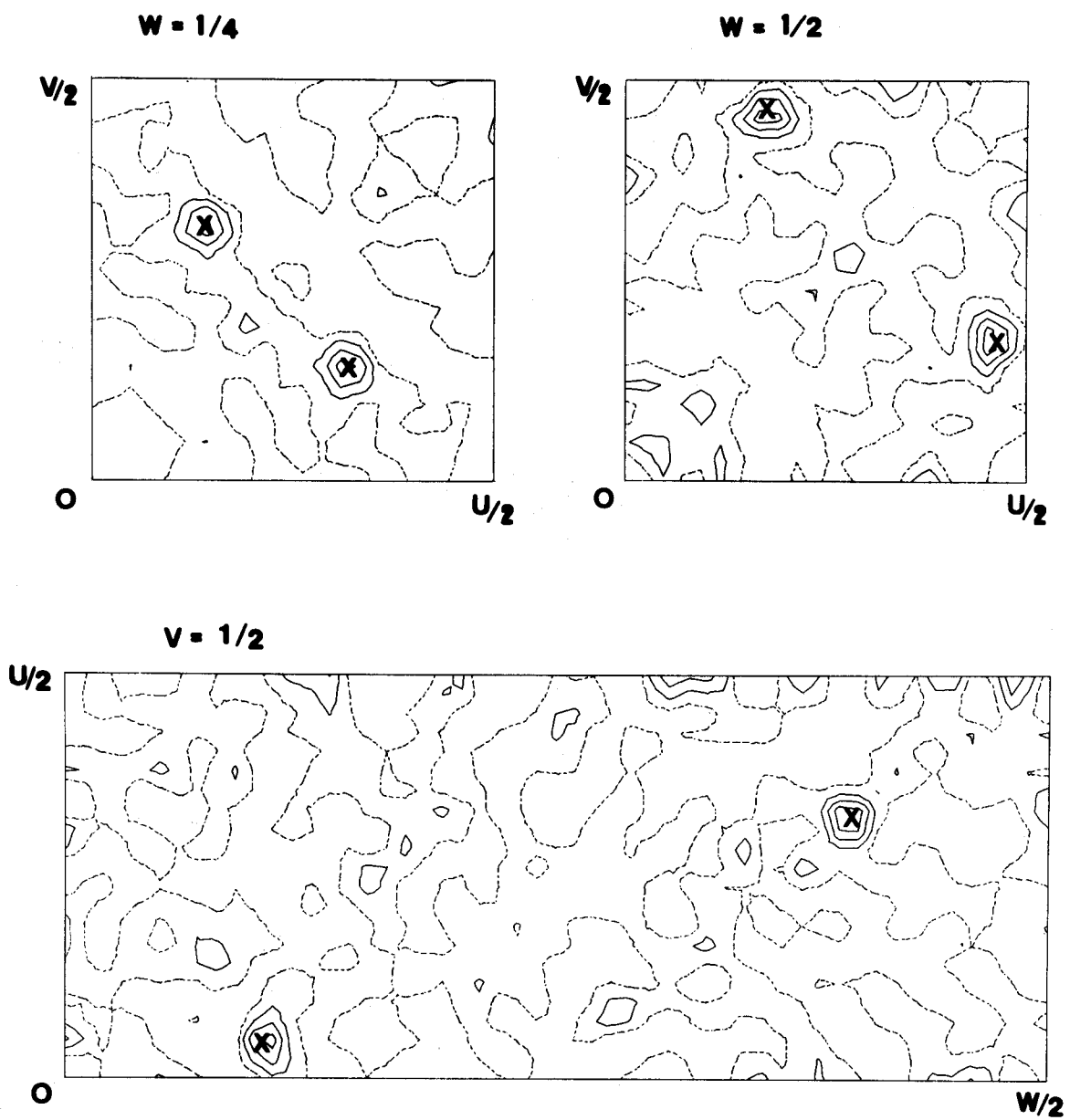
(b) $|F_P(1.746) - F_P(1.040)|^2$

Figure 3-7
(continued)



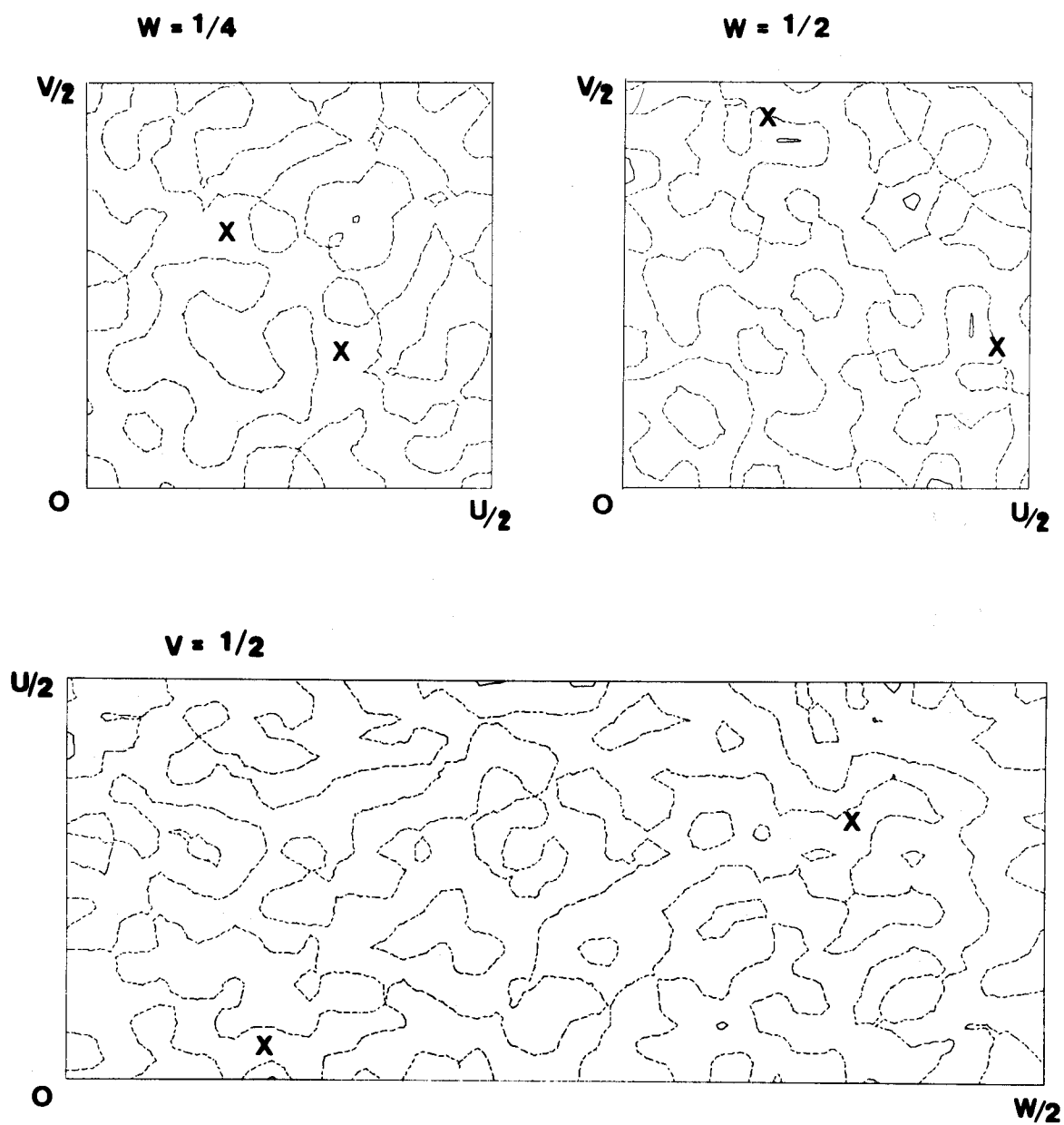
(c) $|F_P^+(1.380) - F_P^-(1.380)|^2$

Figure 3-7
(continued)



(d) $|F_P^+(1.743) - F_P^-(1.743)|^2$

Figure 3-7
(continued)



(e) $|F_P^+(1.746) - F_P^-(1.746)|^2$

Figure 3-7
(continued)

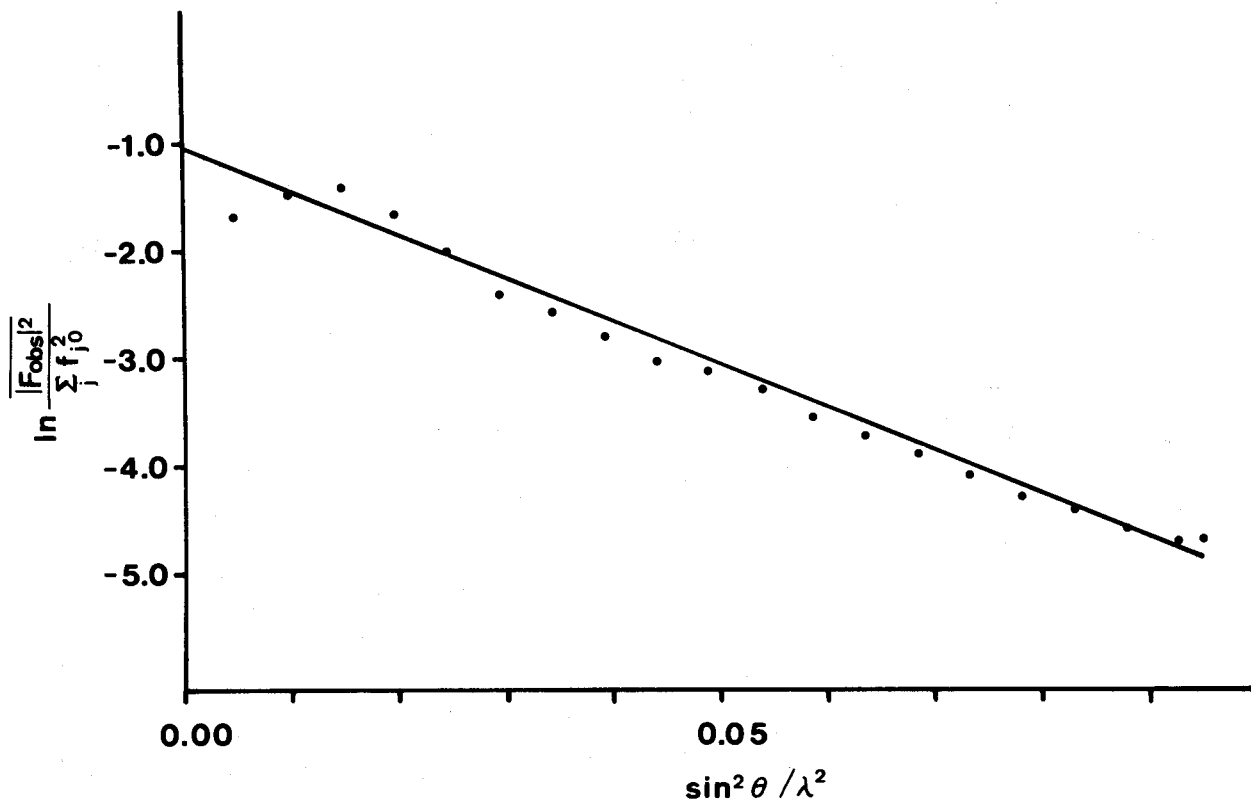


Figure 3-8

Wilson's plot (Wilson, 1949) of data
of cytochrome c553 at 1.380 Å

$K = 1.727$

$B = 19.0$

Wave length (Å)	Scale factor	R^*
1.040	1.723	0.031
1.380	1.727	—
1.743	1.706	0.056
1.746	1.710	0.064

Table 3-6

Scale factors and R -factors for each data sets

$$*) \quad R = \frac{\sum ||F_P(1.380)| - |F_P(\lambda_A)||}{\sum |F_P(1.380)|}$$

3-3. Phase Determination by Multi-Wavelength Anomalous Dispersion Method

3-3-1. Theoretical Background

a. Anomalous scattering effect

X-ray diffraction phenomenon from crystal is caused by the interface of scattering from each atoms in the crystal.

When X-ray is irradiated on a free electron, the influence of the fluctuating electromagnetic field of the incident wave forces the electron into oscillations of the same frequency as the incident wave. All the scattered X-rays from a single electron have the same phase relation to the incident beam, differs by 180 degree, so that the scattering is coherent. Scattering from one atom can be expressed as follows,

$$f = \int_{\text{atom}} \rho(r) \cdot \exp(2\pi i \mathbf{r} \cdot \mathbf{k}) \cdot d\mathbf{v} \quad (3-4)$$

where

$$\mathbf{k} = \mathbf{s}/\lambda$$

and $\mathbf{s}$ shows a scattering vector, $\mathbf{s} = \mathbf{s}_s - \mathbf{s}_i$, difference of the direction from incident, $\mathbf{s}_i$, to scattered beam, $\mathbf{s}_s$. When the direction of scattered beam is same as that of incident beam, f_0 becomes as Z , where Z is the total number of electrons in the atom.

This assumption is correct for free electrons of atoms. But real electrons are bound quite tightly, particularly those in the inner K and L

shells and such a description is only adequate for the lightest of elements (eg. carbon, nitrogen and oxygen). Then atomic scattering factor must write are follows,

$$f = f_0 + \Delta f' + i\Delta f'' \quad (3-5)$$

this means that atomic scattering factor has its real term, $f_0 + \Delta f'$, and its imaginary term, $\Delta f''$. The phase of imaginary part of atomic scattering factor is always $\pi/2$ in front of that of the scattered X-rays. Figure (3-2) showed one example of both real and imaginary term of anomalous dispersion effect, $\Delta f'$ and $\Delta f''$. The real part of anomalous scattering term varies dramatically near its absorption edge, and the imaginary part of anomalous scattering term varies slightly at the wavelength which is shorter than absorption edge and is nearly equal to zero at wavelength which is longer than absorption edge.

Suppose that structure factor amplitudes of the reflection hkl and its inverse reflection $\bar{h}\bar{k}\bar{l}$.

$$F_P(+) = \sum_j f_j \cdot \exp(2\pi i \mathbf{h} \cdot \mathbf{r}_j) \quad (3-6)$$

and

$$F_P(-) = \sum_j f_j \cdot \exp(-2\pi i \mathbf{h} \cdot \mathbf{r}_j) \quad (3-7)$$

where summation is over all the atoms in the unit cell. Therefore

$$F_P(+) = F_P^*(-) \quad (3-8)$$

and

$$|F_P(+)|^2 = |F_P(-)|^2 \quad (3-9)$$

when imaginary part, Af'' , of atomic scattering factor is negligible.

As shown in Figure (3-9), if there are some anomalous scatterers in the crystal, $F_P(+)$ is no longer equal to $F_P(-)$ for the general case. And if the reflection is centric, $F_P(+)$ is equal to $F_P(-)$.

Magnitude of diffraction data collected at the wavelength close to absorption edge varies by the Af' term, and its structure factor is different from the corresponding data which is collected at the wavelength far from absorption edge.

These two kind of differences, both real and imaginary part of diffraction, due to anomalous dispersion effect, can be used for phase determination.

As an analogue of isomorphous replacement method, Figure (3-10) showed a Harker diagram that showed a multi-wavelength anomalous dispersion method.

At first, atomic parameters of anomalous scatterer are refined, then phases were determined.

As the multi-wavelength anomalous dispersion (MAD) technique has just been applied to structure determination of macromolecular crystals, there is no established procedure for this method on both refinement of parameters of anomalous scatterers and phasing procedure. In this study, the similar procedure of isomorphous replacement technique was applied.

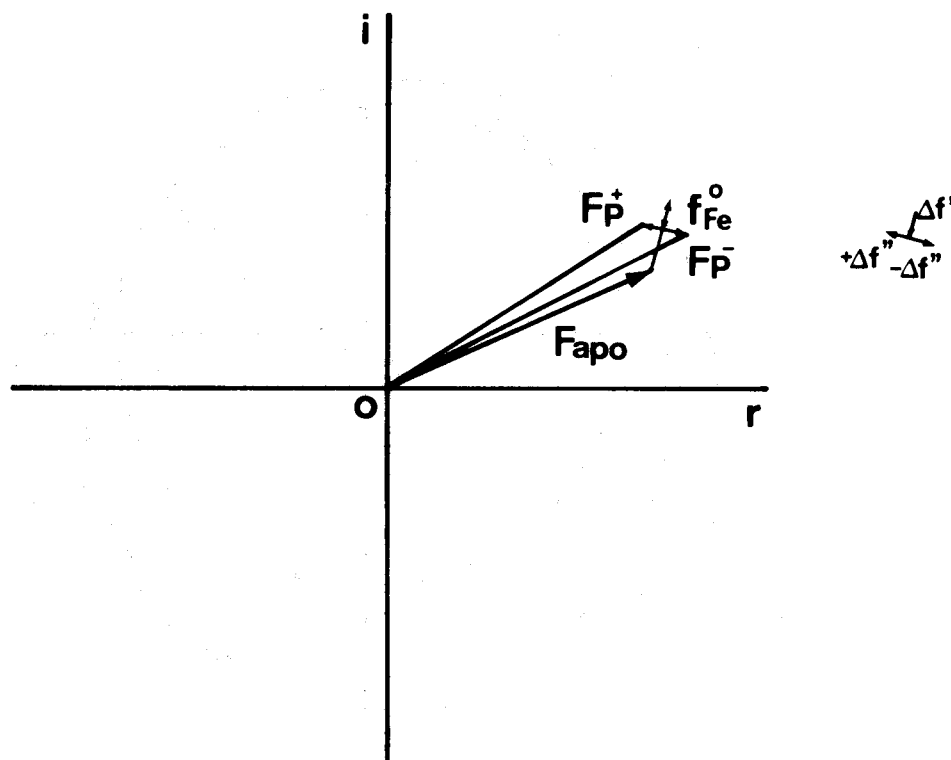


Figure 3-9

Vector diagram showing
native anomalous case

F_{apo} : Structure factor of protein without anomalous scatterer

F_P : Structure factor of protein

f_{Fe} : Structure factor of iron atom (anomalous scatterer)

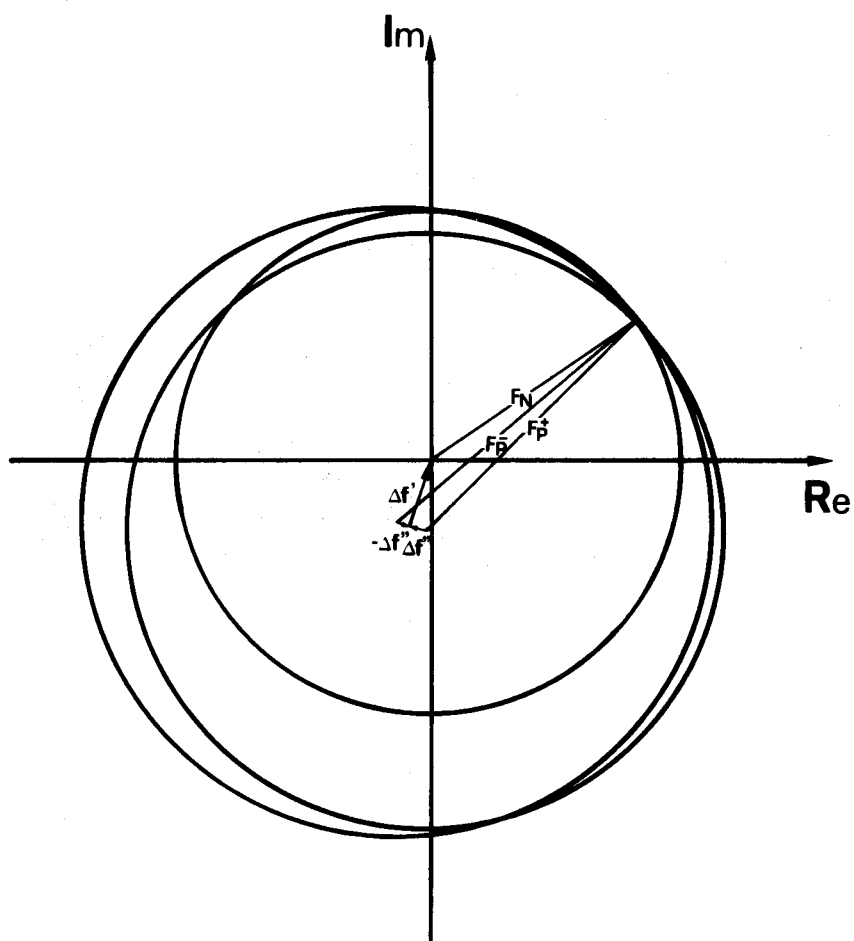


Figure 3-10

Harker diagram showing
MAD case

b. Refinement of atomic parameters of anomalous scatterer

The occupancy parameter of anomalous scatterer may be unity after scale factor is applied to each wavelength data. Thus the coordinates, (x,y,z), and the temperature factor will be refined.

In this study, centric refinement technique was applied to the refinement of atomic parameters. In this method, parameters were refined to minimize the following quantity using only centric reflections.

$$\epsilon^2 = ||F_P^A - F_P^0| - |\sum A f_j' B_j \cos 2\pi i(hx_j + ky_j + lz_j)|||^2 \quad (3-10)$$

where F_P^A is an observed structure factor with anomalous effect, F_P^0 is an observed structure factor without anomalous scattering effect and $A f_j'$, B_j , (x_j, y_j, z_j) are real part of anomalous scattering term, temperature factor, of anomalous scatterer and its coordinates, respectively. The least-squares refinement was applied to minimize the equation (3-10).

For the data which was only used its imaginary part of anomalous scattering effect, refinement of atomic parameters was proceeded described by Hendrickson (Hendrickson, 1981). This method minimize followings,

$$\epsilon^2 = |\Delta F_P(\pm)(\text{obs}) - (-2 \sum_j A f_j'' B_j \sin(hx_j + ky_j + lz_j))|^2 \quad (3-11)$$

for only large $\Delta F_P(\pm)(\text{obs})$ reflections.

c. Phase Calculation

Phase determination procedure was according to Blow and Crick's best phase calculation (see 2-2-1).

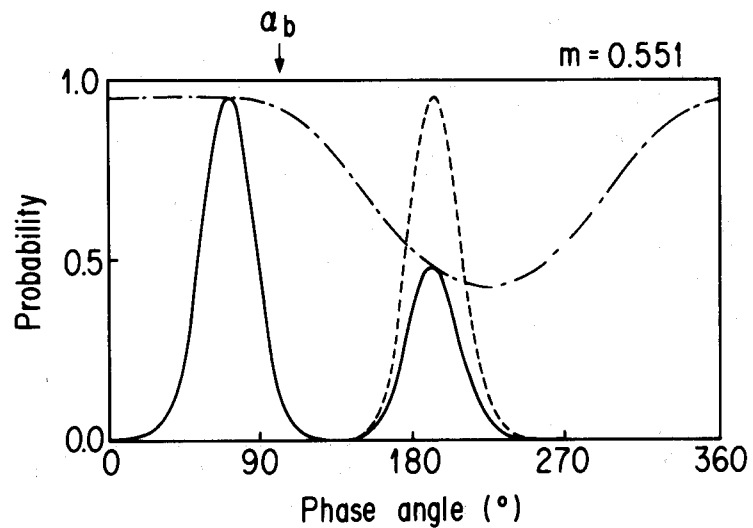
Apparently, as shown in Figure (2-5), the unique correct phase can not be determined by the single isomorphous replacement method. Figure (3-11) showed an example of phase distribution of single isomorphous case. The dashed line showed a distribution of isomorphous term and dot-dashed line showed a distribution of anomalous term. In this case, if the root-mean squares error of isomorphous term, E_{iso} , and the root-mean squares error of anomalous term, E_{ano} , are same value, it could not possible to select correct phase from two possible phases of isomorphous term. North showed that a phase calculation with a phase calculation with an assumption of $E_{ano}=1/3E_{iso}$ could select the correct phase without ambiguity (North, 1965). On the other hand, in the multi-wavelength anomalous dispersion method, both magnitudes of real and imaginary differences are close to each other. As shown in Figure (3-12(b)), calculation with the assumption of $E_{ano}=1/3E_{iso}$ does not give correct phase, although the figure of merit is greater than that of with $E_{ano}=E_{iso}$. Using the assumption that E_{ano} is same as E_{iso} gives better result as shown in Figure (3-12(a)).

Phase probability of real part of anomalous difference is given as,

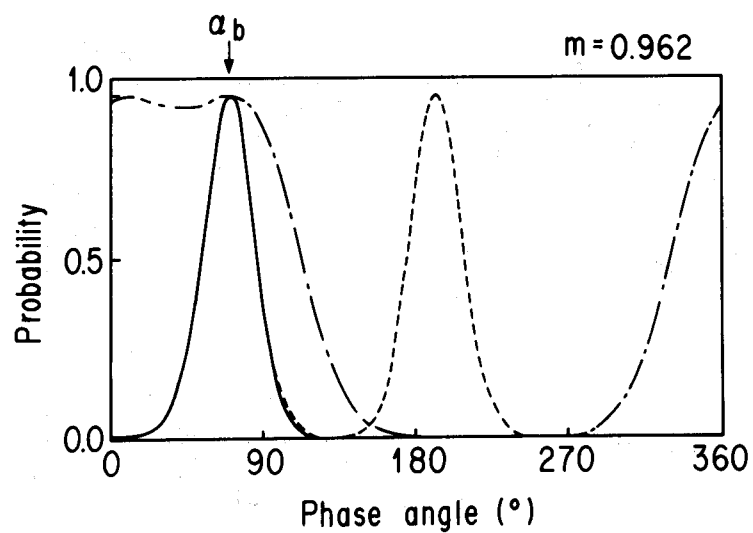
$$\epsilon_{real}(\alpha) = |F_P^A| - (|F_P^0|^2 + |\Delta f'|^2 - 2 \cdot |F_P^0| \cdot |\Delta f'| \cdot \cos(\phi - \alpha)) \quad (3-12)$$

$$P_{real}(\alpha) = \prod N \cdot \exp(-\epsilon_{real}^2(\alpha)/2E^2) \quad (3-13)$$

and imaginary part was given as,



a



b

Figure 3-11

An example of phase distribution of SIRA case

(a) $E_{ano} = E_{iso}$

(b) $E_{ano} = 1/3 E_{iso}$

Assumption of $E_{ano} = 1/3 E_{iso}$ gives
correct phase without ambiguity
(North, 1965)

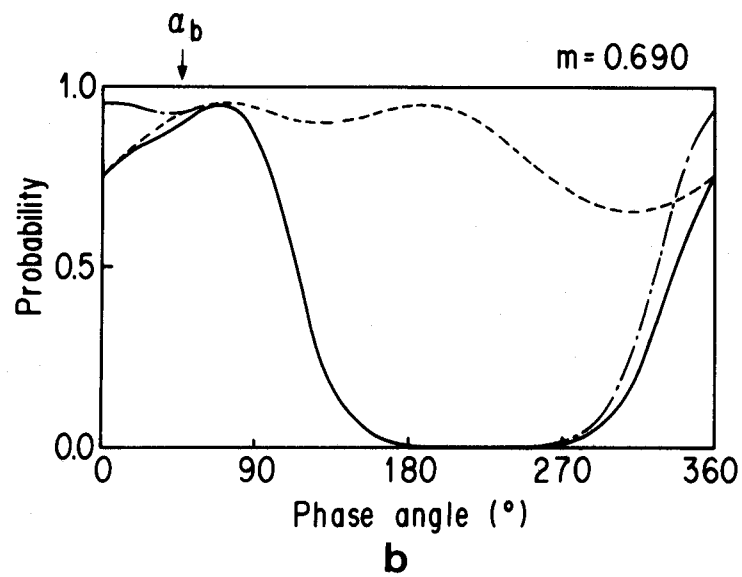
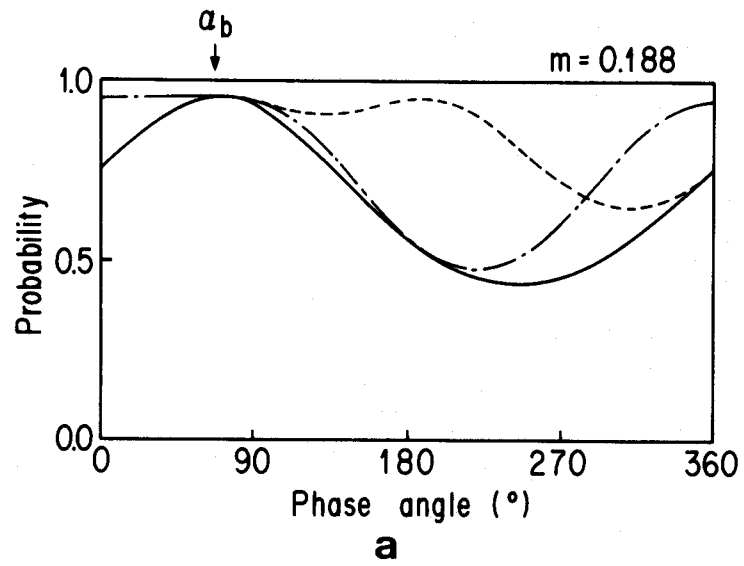


Figure 3-12

An example of phase distribution of MAD case

- (a) $E_{ano} = E_{iso}$
- (b) $E_{ano} = 1/3 E_{iso}$

Assumption of $E_{ano} = E_{iso}$ gives better result
than assumption of $E_{ano} = 1/3 E_{iso}$

$$\begin{aligned} \epsilon_{\text{imag}}(\alpha) = & -\Delta F_P(\pm) \\ & - (2 \cdot |F_P^0| \cdot |\Delta f''| / |F_P(\pm)| \cdot |\Delta f'|) \cdot (b \cdot \cos \alpha - a \cdot \sin \alpha) \end{aligned} \quad (3-14)$$

$$P_{\text{imag}}(\alpha) = \prod N \cdot \exp(-\epsilon^2_{\text{imag}}(\alpha)/2E^2) \quad (3-15)$$

$$\Delta F_P = |F_P^+| - |F_P^-|, \quad a = \Delta f' \cdot \cos \psi, \quad b = \Delta f' \cdot \sin \psi$$

Total phase probability distribution is given as the product of these terms as followings,

$$P(\alpha) = \prod P_{j, \text{real}}(\alpha) \cdot \prod P_{j, \text{imag}}(\alpha) \quad (3-16)$$

3-3-2. Refinement of Atomic Parameters

Scale factors for each data were determined from statistics between F_P^A and $F_P^0(1.380\text{\AA})$ (see Chapter 3-2-3).

Atomic parameters corresponded to data at 1.743 Å and 1.746 Å were refined by centric refinement technique. The real part of anomalous scattering term, $\Delta f'$, varies dramatically near the absorption edge, and it was very difficult to determine absolute wavelength and correct absorption edge using the instruments at BL-6A2. So in these refinement procedure, real part of anomalous scattering term, $\Delta f'$, was refined as one parameter of least-squares procedure. The least-squares procedure was used in the refinement program in the BOSS system (REFINE).

Atomic parameters corresponding to data at 1.380 Å were refined according to Hendrickson's procedure (Hendrickson *et al.*, 1981). Imaginary part, $\Delta f''$, does not vary dramatically, so it was not refined. The imaginary

part of anomalous scattering term was used the value calculated by Cromer and Liberman's method (Sasaki, 1984).

All atomic parameters are summarized in Table (3-7). These refined parameters for each data were treated as independent derivatives although these values should be same.

3-3-3. Phasing

The data sets collected at 1.380 Å, 1.743 Å and 1.746 Å were treated as three different derivatives, and best phases were calculated according to Blow and Crick's treatment (Blow and Crick, 1985) and the program PHASE in the BOSS system was used for phase calculation.

Phase probability distribution for the data set at 1.380 Å was calculated using only the imaginary part of anomalous difference term.

The data below 2.2 Å resolution were used for the best phase calculation because the phasing power, $\text{r.m.s.}|F_c|/\text{r.m.s.}|E|$, was greater than 1.0 below 2.2 Å resolution data. The mean value of the figure of merit at this resolution was 0.597 for 4995 independent reflections (93% of total reflections at 2.2 Å resolution). The result of phasing is summarized in Figure (3-13).

3-4. Model Building

The electron density map calculated using crystallographic phases

Wave length (Å)	x	y	z	B	$\Delta f'$
1.743	0.41311	0.76813	0.17569	9.33	-5.62
		$R = 0.586$		$\langle E \rangle = 10.01$	
1.746	0.41576	0.76628	0.17594	8.01	6.15
		$R = 0.599$		$\langle E \rangle = 11.40$	
1.380	0.41486	0.76709	0.17599	9.42	
				$\langle E \rangle = 7.15$	

Table 3-7

Atomic parameters of MAD procedure

Three different wavelength data are
treated as different derivatives

Parameters are refined against data
from 10.0 to 2.2 Å resolution

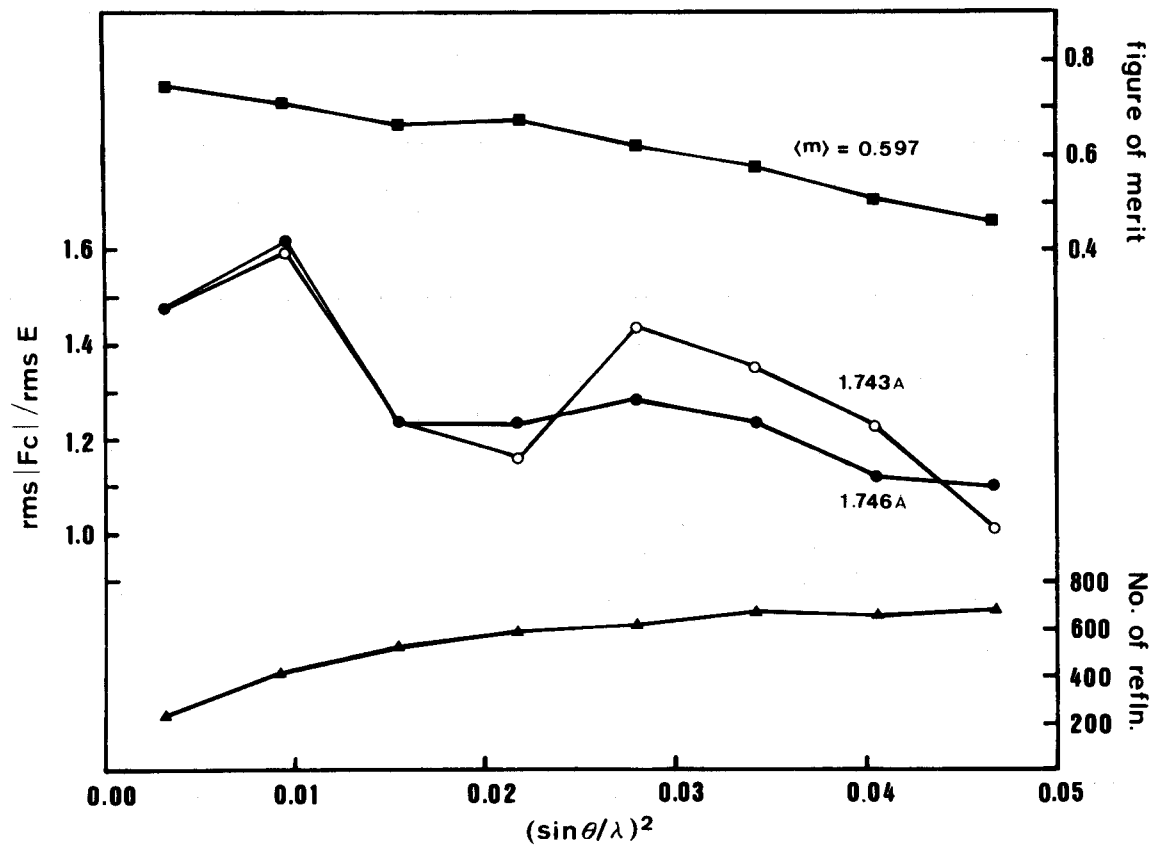


Figure 3-13

The radial distribution of the ratio of r.m.s. $|F_c|$ / r.m.s. E , figure of merit and number of reflections

- : Figure of merit
- : r.m.s. $|F_c|$ / r.m.s. $|E|$ of 1.743 Å data
- : r.m.s. $|F_c|$ / r.m.s. $|E|$ of 1.746 Å data
- ▲ : Number of reflections

determined by the multiple-wavelength anomalous dispersion method at 2.2 Å resolution had good quality to interpret main chain folding on the mini-map.

The model building procedure was carried out using the model fitting program FRODO (Jones, 1978) on the three-dimensional graphics display PS340, Evans & Sutherland Co.LTD. The author used the Rice university version of FRODO, PS300 FRODO.

Interpretation of the electron density map and model building procedure was not so difficult as many part of side chains were found on the electron density map. Only a few part showed very weak electron density and these part were difficult to assign atomic models. These region were constructed considering geometries of backbone atoms. Interpretation of $2F_o - F_c$ map calculated using atomic coordinates of the model showed unambiguous electron density for interpretation.

Figure (3-14) shows MAD-map which was used for model building.

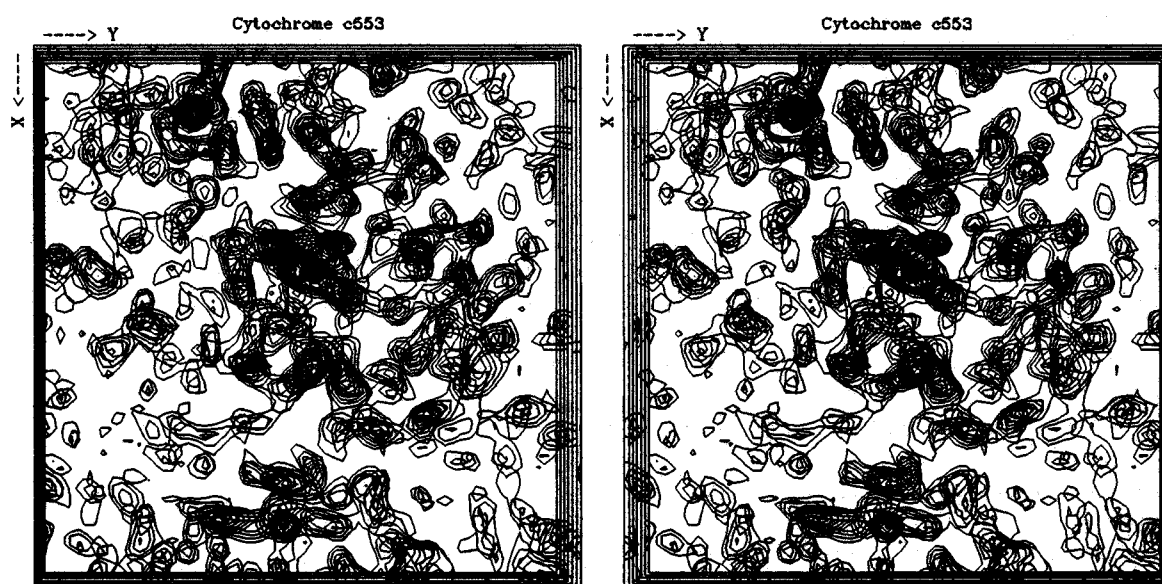


Figure 3-14

Electron density map using MAD phases

(a) Stacking map calculated by MAD method

The map was obtained from MAD phase information ($m=0.60$). This map is at 2.2 Å with contouring of levels at 20,30,40,... in arbitrary unit. Seven sections, taken at 0.99 Å intervals from section 15/104 to 21/104, are superimposed.

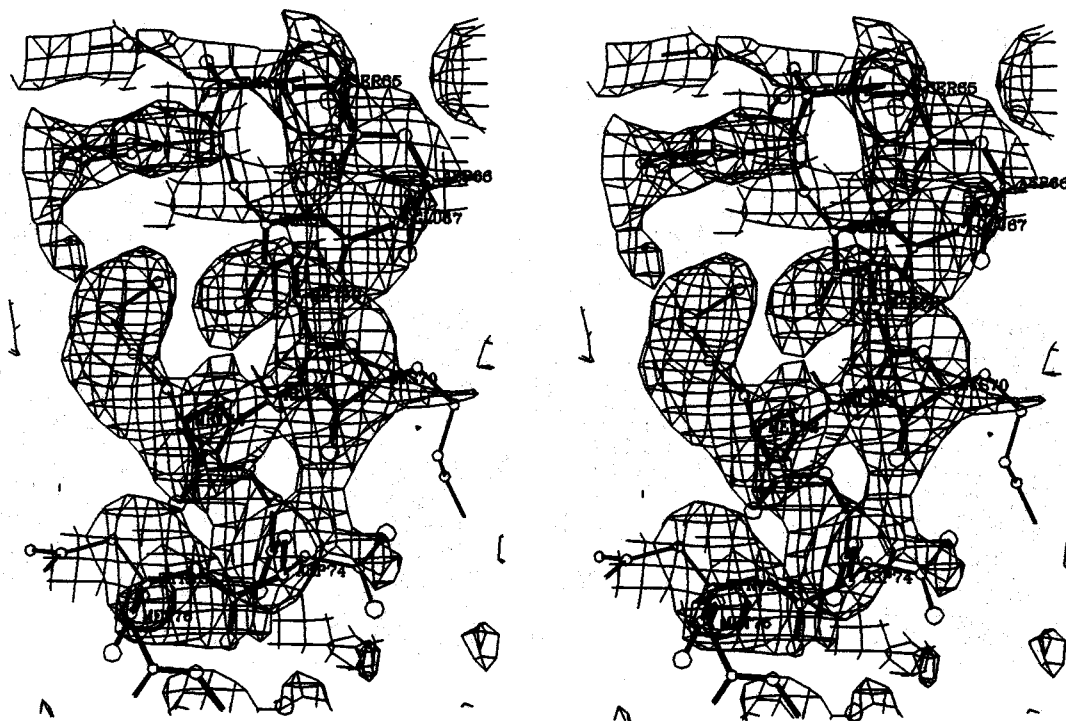


Figure 3-14
(continued)

(b) Electron density map around C-terminal helix

MAD map showing C-terminal helix, from Tyr64 to Tyr75, superimposed in MAD-map contoured at arbitrary level. Refined atomic coordinate ($R=0.226$) are superimposed. Intervals of contouring mesh is about 0.65 Å.

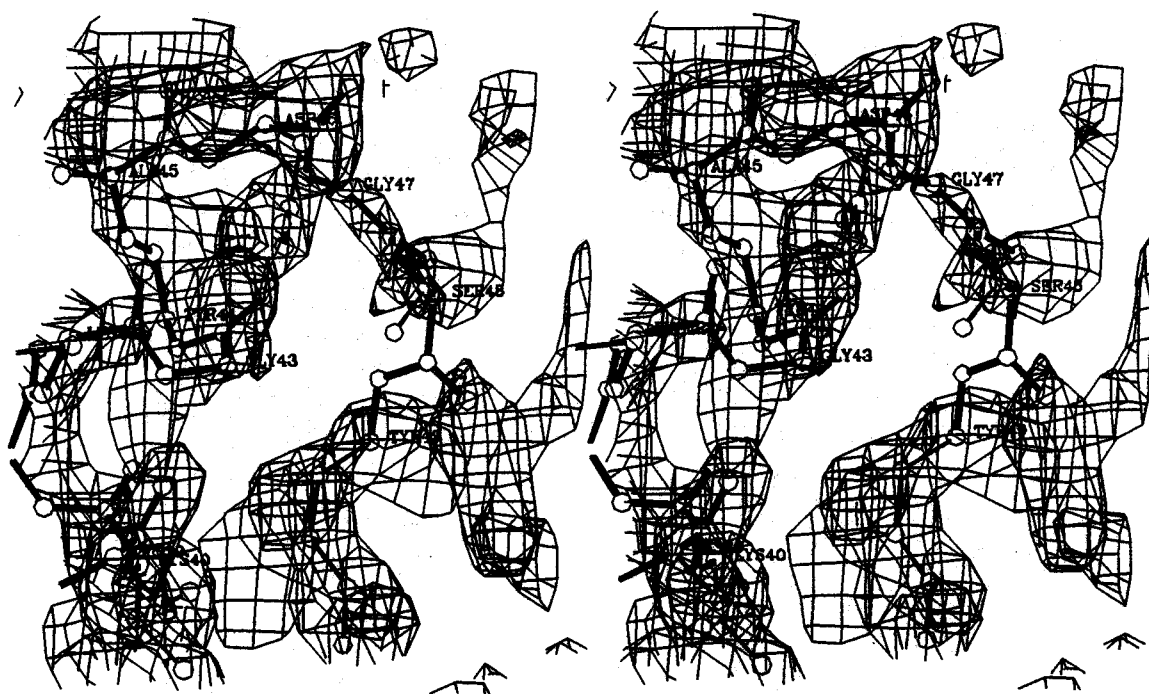


Figure 3-14
(continued)

(c) Electron density map shows the short loop
which is in the bottom of the molecule

MAD map showing the region from Lys42 to Tyr49 superimposed
in MAD-map contoured at arbitrary level. Refined atomic
coordinate ($R=0.226$) are superimposed. Intervals of contouring
mesh is about 0.65 Å. This region is the most difficult region
to make an atomic model.

4. REFINEMENT STAGE OF STRUCTURE ANALYSIS

4-1. Refinement

4-1-1. Theoretical Background

An atomic structure model which is build by the interpretation of the electron density map has some errors, such as misinterpretation of the map, fitting error to the electron density map, structure distortion, lack of solvent structure, disorder of atoms. It must be neccesary to refine the atomic parameters of the model.

In the X-ray structure analysis, observed structure factor, F_{obs} , is the only observation. So it is necessary to minimize the difference between observed and calculated structure factors.

For usual structure analysis of small molecules, the refinement proceeds to minimize the following quantity,

$$\epsilon^2 = \sum W_j \cdot |F_j(obs) - F_j(calc)| \quad (4-1)$$

where W_j is a weight for each reflections that is often used as the inverse of the deviation of the observed structure factor, $1/\sigma_F$. Number of observation should exceed number of parameters those are to be refined by a factor of ten.

However macromolecular crystal does not diffract towards so high Bragg

angle, and the number of observations might not be enough for normal refinement unlike small molecular crystallography. The ratio of observation via parameters ratio is about two at 2.0 Å resolution data, and that means the structure is underdetermined for the refinement.

Hendrickson and Konnert proposed the restrained parameter least-squares refinement procedure for macromolecular crystal structure analysis (Hendrickson and Konnert, 1980). Their program, PROLSQ, is one of the most famous and the most useful technique for refinement of macromolecular structure, and this method was applied to the refinement of the structure analysis of cytochrome c553.

This method minimize the following quantity,

$$\begin{aligned}
 \Phi = & \sum_j W_j^F \cdot |F_j^O - F_j^C|^2 \\
 & + \sum_j W_j^B \cdot |D_j^I - D_j^M|^2 \\
 & + \sum_j W_j^A \cdot |D_j^I - D_j^M|^2 \\
 & + \sum_j W_j^P \cdot |D_j^I - D_j^M|^2 \\
 & + \sum_j W_j^C \cdot |V_j^I - V_j^M|^2 \\
 & + \sum_j W_j^V \cdot |D_j^I - D_j^M|^2 \\
 & + \sum_j W_j^T \cdot |B_j^I - B_j^M|^2
 \end{aligned} \tag{4-2}$$

where the first term is the diffraction term which is the term to minimize in the refinement procedure for small molecular crystal, same function as the equation (4-1). The second term minimizes the deviation of bond distances from their ideal values. The third term minimizes the deviation of angle-related distances. The forth term minimizes the deviation of planarity. The fifth term minimizes the deviation of chiral center volumes.

The sixth term prevents the unreasonably short non-bonded contacts. The seventh term minimizes the unreasonably discrepancy between the thermal parameters of the neighboring atoms those are covalently bonded, and this is known as a "rigid motion".

All ideal values, those are shown as a superscript "I", are determined from small molecular crystal structures. The superscript "M" means the current value of the parameter of the present model. Superscripts "O" and "T" of the last term mean original and target temperature factors. And superscripts "O" and "C" of the first term means observed and calculated structure factors. The term "W" denotes the weighting factor for each terms.

The least-squares procedure is proceeded according to minimization of the summation of these parameters. It is obvious that minimization of Φ value is depends on the balance of weighting terms for these terms. It is necessary to reduce the discrepancy between observed and calculated structure factors, which is described as a crystallographic *R*-factor,

$$R = \frac{\sum |F_o - F_c|}{\sum |F_o|} \quad (4-3)$$

with stereo chemically reasonable values of other parameters.

In the usual refinement procedure of protein structure analysis, the least-squares refinement procedure often falls to local minimum. Thus it is necessary to be jumped out from a local minimum. It is important to revise the atomic model according to the residual Fourier maps, $(2F_o - F_c)$ or $(F_o - F_c)$ maps.

4-1-2. Refinement Survey

The starting atomic coordinates used for the refinement were derived from the model which was built by the interpretation of 2.2 Å electron density map calculated using MAD phases. At the end of each refinement stage, the $(2F_o - F_c)$ -map was calculated using the atomic coordinates those were derived at their stage, and misplaced fragments were manually corrected using the three-dimensional color graphics display and the model building program FRODO to be jumped out from the local minimum.

Sixty three water molecules are included in the last stage of the refinement. And finally, the crystallographic R value decreased to 0.226 using 10903 reflections between 6.0 and 1.6 Å resolution (83% of acceptable reflections) which have values grater than 3σ and also greater than 10. Disorder structure does not consider in this stage.

Weighting parameters at the final stage of the refinement were summarized in Table (4-1). Constrains for temperature factor variation does not applied at the final stage. Figure (4-1) shows the distribution of the final R factor against Bragg angle of reflections.

All atomic parameters are listed in Appendix B. Temperature factor of the carbonyl oxygen atom of Asp46 shows relatively high value, the omit-map of this region shows possibility of rotating movement of the peptide plane between Asp46 and Gly47.

Figure (4-2) shows a $2F_o - F_c$ map using the atomic parameters at the final stage, it shows same region as shown in Figure (3-15).

Restraints		σ	r.m.s deviation	No. of parameters
Distances (total)				1748
Bond distance	Å	0.015	0.025	643
Angle distance	Å	0.030	0.042	859
Planer distance	Å	0.040	0.058	244
Heme coordination	Å	0.020	0.128	2
Planar group	Å	0.020	0.019	553
Chiral group	Å ³	0.150	0.212	79
Non-bonded contacts (total)				470(2218)
Single-torsion	Å	0.500	0.170	204
Multiple-torsion	Å	0.500	0.256	205
Possible H-bond	Å	0.500	0.209	61
Torsion angles (total)				204(374)
Planar (ϕ) °		3.0	2.9	79
Staggered ($\pm 60, 180$) °		15.0	20.3	117
Orthonormal (± 90) °		20.0	17.3	8
Isotropic thermal factors (No restrain)				(Total) 1504
Main-chain bond	Å ²	-	8.985	365
Main-chain angle	Å ²	-	8.928	468
Side-chain bond	Å ²	-	15.319	278
Side-chain angle	Å ²	-	17.150	393

$R = 0.226$ (10903 refln.) 6.0 Å - 1.6 Å
 (F_{min}=10.0, F_{≥ 3σ})

Table 4-1

Weighting parameters for refinement of
 cytochrome c553 at the final stage of refinement

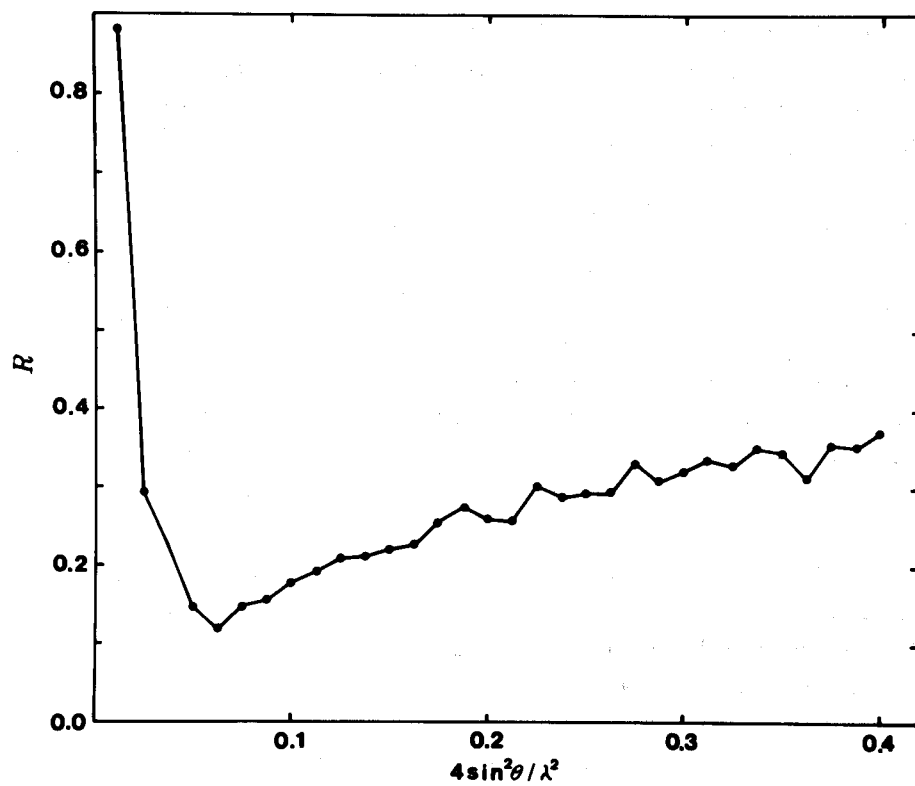


Figure 4-1

The final R factor versus $4(\sin\theta/\lambda)^2$

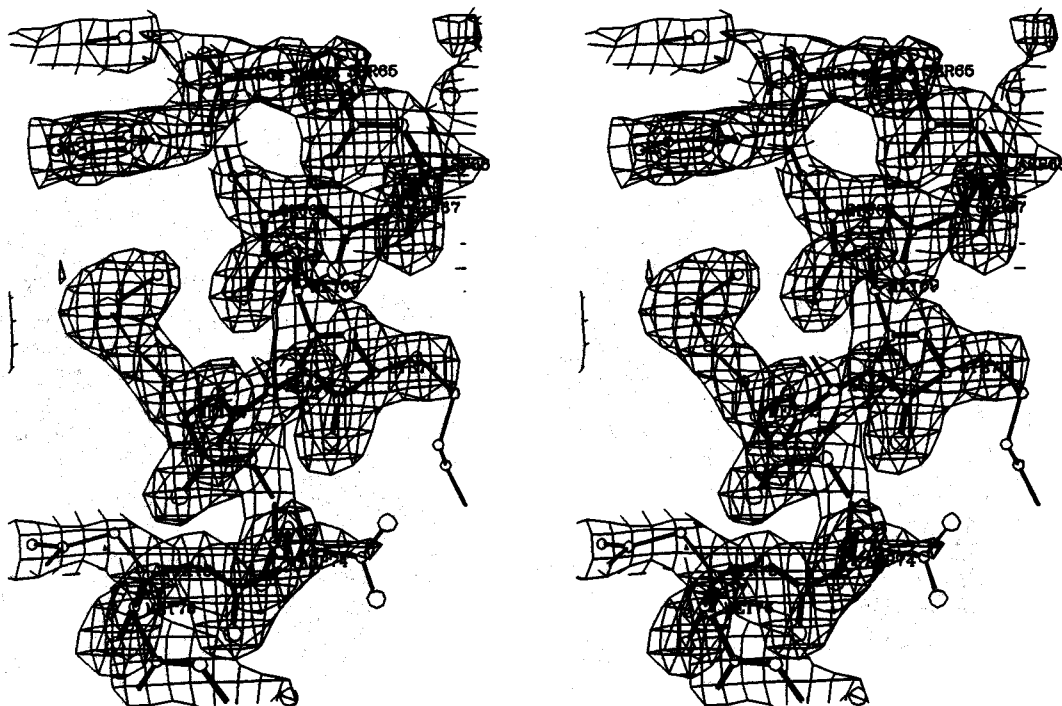


Figure 4-2

$2F_o - F_c$ map at the final stage
using 1.6 Å resolution data

(a) Electron density map around C-terminal helix

The map was obtained from calculated phase information ($R=0.226$) with coefficient of $2F_o - F_c$. Contouring level is arbitrary, and interval of contouring mesh is about 0.65 Å. (see, Fig(3-14(b)))

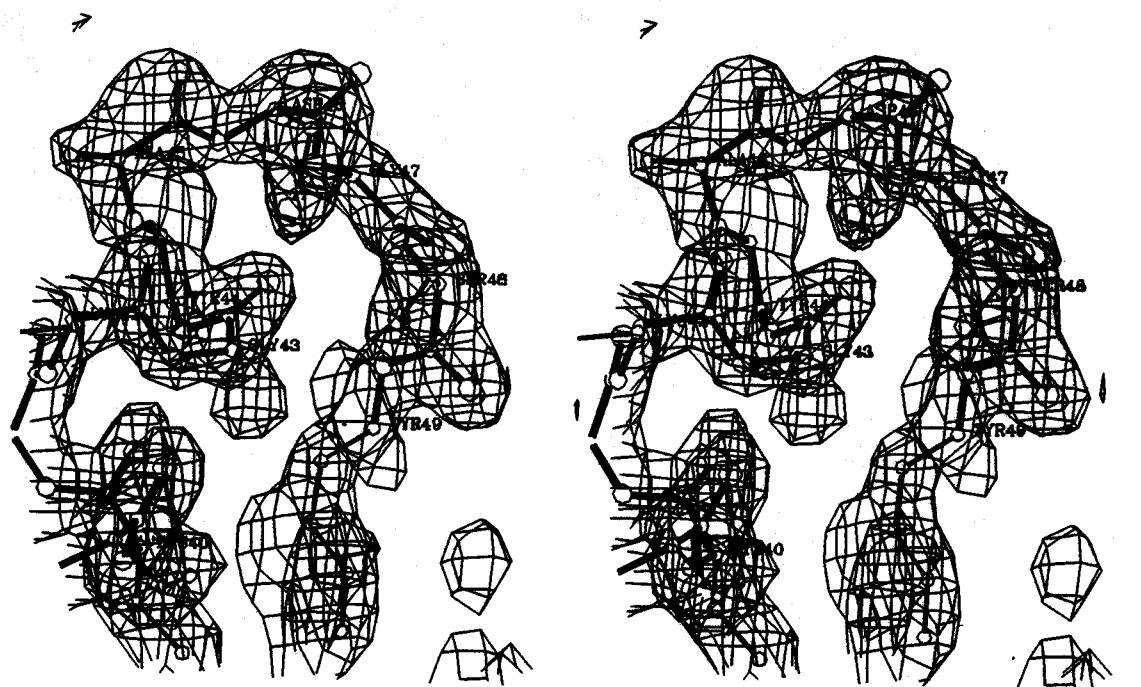


Figure 4-2
(continued)

(b) Electron density map shows the short loop
which is in the bottom of the molecule

The map was obtained from calculated phase information ($R=0.226$) with coefficient of $2F_o-F_c$. Contouring level is arbitrary, and interval of contouring mesh is about 0.65 Å. (see, Fig(3-14(c)))

5. RESULT AND DISCUSSION

5-1. Refined structure

5-1-1. Molecular Packing

Packing of molecules contacted to a molecule (MOL1) are shown in Figure (5-1). There are one molecule in an asymmetric unit, and it was supported by the calculated V_M value (see Chapter 2-1-2). The MOL1 is contacted to the other symmetry-related molecules by hydrogen bonds. Intermolecular contacts are listed in Table (5-1).

The closest intermolecular iron-iron distance is 18.9 Å (MOL1 to MOL3) and other intermolecular iron-iron distances which are shorter than 30.0 Å are 23.9 Å (MOL1 to MOL4) and 29.8 Å (MOL1 to MOL2, MOL1 to MOL5). The closest intermolecular iron-iron distances in ferricytochrome *c* and ferrocytochrome *c* from albacore, ferrocytochrome *c* from bonito and cytochrome *c5* from *Azotobacter vinelandii* are 24.2, 21.7, 22.2 and 16.4 Å, respectively (Higuchi *et al.*, 1984, Carter *et al.*, 1985). The closest iron-iron interaction of cytochrome *c5* is corresponding to dimer interaction of these molecules (Carter *et al.*, 1985). A cytochrome *c553* molecule, therefore, has relatively close heme-heme interactions within neighboring molecules.

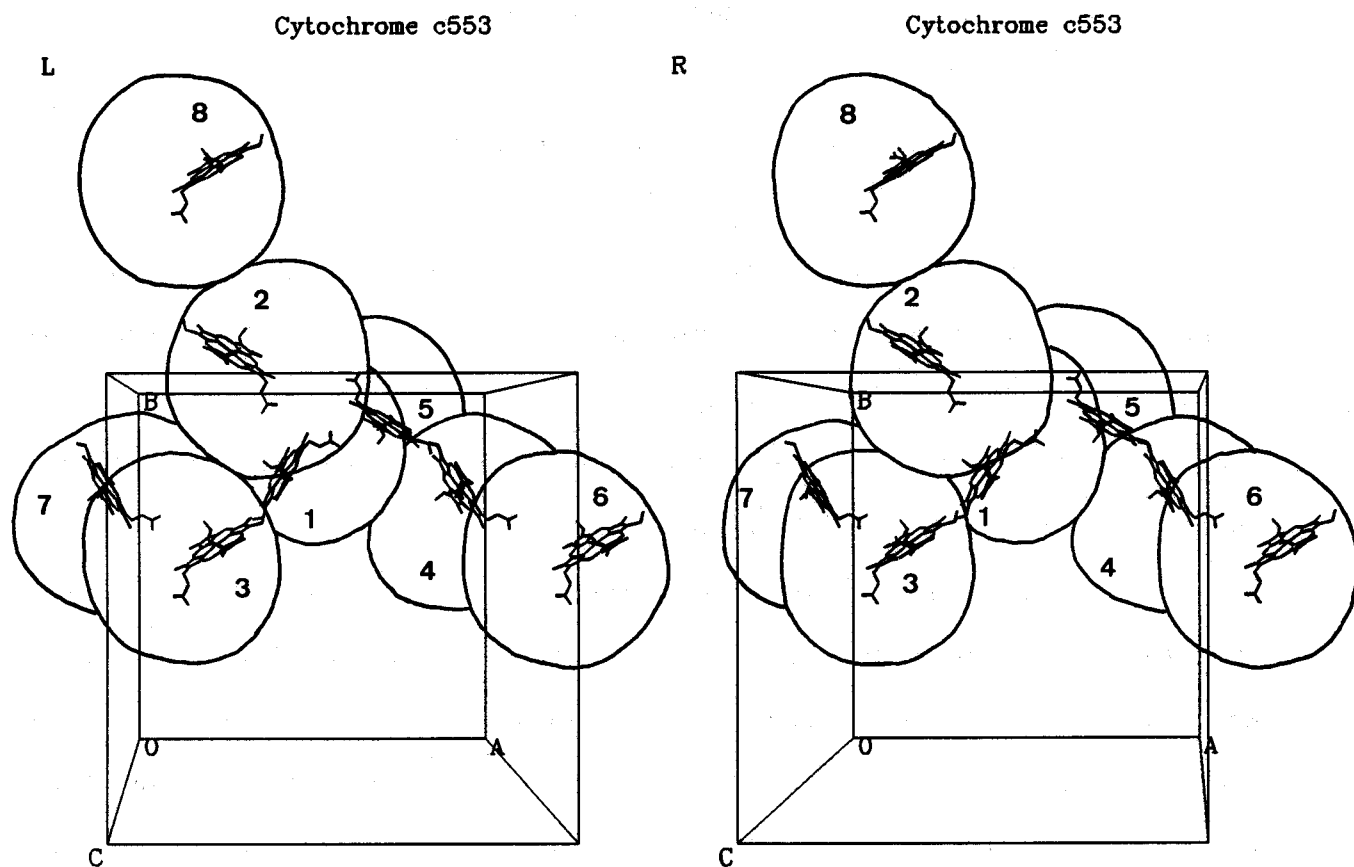


Figure 5-1

A schematic drawing of the crystal structure of cytochrome c553

The envelopes show boundaries of molecules

Source atoms			symmetry operation	target atoms			distance (Å)
Asp	2	Oδ1	SYM2	Lys	40	Nζ	2.72
Lys	8	O	SYM3	Wat	99	O	2.80
Ser	9	O	SYM3	Ser	9	O	3.10
Ser	9	O	SYM3	Wat	108	O	2.52
Gln	21	Nε2	SYM4	Lys	62	Nζ	2.84
Gln	21	Oε1	SYM4	Wat	87	O	3.07
Gly	24	O	SYM4	Lys	62	Nζ	2.72
Gly	26	O	SYM4	Asn	59	Nδ2	2.83
Gly	26	N	SYM5	Wat	105	O	3.07
His	27	Nε2	SYM5	Wat	83	O	3.02
Lys	30	Nζ	SYM6	Glu	67	Oε2	2.50
Lys	40	Nζ	SYM5	Asp	2	Oδ1	2.72
Gly	50	N	SYM5	Wat	96	O	2.94
Gly	51	O	SYM7	Wat	90	O	2.77
Asn	59	Nδ2	SYM7	Gly	26	O	2.83
Asn	59	Nδ2	SYM3	Wat	83	O	3.02
Asn	59	Oδ1	SYM3	Wat	124	O	2.59
Leu	60	Cδ2	SYM3	Wat	124	O	2.95
Lys	62	Nζ	SYM7	Gln	21	Nε2	2.84
Lys	62	Nζ	SYM7	Gly	24	O	2.72
Arg	63	O	SYM3	Arg	63	Nη1	3.01
Arg	63	Nη1	SYM3	Arg	63	O	3.01
Arg	63	Nη1	SYM3	Glu	68	Oε2	2.84
Arg	63	Nη2	SYM3	Glu	68	Oε1	2.67
Ser	65	Oγ	SYM2	Wat	85	O	2.84

Table 5-1

Intermolecular contacts

SYM1 x, y, z ; SYM2 $1/2+y, 1/2-x, 1/4+z$; SYM3 $-y, -x, 1/2-z$;
SYM4 $1/2+x, -1/2-y, 1/4-z$; SYM5 $1/2-y, -1/2+x, -1/4+z$;
SYM6 $1-y, -x, 1/2-z$; SYM7 $-1/2+x, -1/2-y, 1/4-z$;
SYM8 $-y, 1-x, 1/2-z$

These symmetry operations above are
corresponding to Figure (5-1)

Source atoms			symmetry operation	target atoms			distance (Å)
Glu	67	Oε ₂	SYM8	Lys	30	Nζ	2.50
Glu	68	Oε ₁	SYM3	Arg	63	Nη ₂	2.67
Glu	68	Oε ₂	SYM3	Arg	63	Nη ₁	2.84
Hem	80	O2D	SYM5	Wat	82	O	2.89
Wat	82	O	SYM2	Hem	80	O2D	2.89
Wat	82	O	SYM2	Wat	93	O	2.91
Wat	83	O	SYM2	His	27	Nε ₂	3.02
Wat	83	O	SYM3	Asn	59	Nδ ₂	3.02
Wat	85	O	SYM5	Ser	65	Oc	2.84
Wat	85	O	SYM5	Wat	103	O	2.73
Wat	86	O	SYM3	Wat	104	O	3.19
Wat	87	O	SYM7	Gln	21	Oε ₁	3.07
Wat	87	O	SYM3	Wat	105	O	3.11
Wat	89	O	SYM5	Wat	113	O	2.96
Wat	90	O	SYM4	Gly	51	O	2.77
Wat	93	O	SYM5	Wat	82	O	2.91
Wat	96	O	SYM2	Gly	50	N	2.94
Wat	98	O	SYM3	Wat	94	O	3.04
Wat	99	O	SYM3	Lys	8	O	2.80
Wat	99	O	SYM3	Wat	99	O	2.74
Wat	103	O	SYM2	Wat	85	O	2.73
Wat	104	O	SYM3	Wat	86	O	3.19
Wat	105	O	SYM2	Gly	26	N	3.07
Wat	105	O	SYM3	Wat	87	O	3.11
Wat	108	O	SYM3	Ser	9	Oη	2.52
Wat	113	O	SYM2	Wat	89	O	2.96
Wat	113	O	SYM2	Wat	141	O	3.09
Wat	115	O	SYM3	Wat	115	O	2.54
Wat	118	O	SYM2	Wat	123	O	2.99
Wat	123	O	SYM5	Wat	118	O	2.99
Wat	124	O	SYM3	Asn	59	Oδ ₁	2.59
Wat	124	O	SYM3	Leu	60	Cδ ₂	2.95
Wat	124	O	SYM3	Wat	94	O	3.02
Wat	132	O	SYM3	Wat	92	O	3.08
Wat	141	O	SYM5	Wat	113	O	3.09

Table 5-1
(continued)

5-1-2. Secondary structure, Main chain folding and Heme packing

Figure (5-2(a)) shows main chain atoms(C,N,O,C α ,OXT), heme group atoms and side chain atoms bonded to heme group (Cys10, Cys13, His14, Met57) viewed along z-axis of crystallographic unit cell. N-terminus is located at the top of the molecule, and heme group is nearly perpendicular to the paper.

Figure (5-2(b)) shows same atoms as Figure (5-2(a)), but the view point is different from that in Figure (5-2(a)). This direction of view is always used to show a molecular structure of cytochromes c. The heme group is located perpendicular to the paper. N-terminus is located at the top of the molecule on this drawing and N-terminal six residues keep α -helical conformations. C-terminal region is located behind the molecule. C-terminal thirteen residues also have α -helical conformations, and C-terminus terminates at the right-side of the molecule in this figure.

Figure (5-3) shows all atoms except for solvent molecules from the same view point of Figure (5-2(b)). All side chain atoms are colored according to the kind of residues. Magenta shows acidic and hydrophilic residues, eg. aspartic acid and glutamic acid. Cyan shows basic and hydrophilic residues, eg. histidine, lysine and arginine. Red shows aromatic and hydrophobic residues, eg. phenylalanine and tyrosine. And red also shows heme group. Yellow shows aliphatic and hydrophobic residues, eg. leucine, methionine and valine. Green shows neutral residues, eg. asparagine, glutamine, cysteine, threonine, serine, alanine and glycine.

As shown in Figure (5-4), this cytochrome c₅₅₃ has many residues those conformations are α -helix type.

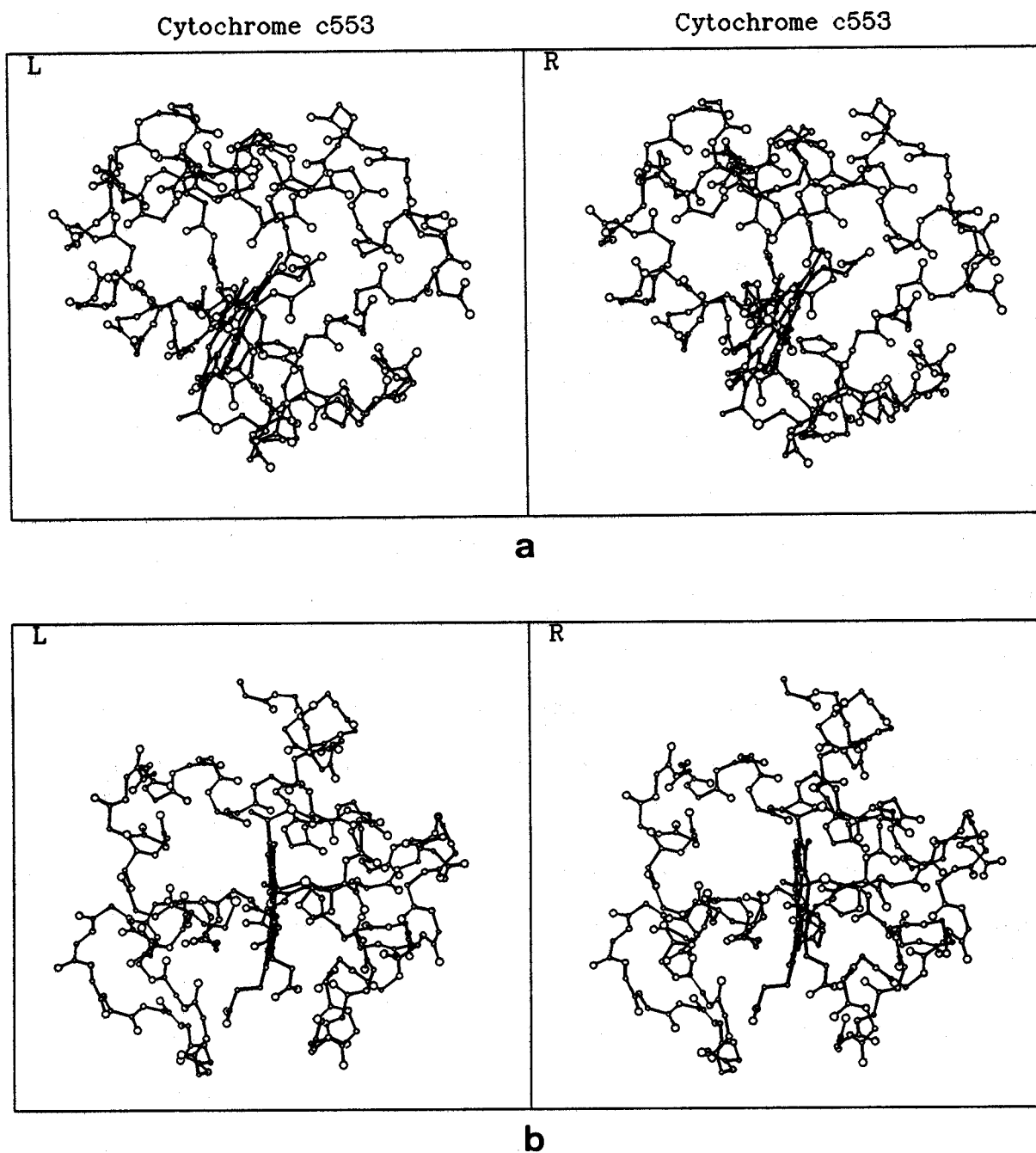


Figure 5-2

Folding pattern of cytochrome c553

Showing main chain, heme group and side chain atoms which bond to heme group (Cys10, Cys13, His14, Met57)

- (a) Viewing along crystallographic z-axis
- (b) Viewing from the heme cleavage

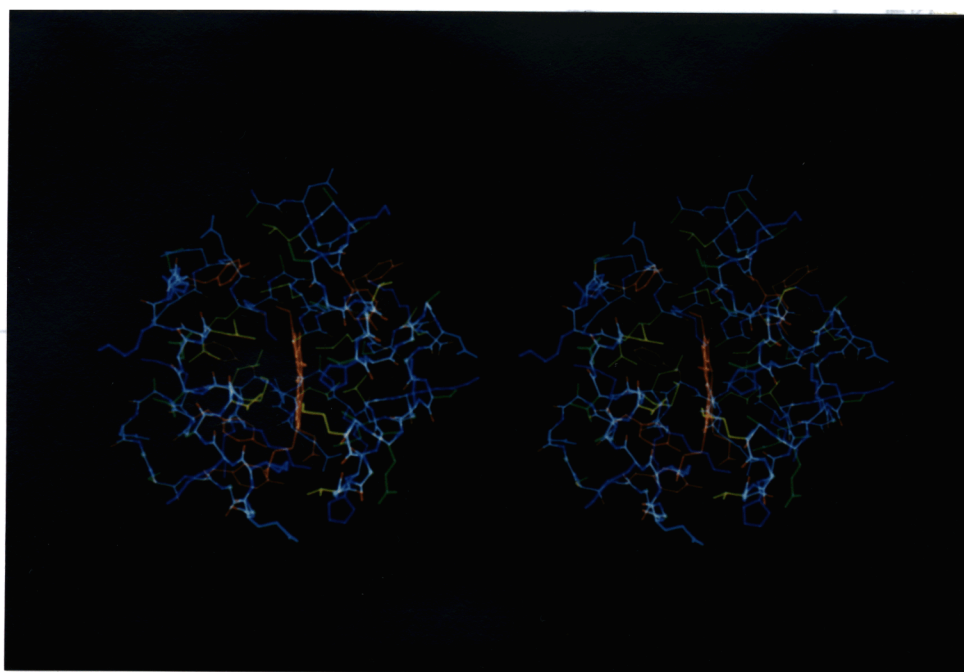


Figure 5-3

Wire-model of cytochrome c553 showing all atoms
except for water molecules

Blue : Lys, Arg, His ; Cyan : Asp, Glu
Red : Phe, Tyr, Hem ; Yellow : Leu, Met, Val
Green : Asn, Gln, Cys, Thr, Ser, Ala, Gly

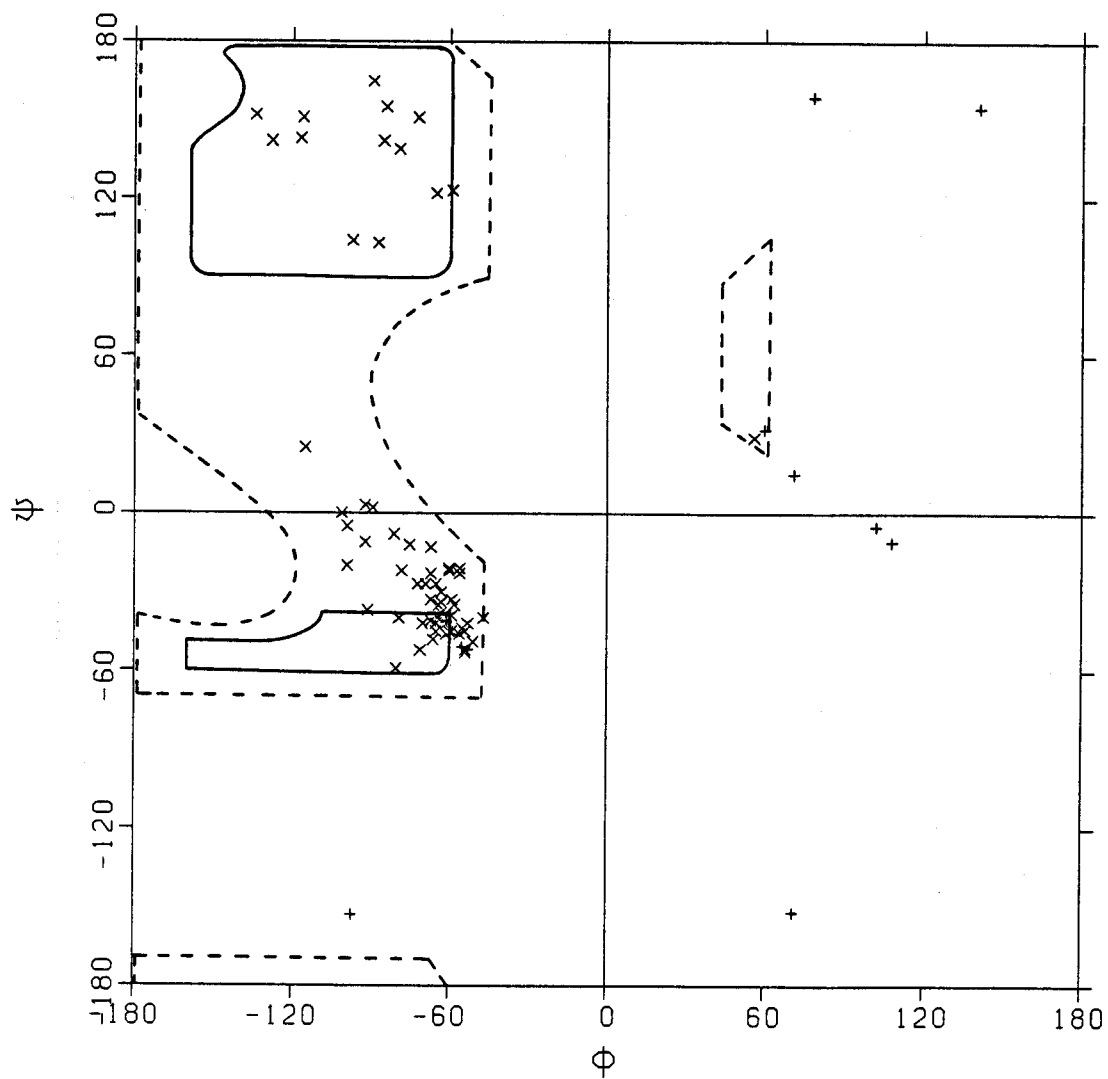


Figure 5-4

Ramachandran's plot of cytochrome c553
(Ramakrishnan and Ramachandran, 1965)

+ Glycine residue
x Other residue

Considering hydrogen bonds and conformational torsion angles of main chain atoms, following nine secondary structures were identified.

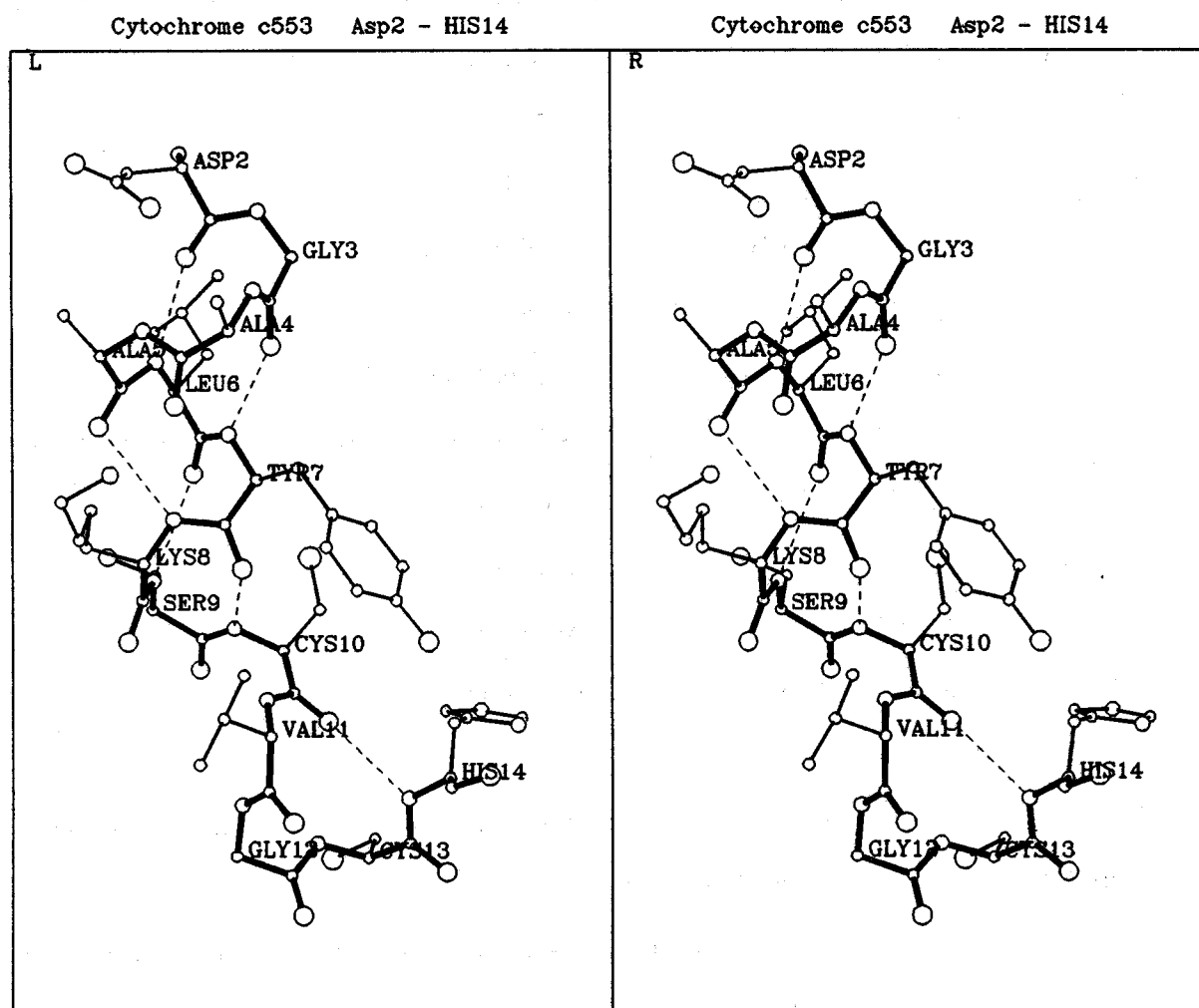
·Asp2 - Tyr7	α -helix
·Ala5 - Cys10	3.10-helix
·Val11 - His14	α -helix
·Gly15 - Gly18	β -turn(type I)
·Gln21 - Val25	left-handed 3.10-helix (β -turn(type III))
·Lys33 - Gly47	α -helix
·Lys54 - Asn59	α -helix
·Ser65 - Ser77	α -helix
·Met76 - Leu79	β -turn(type I)

Above secondary structures with showing hydrogen bonds are shown in Figure (5-5(a)) to Figure (5-5(g)).

The classification of β -turns above is according to the classification proposed by Chou and Fasman (Chou and Fasman, 1977). Two β -turns are identified in cytochrome c553 molecule, and they keep type I conformation.

There are five α -helices and two 3.10-helices, one is right-handed and the other is left-handed, in cytochrome c553 molecule as shown above. Forty five of seventy nine residues have at least one hydrogen bond of a carbonyl or amido group in an α -helix conformation. There are no β -sheet conformation in this molecule.

Two α -helices of N- and C-terminal residues, large cleavage at the center of the molecule where heme group is located and overall main chain folding pattern shows "cytochrome c folding".

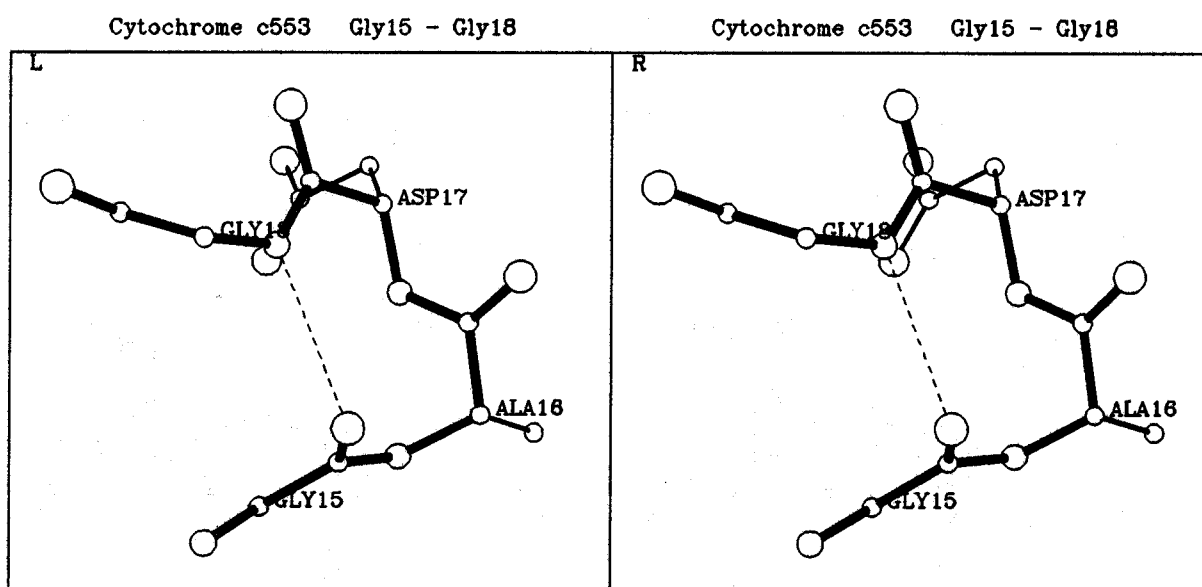


(a) Asp2 - His14

Figure 5-5

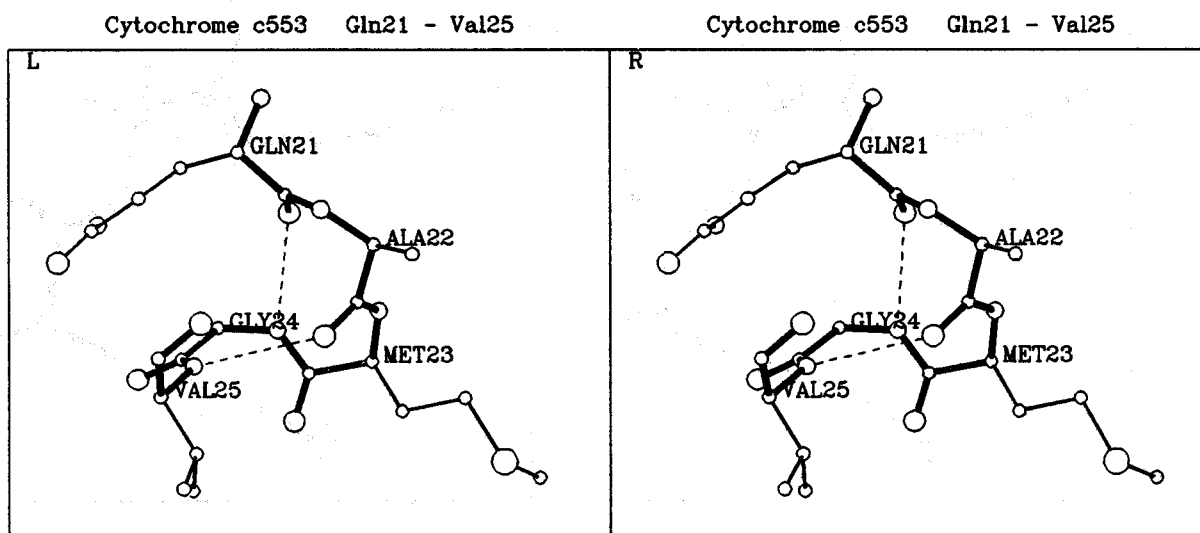
Identified secondary structures of cytochrome c553

Dashed lines show hydrogen bonds



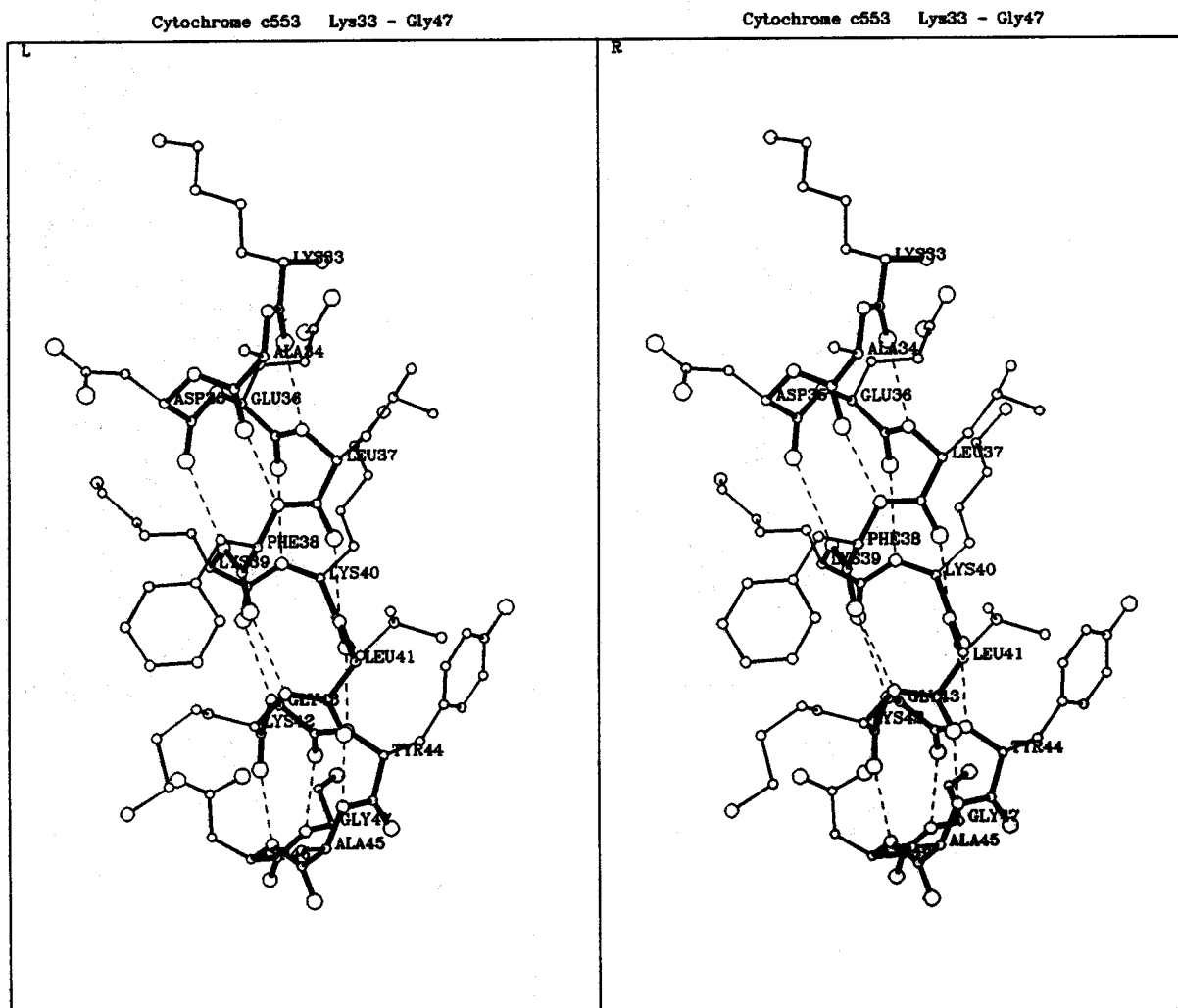
(b) Gly15 - Gly18

Figure 5-5
(continued)



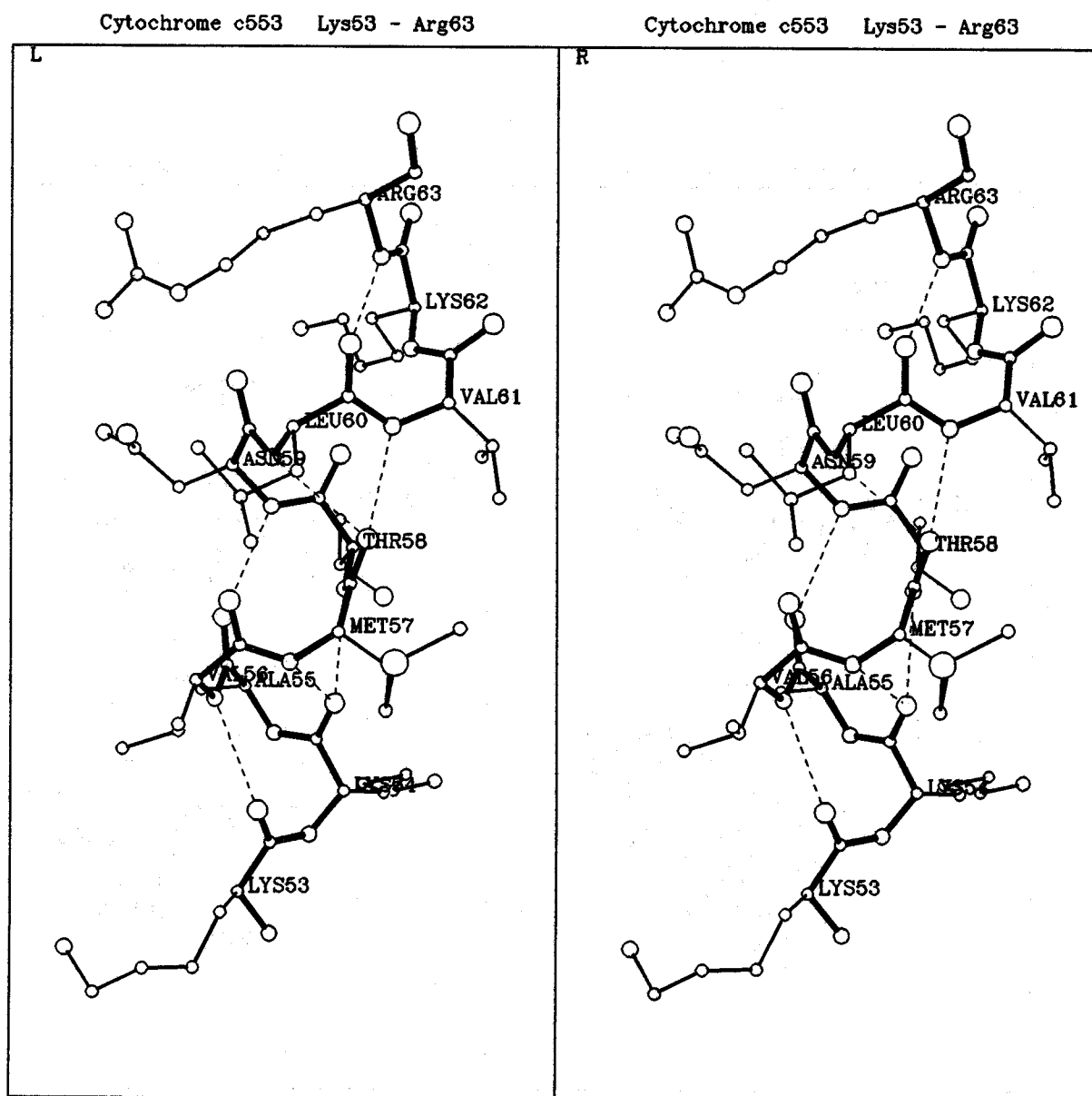
(c) Gln21 - Val25

Figure 5-5
(*continue*)



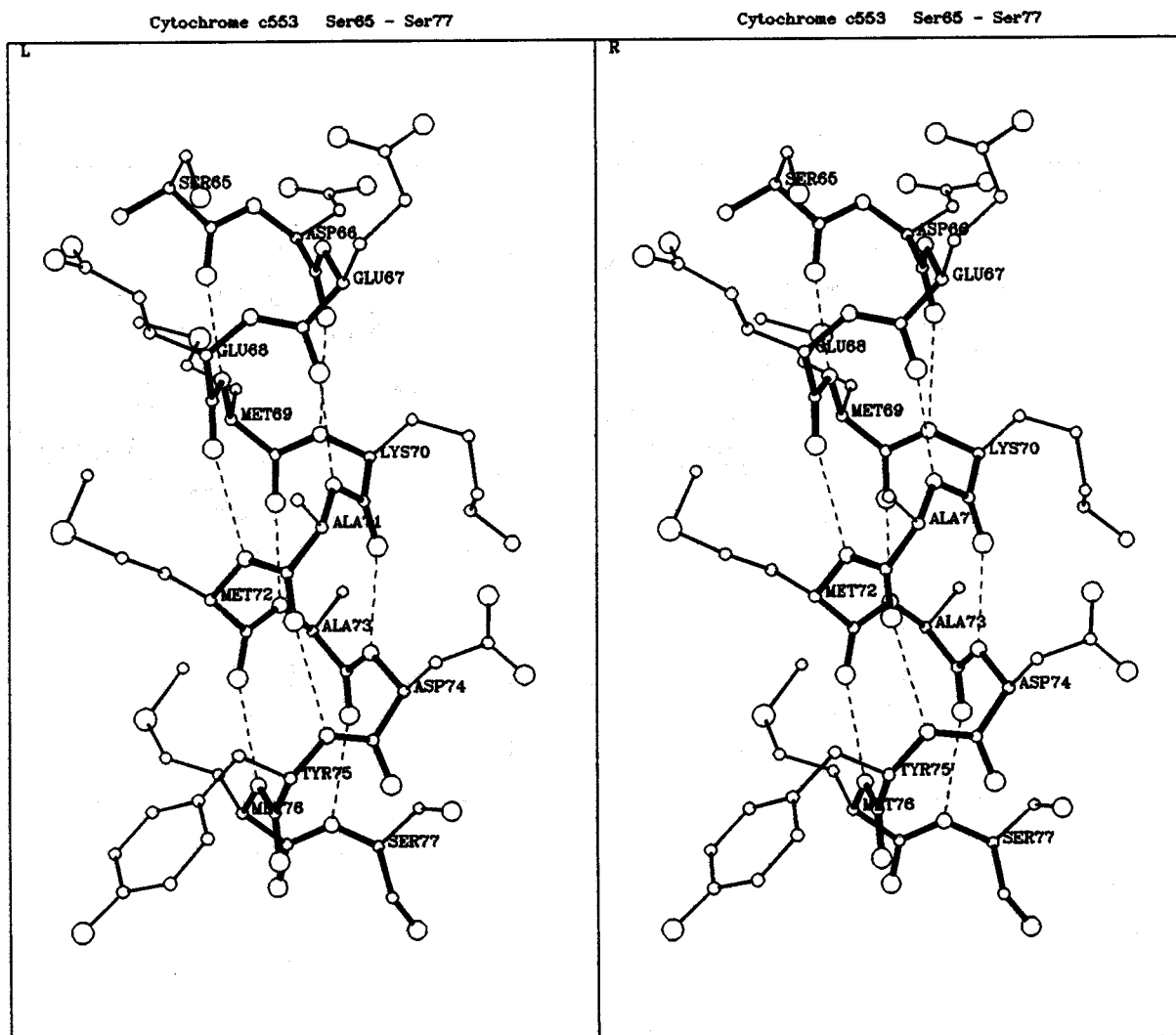
(d) Lys33 - Gly47

Figure 5-5
(continued)



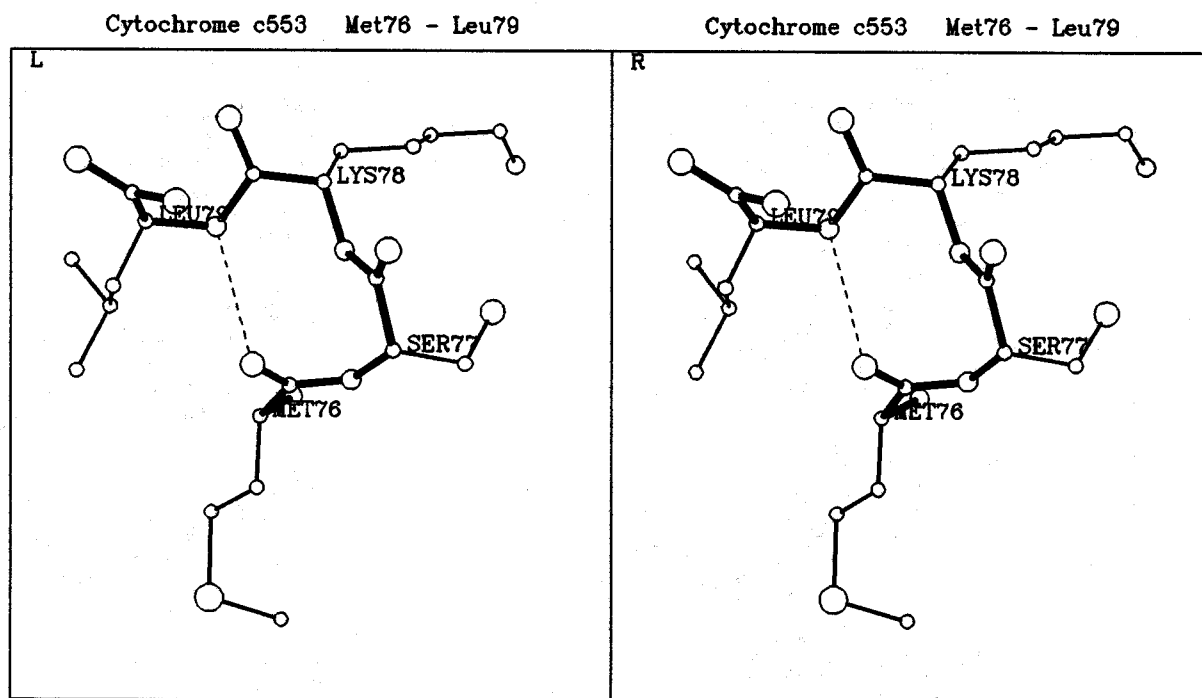
(e) Lys53 - Arg63

Figure 5-5
(continued)



(f) Ser65 - Ser77

Figure 5-5
(continued)



(g) Met76 - Leu79

Figure 5-5
(continued)

The heme group is covalently bonded to amino acid chain with two residues and coordinated with two residues, just same as other cytochromes *c*. Two reduced vinyl groups of the heme group are bonded to Cys10(S γ to CAB) and Cys13(S γ to CAC). The fifth coordinate of iron of heme is His14(N ϵ 2) and the sixth coordinate is Met57(S δ). The propionate side-chains of the heme group make hydrogen bond to polypeptide chain and are fixed.

Main chain folding pattern was summarized as follows,

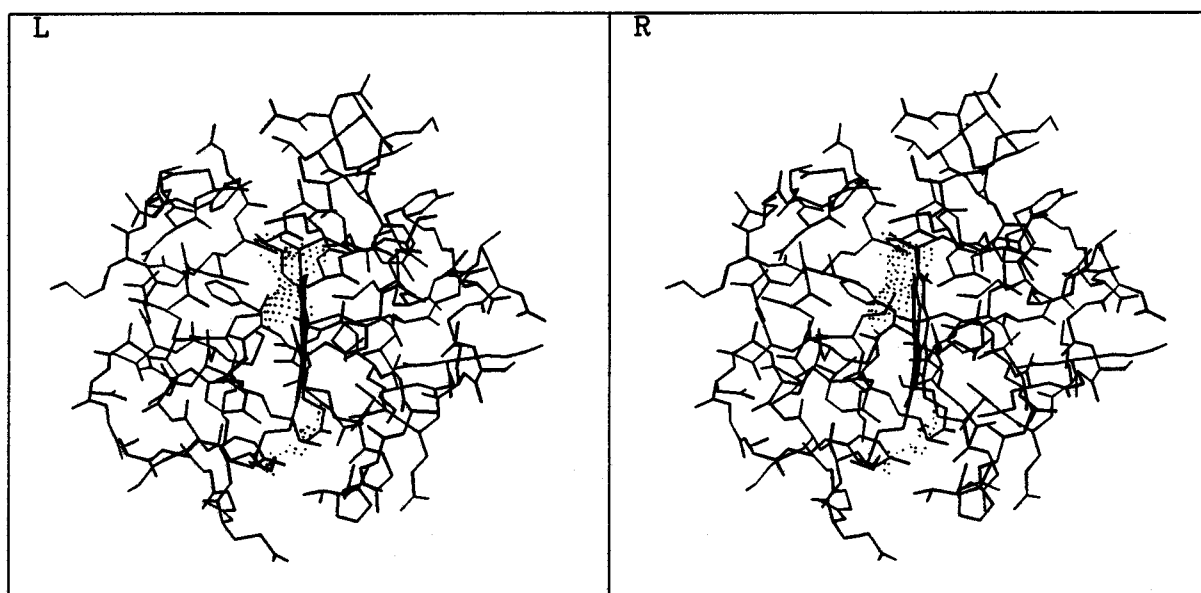
1. N-terminal α -helix which goes down from the top of the molecule
(Ala1 to Tyr7)
2. Covalently bonds to reduced vinyl groups of the heme group by
S γ atoms of cysteins
(Cys10 and Cys13)
3. Coordinates to heme iron by N ζ atom of histidine as the fifth
coordinate
(His14)
4. Small loop at the right side of the molecule
(Gly15 to Gly18)
5. Goes down to the right bottom, and makes loop,
covering right bottom of the molecule
(Lys20 to Val29)
6. Goes across the behind of the molecule
(Lys30 to Gln32)
7. α -helix at the left-back side of the molecule,
covering the back of the molecule

- (Lys33 to Gly47)
8. The loop covering left-bottom side of the molecule
(Ser48 to Lys53)
9. Short distorted α -helix covering left side of the molecule,
and coordinates to heme iron by S δ of methionine 57
(Cys54 to Asn59)
10. Short extended conformation
(Val61 to Tyr64)
11. C-terminal α -helix from left side to right side of
the behind of the molecule
(Ser65 to Leu79)

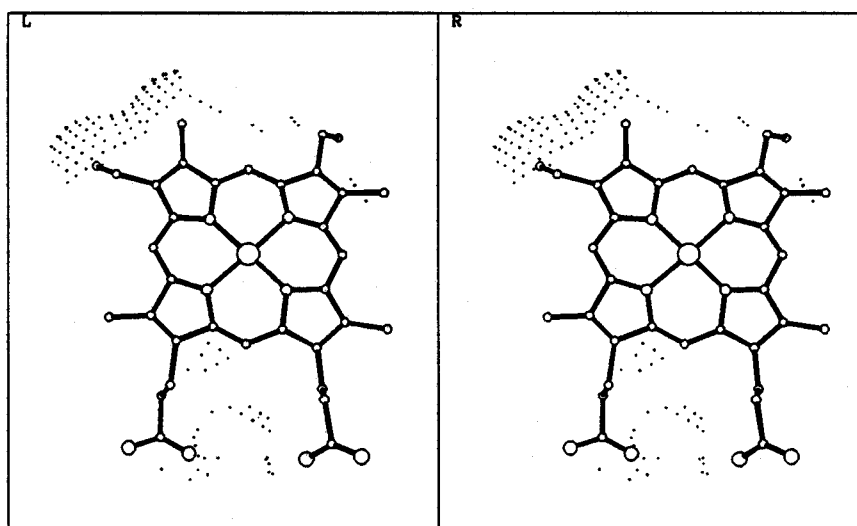
5-1-3. Heme Environment

The heme group is covalently bonded to the polypeptide chain with four residues same as other cytochromes *c*. The heme group is displaced at the center of the molecule and some part of the heme group is expressed to the molecular surface. Figure (5-6) showed contact and re-entrant surface area of heme group (Conolly, 1983). There are three regions of heme group those are exposed to molecular surface. Two propionates are both exposed, the side chain of pyrrole D is more exposed than that of pyrrole A. Both propionates are fixed by hydrogen bonds. These hydrogen bonds are summarized in Table (5-2).

Senn *et al.* suggested the conformation of the side chain of Met57 of reduced form of cytochrome *c*553 from *Desulfovibrio vulgaris* using NMR tech-



a



b

Figure 5-6

Contact and reentrant surface area
of heme group

Propionate A

O1A	Gln32 Nε2	2.72 Å
	Lys40 Nζ	2.66
O2A	Tyr49 Oη	2.69
	Wat93 O	2.65

Propionate D

O1D	Lys53 N	2.86
	Lys54 N	3.08
O2D	Tyr49 Oη	2.69
	Gly51 N	2.75

Table 5-2

Possible hydrogen bonds to heme propionates

nique (Senn *et al.*, 1983). Figure (5-7(a)) and (5-7(b)) showed predicted and determined conformations of the side chain atoms of Met57. Although over all conformations of both model are similar to each other, positions of C β and C γ of Met57 are relatively different.

5-1-4. Hydrogen Bonds

Intramolecular hydrogen bonds, excluding water molecules, are listed in Table (5-3). There are ninety five hydrogen bonds within the range from 2.3 Å to 3.2 Å. The bonds are categorized into four groups : main chain - main chain, main chain - side chain, side chain - side chain, heme - other residue.

In Table (5-3(a)), hydrogen bonds in α -helices and β -turns are marked by (α), (T), respectively. There are three salt-bridges, marked by (S) in Table (5-3(c)) between the lysine N ζ moiety and the carboxyl groups of aspartic or glutamic acids on the molecular surface.

5-1-5. Water Molecules

Sixty three peaks showed on both MAD-map and D-Fourier map were assigned to water molecules. These waters are incorporated in the refinement procedure. The temperature factors of water molecules are in the range from 19.2 to 77.2 Å². Twelve water molecules have temperature factors greater than 50 Å². All of these water molecules are assumed as fully occupied sol-

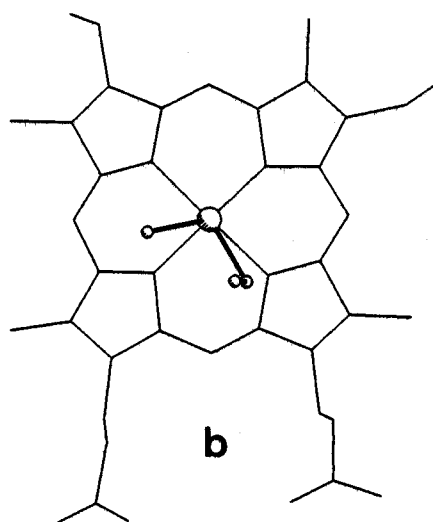
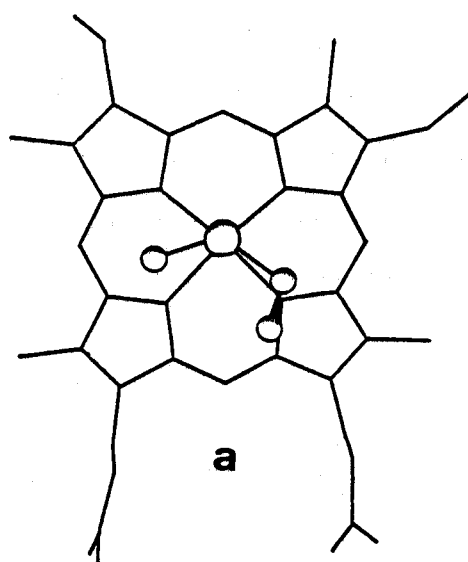


Figure 5-7

Heme group and axial methionine of cytochrome c553

(a) predicted conformations
(Senn *et al.*, 1983)

(b) determined conformations

(a) Main chain - main chain interactions

D 2 O	A 5 N	3.15	D 2 O	L 6 N	2.95 (α)
G 3 O	Y 7 N	2.89 (α)	L 6 O	K 8 N	3.08
L 6 O	S 9 N	3.22 (α)	Y 7 O	S 9 N	3.05
Y 7 O	C 10 N	2.85 (α)	C 10 O	H 14 N	3.06
V 11 O	G 15 N	3.02	G 15 O	G 18 N	3.10
G 18 O	V 29 N	3.18	G 18 O	K 30 N	3.12
K 20 O	A 22 N	3.20	Q 21 O	M 23 N	3.17
Q 21 O	G 24 N	3.03	A 22 O	G 24 N	3.01
A 22 O	V 25 N	3.20	K 33 O	D 35 N	3.18
K 33 O	E 36 N	2.84	K 33 O	L 37 N	2.93 (α)
A 34 O	E 36 N	3.24	A 34 O	F 38 N	2.83 (α)
D 35 O	K 39 N	2.99 (α)	E 36 O	K 40 N	3.12 (α)
L 37 O	L 41 N	3.02 (α)	F 38 O	L 41 N	3.19
F 38 O	K 42 N	3.04 (α)	K 39 O	G 43 N	2.79 (α)
K 40 O	G 43 N	2.96	K 40 O	Y 44 N	2.97 (α)
L 41 O	Y 44 N	3.19	K 42 O	A 45 N	2.94
K 42 O	D 46 N	3.11 (α)	G 43 O	G 47 N	2.85 (α)
G 43 O	S 48 N	3.00	G 43 O	Y 49 N	3.14
K 53 O	A 55 N	3.10	K 53 O	V 56 N	3.03
K 54 O	V 56 N	3.18	K 54 O	M 57 N	2.99
K 54 O	T 58 N	3.06 (α)	A 55 O	N 59 N	3.18 (α)
M 57 O	L 60 N	3.08	M 57 O	V 61 N	2.94 (α)
L 60 O	K 62 N	3.08	L 60 O	R 63 N	2.87
V 61 O	R 63 N	3.14	S 65 O	E 68 N	3.09
S 65 O	M 69 N	2.76 (α)	D 66 O	M 69 N	3.19
D 66 O	K 70 N	3.05 (α)	E 67 O	A 71 N	2.88 (α)
E 68 O	M 71 N	3.17	E 68 O	M 72 N	2.89 (α)
M 69 O	M 72 N	3.08	M 69 O	A 73 N	2.72 (α)
K 70 O	A 73 N	3.24	K 70 O	D 74 N	2.80 (α)
A 71 O	Y 75 N	3.11 (α)	M 72 O	M 76 N	2.79 (α)
A 73 O	S 77 N	2.95 (α)	Y 75 O	S 77 N	3.22
Y 75 O	K 78 N	2.95	M 76 O	K 78 N	3.10
M 76 O	L 79 N	2.93			

Table 5-3

Intramolecular hydrogen bonds

(b) Main chain - side chain interaction

G 12 O	K 20 N ζ	3.01	A 5 N	D 2 O γ_2	3.17
G 15 O	Y 7 O ζ	2.60	G 26 O	Q 21 O ϵ_1	2.84
A 28 O	Q 32 N ϵ_2	3.06	K 42 O	D 46 O δ_2	3.05
G 43 O	D 46 O δ_2	2.53	Y 44 O	T 58 O γ_1	2.52
G 47 N	D 46 O δ_2	3.14	G 47 O	K 54 N ζ	3.23
S 48 N	D 46 O δ_2	3.23	N 59 O	R 63 N ϵ	3.02
E 68 N	S 65 O γ	3.18	S 65 N	E 68 O ϵ_2	2.95
K 70 O	D 74 O δ_2	3.06	D 74 O	S 77 O γ	2.69

(c) Side chain - side chain interaction

D 17 O δ_1	S 19 O γ	2.90	Q 32 O ϵ_1	E 36 O ϵ_2	3.06
Q 32 O ϵ_1	K 40 N ζ	2.74 (S)	K 42 N γ	D 66 O δ_1	3.23 (S)
K 42 N γ	D 66 O δ_2	3.02 (S)	D 46 O δ_2	S 48 O γ	3.10
N 59 O δ_1	R 63 N ζ_2	3.12			

(d) heme - other residue interaction

X 80 O1A	Q 32 N ϵ_2	2.72	X 80 O1A	K 40 N ζ	2.66
X 80 O2D	Y 44 O γ	2.51	X 80 O2A	Y 49 O γ	2.69
X 80 O2D	G 51 N	2.75	X 80 O1D	G 53 N	2.86
X 80 O1D	K 54 N	3.08			

Table 5-3
(continued)

vent molecules, and disordered water molecules are not included in this model. Table (5-4) shows hydrogen bonds which are bonded to water molecules. Sixty four hydrogen bonds between water molecule and protein molecule are identified.

5-2. Comparion of the Sturucture of Cytochrome *c* families

5-2-1. Folding Pattern and Charge Distribution

Figure (5-8(a)) to Figure (5-8(f)) show folding patterns of six different² cytochromes *c*. They are cytochrome *c*₂ (2C2C; Salemme *et al.*, 1973), as an example of L-class cytochrome *c*, cytochrome *c* from albacore (3CYT; Takano and Dickerson, 1980), as an example of M-class mitochondrial cytochrome *c*, cytochrome *c* from rice (1CCR; Ochi *et al.*, 1983), as an example of plant cytochrome *c*, cytochrome *c*₅₅₁ from *Pseudomonas aeruginosa* (351C; Matsuura *et al.*, 1982), as an example of S-class cytochrome *c*, cytochrome *c*₅ from *Azotobacter vinelandii* (1CC5; Carter *et al.*, 1985) and cytochrome *c*₅₅₃ from *Desulfovibrio vulgaris* Miyazaki.

All these cytochromes *c* have similar folding pattern called "cytochrome *c* folding". All of them have identical two α -helices at C-terminal and N-terminal and they cross right angle at the top of the

2. All coordinates are supplied by the Brookheaven Protein Data Bank

(Bernstein *et al.*, 1977)

(a) Main chain - water molecule interactions

A 1 N	Wat 82	2.56	A 1 O	Wat 81	2.91
A 1 O	Wat 96	2.73	G 3 N	Wat 81	2.82
A 4 O	Wat 102	2.71	A 5 O	Wat 98	2.59
A 5 O	Wat 132	2.90	L 6 O	Wat 88	2.90
K 8 O	Wat 129	2.75	S 9 O	Wat 99	2.79
S 9 O	Wat 108	3.10	G 12 N	Wat 99	2.86
A 16 O	Wat 120	2.80	G 18 O	Wat 97	2.90
K 20 O	Wat 97	2.69	Q 21 N	Wat 140	3.15
A 22 N	Wat 111	3.17	M 23 N	Wat 95	2.97
V 25 O	Wat 114	2.84	H 27 N	Wat 93	3.05
H 27 O	Wat 111	2.97	A 28 O	Wat 141	2.61
V 29 N	Wat 97	3.05	G 31 N	Wat 110	2.77
A 34 N	Wat 109	3.13	D 35 N	Wat 91	3.04
K 39 O	Wat 125	2.82	G 50 O	Wat 123	2.36
E 52 N	Wat 85	3.01	A 55 N	Wat 84	2.91
V 56 O	Wat 94	2.94	N 59 O	Wat 87	2.77
K 62 O	Wat 130	2.74	R 63 o	Wat 104	2.95
Y 64 O	Wat 119	2.77	D 66 N	Wat 100	3.10
E 67 O	Wat 92	2.86	A 71 O	Wat 133	2.93
Y 75 O	Wat 122	2.94	M 76 O	Wat 110	2.70
L 79 OXT	Wat 110	2.81	L 79 OXT	Wat 127	2.66

Table 5-4

Hydrogen bonds with water molecules

(b) Side chain - water molecule interaction

D 2 O δ_1	Wat 126	2.82	K 8 N η	Wat 102	2.71
S 9 O γ	Wat 88	2.83	H 14 N δ_1	Wat 97	2.87
H 14 N δ_1	Wat 111	3.15	D 17 O δ_2	Wat 128	2.99
H 27 N δ_1	Wat 89	2.31	K 30 N ζ	Wat 90	2.94
K 30 N ζ	Wat 128	2.57	E 52 O ϵ_2	Wat 85	2.66
K 54 N ζ	Wat 123	3.04	Y 64 O η	Wat 88	2.60
Y 64 O η	Wat 115	2.79	D 66 O δ_1	Wat 119	3.04
E 67 O ϵ_1	Wat 116	2.10	E 67 O ϵ_2	Wat 116	2.85
E 68 O ϵ_1	Wat 83	2.77	E 68 O ϵ_1	Wat 105	2.85
E 68 O ϵ_2	Wat 101	3.09	Y 75 O η	Wat 102	2.97
Y 75 O η	Wat 120	2.59			

(c) Heme - water molecule chain interaction

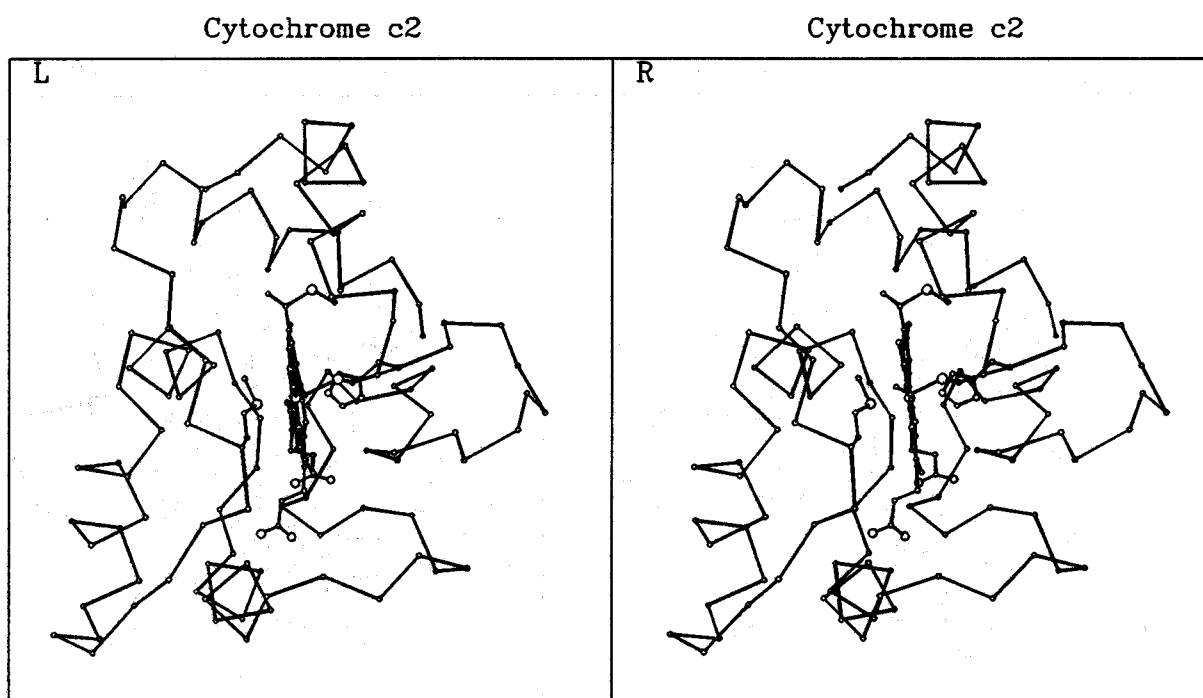
X 80 O2A	Wat 93	2.65
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Table 5-4
(continued)

(d) Water molecule - water molecule chain interaction

Wat 81	Wat 133	2.65	Wat 82	Wat 103	2.80
Wat 86	Wat 87	3.16	Wat 88	Wat 124	2.90
Wat 90	Wat 128	2.39	Wat 92	Wat 96	2.98
Wat 92	Wat 106	2.82	Wat 98	Wat 132	2.81
Wat 98	Wat 142	3.03	Wat 100	Wat 116	3.11
Wat 102	Wat 121	2.97	Wat 101	Wat 117	2.56
Wat 101	Wat 135	2.54	Wat 105	Wat 135	2.78
Wat 105	Wat 135	2.78	Wat 108	Wat 115	2.60
Wat 111	Wat 114	2.63	Wat 113	Wat 126	3.11
Wat 120	Wat 121	2.58	Wat 124	Wat 132	2.77
Wat 130	Wat 134	2.70			

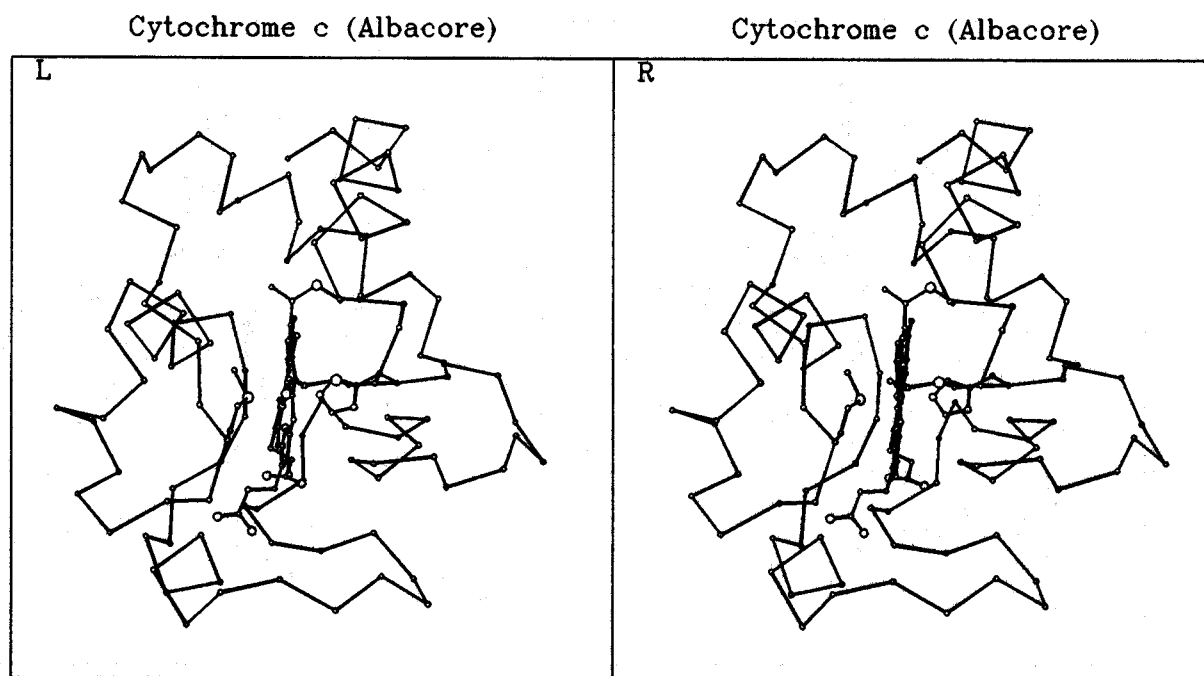
Table 5-4
(continued)



(a) cytochrome c2

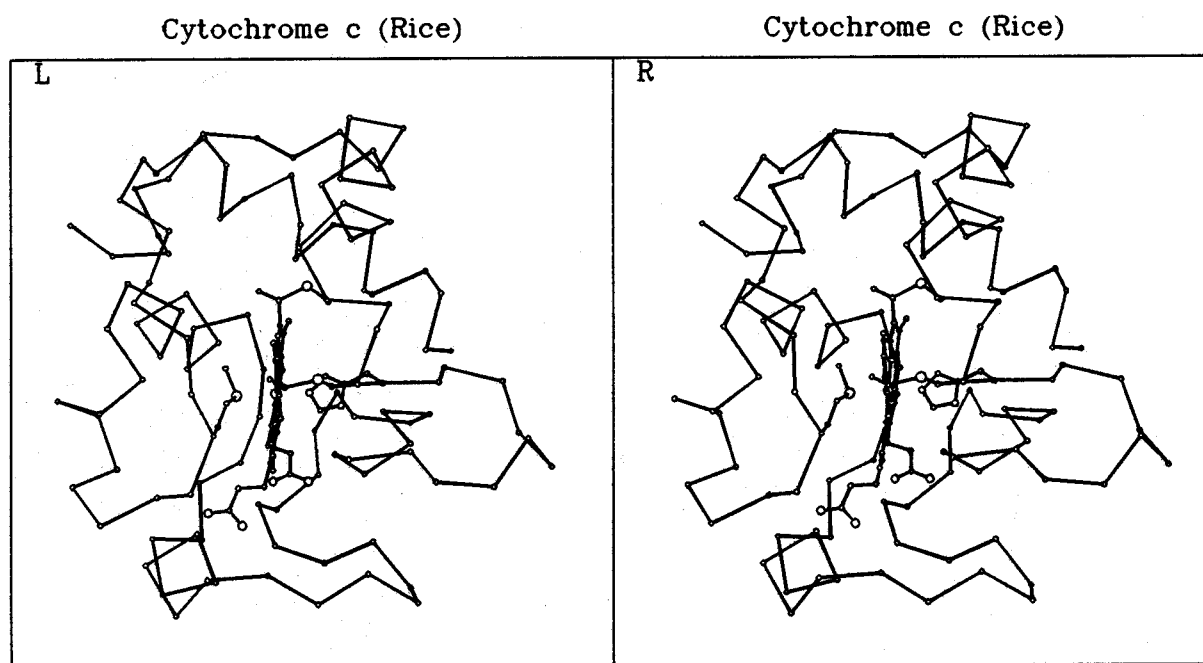
Figure 5-8

Folding patterns of different cytochromes c



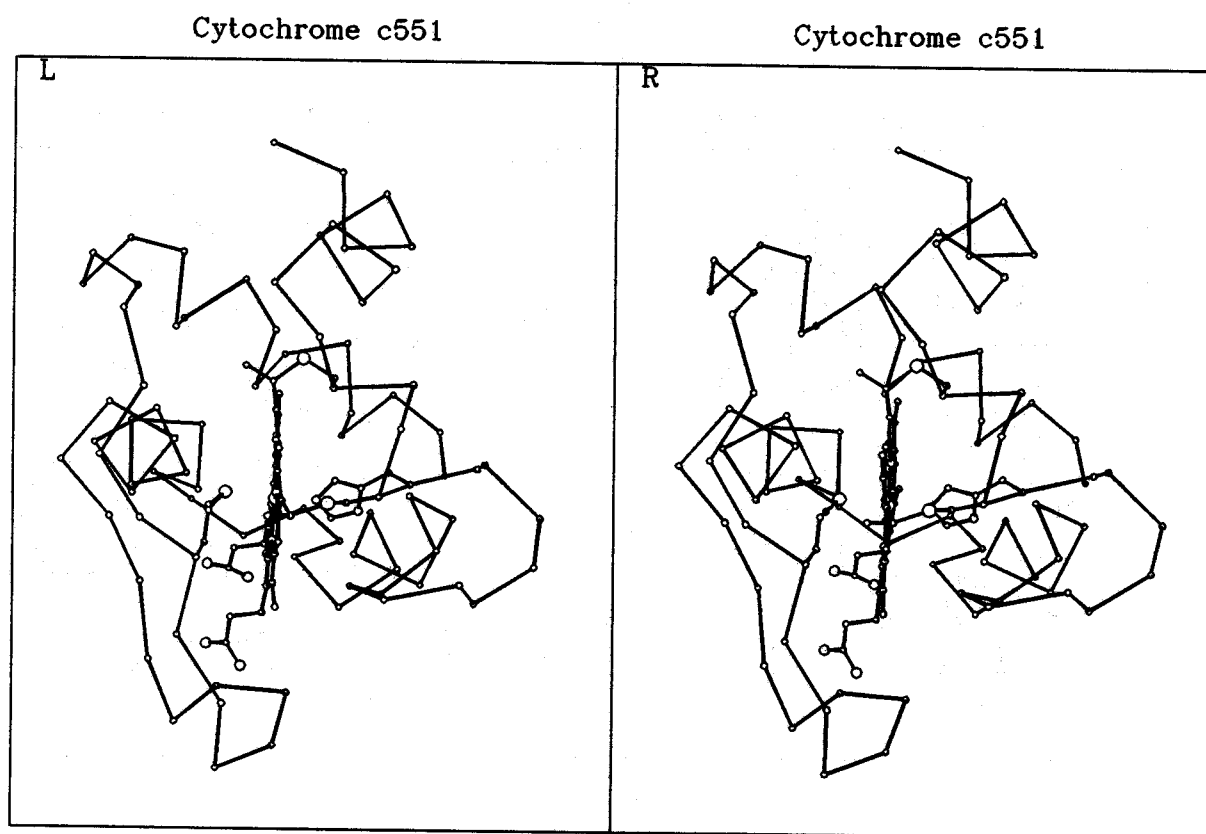
(b) cytochrome c from albacore

Figure 5-8
(continued)



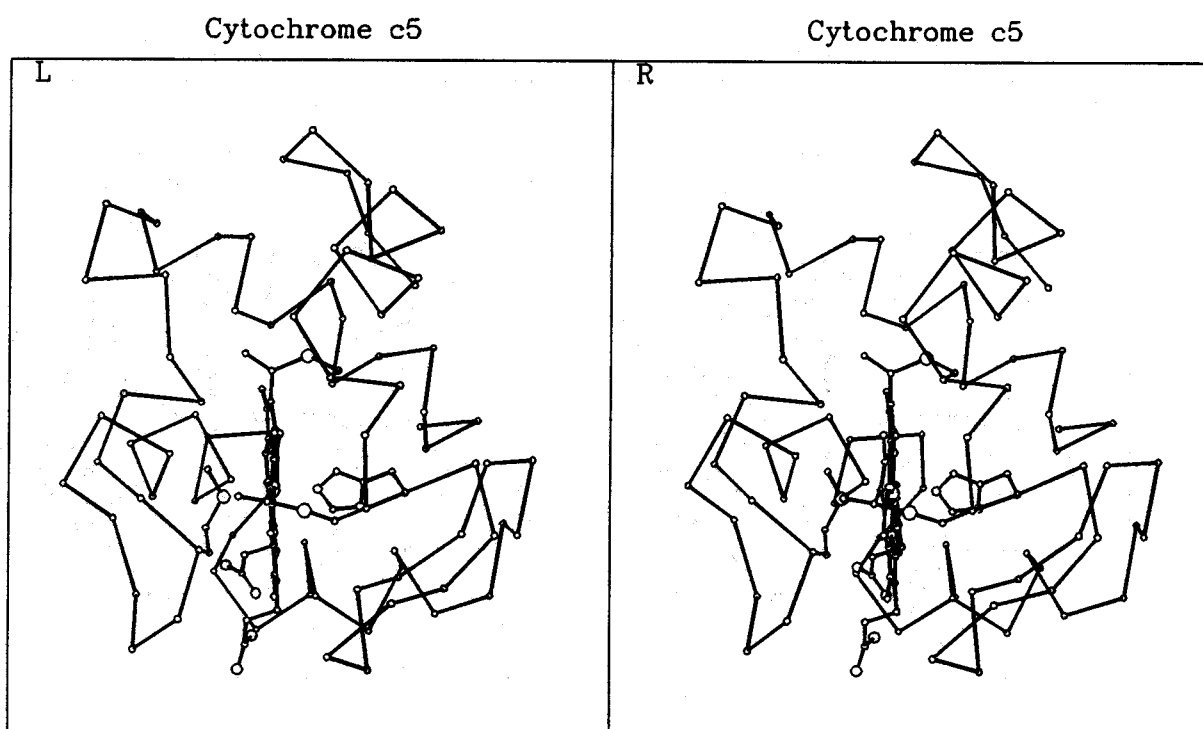
(c) cytochrome c from rice

Figure 5-8
(continued)



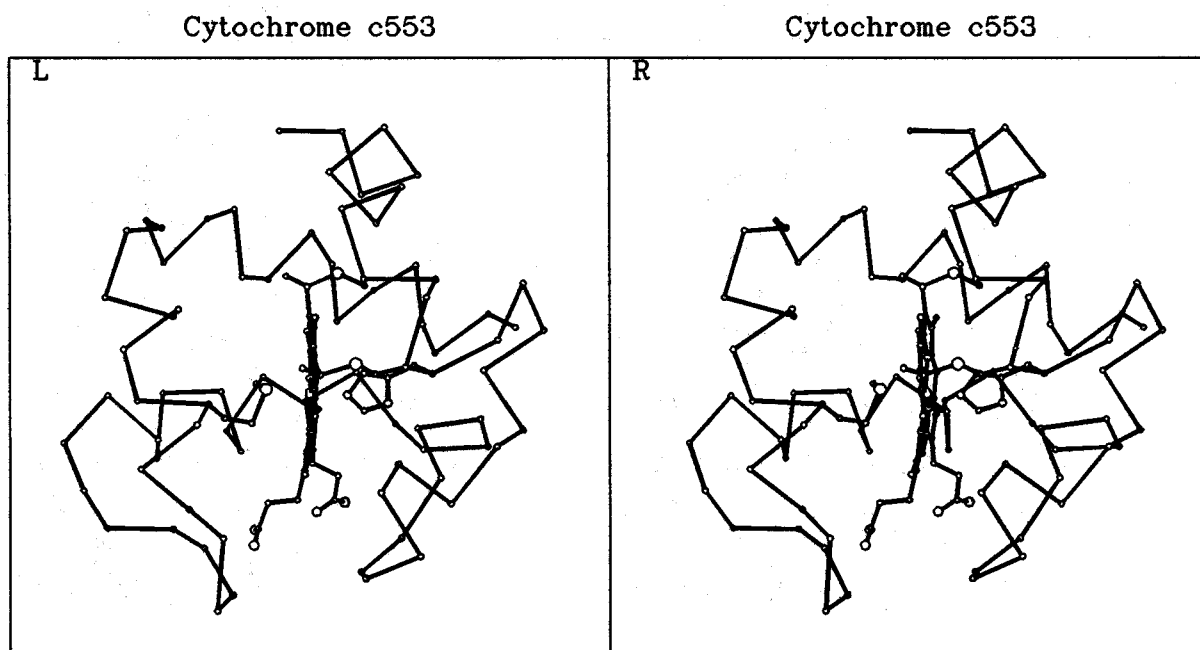
(d) cytochrome c551

Figure 5-8
(continued)



(e) cytochrome c5

Figure 5-8
(continued)



(f) cytochrome c553

Figure 5-8
(continued)

molecule. The upper part of the molecules highly conserved in cytochrome *c* superfamily. And covalently bonded and coordinated residues to heme group, Cys, Cys, His, Met, are also highly conserved.

In the contrast, the lower part of the molecule is quite different from each other, the lower part of L- and M-class cytochrome *c* is covered by the middle part of the amino acid chain and propionates of heme group are buried from the molecular surface. Cytochrome *c*₅₅₁, one kind of S-class cytochrome *c*, lost the long middle part of amino acid chain corresponding to L- and M-class cytochromes *c* and its folding pattern, especially the lower part of the molecule, is quite different from other L- and M-class cytochromes *c*. But, although folding pattern is quite different from other classes of cytochromes *c*, two propionates of heme group is covered by polypeptide chain as shown in Figure (5-8(d)). In the contrast, as shown in Figure (5-8(e)), two propionates of heme group of cytochrome *c*₅ are not covered by polypeptide main chain. Folding pattern of cytochrome *c*₅₅₃ is similar to that of cytochrome *c*₅. The main difference between cytochrome *c*₅₅₃ and cytochrome *c*₅ is the lack of the back right side α -helix for cytochrome *c*₅₅₃. This part of cytochrome *c*₅₅₃ has an extended random conformations, and this helical conformation is conserved in cytochrome *c*₅₅₁ molecule.

Assuming that cytochrome *c*₅₅₃ has the primitive folding pattern of cytochromes *c* superfamily, the following hypothesis can be built up.

1. Cytochrome *c*₅ : Insertion of the short helix at the right bottom of cytochrome *c*₅₅₃
2. Cytochrome *c*₅₅₁ : Insertion of the short loop at the left bottom of cytochrome *c*₅₅₃ and cover the bottom cleaves of the molecule

3. Cytochrome *c* : Insertion of the long loop at the bottom to cytochrome *c*₅₅₃ and covers the bottom of the molecule

4. Cytochrome *c*₂ : Insertion of the left loop to M-class cytochrome *c*

The hypothesis of the evolution of cytochrome *c* superfamily considering amino acid sequences and folding patterns will be discussed in chapter 5-2-3.

Folding patterns of cytochrome *c* is not same among cytochrome *c* superfamily, but overall folding patterns are similar and the bottom are relatively different from each other.

Figure (5-9) shows the space-filling model of cytochrome *c*₅₅₃ and it is colored according to kinds of residues. Blue color shows positively charged residues, such as lysine, arginine and histidine. Cyan color shows negatively charged residues, such as aspartic acid and glutamic acid.

Six lysine residues are surrounded the exposed edge of the heme group and positively charged residues are relatively rich in this side. In the other hand, there are many negatively charged residues in the opposite side. This charge distribution is also found in the other cytochromes *c* (Dickerson, 1972).

5-2-2. Interactions of Cytochrome *c*₅₅₃

Cytochromes *c* have many lysine residues and they are basic proteins. For example, cytochrome *c* from tuna has sixteen lysine residues of 103 amino acid polypeptide chain. Cytochrome *c*₅₅₃ has also thirteen lysine residues

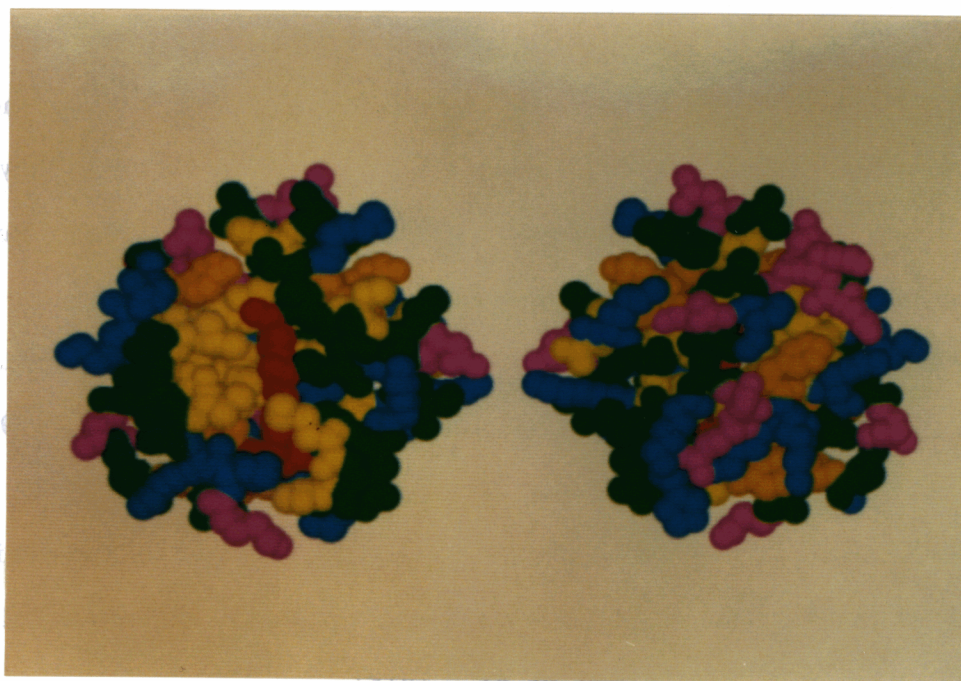


Figure 5-9

Space-filling models of cytochrome c553

These two models show opposite side of molecule each other
The left one shows heme cleaves

Blue : Lys, Arg, His ; Cyan : Asp, Glu
Orange : Phe, Tyr ; Yellow : Leu, Met, Val
Green : Asn, Gln, Cys, Thr, Ser, Ala, Gly
Red : Hem

in 79 amino acid polypeptide chain, and isoelectric point of cytochrome c553 is 10.5.

Many lysine residues of cytochromes *c* are conserved in many different species. The reactions of naturally occurring variants of eukaryotic cytochrome *c* with its oxidase or reductase have implicated the positively charged lysine residues around the exposed edge of the heme in the mechanism of interaction (Ferguson-Miller *et al.*, 1979). Horse heart cytochrome *c* contains nineteen lysine residues. Millett and his colleague have prepared nine cytochrome *c* derivatives singly-modified at lysines with trifluoroacetate (TFA) and six singly-modified at lysines with trifluoromethyl-phenyl carbamate (TFC) (Ahmed *et al.*, 1978 ; Smith *et al.*, 1977, 1980). Both TFA and TFC neutralized the positively charged amino group of lysine. Modification of Lys13 produces a four to seven-fold reduction in the electron transfer rates of cytochrome *c* with the oxidase (Smith *et al.*, 1977) and the reductase (Ahmed *et al.*, 1978).

Margoliash and his colleagues have prepared ten cytochrome *c* derivatives singly-modified at lysine by 4-carboxyl-2,6-dinitrophenol (CDNP) (Ferguson-Miller *et al.*, 1978 ; Osheroff *et al.*, 1980). CDNP replaces the positively charge of lysine by a negatively charge. The effects of CDNP are qualitatively similar to those of TFA and TFC but much more pronounced. The lysine residue with greatest effect on the reaction rates or binding constants are at positions 13, 72, 86, 87 and 8 for the oxidase and 13, 86, 87, 27 for the reductase.

Reider and Bosshard have used differential chemical modification to identify the lysine residues of horse heart cytochrome *c* involved in interactions with the oxidase or the reductase (Reider and Bosshard, 1980). The lysine

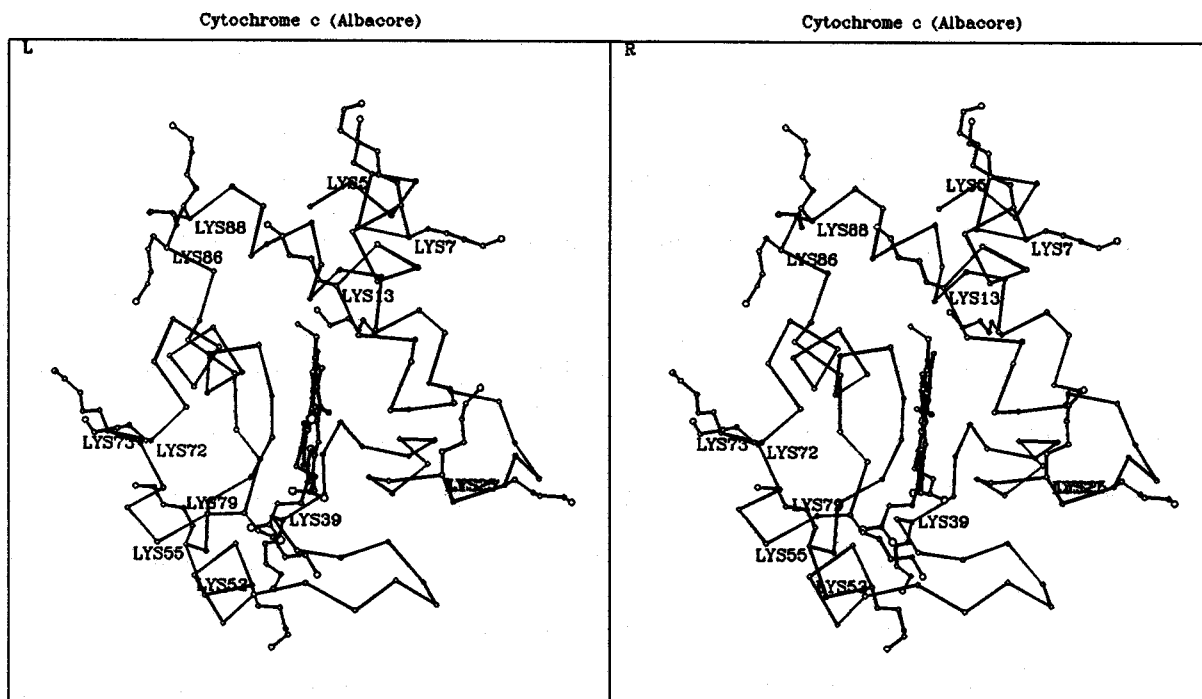
residues afforded the greatest protection by cytochrome *c* oxidase are numbers 13, 86, 87, 8, 72, 73 and by purified cytochrome *c* reductase are numbers 86, 87, 13, 8, 79, 5.

All three studies of the cytochrome *c* interaction surface implicate lysine residues surrounding the exposed edge of the heme group. Lysines 13, 86 and 87 interact strongly with both the oxidase and the reductase as shown by all of the studies. Lysine 72 appears to interact strongly with the oxidase but less strongly with the reductase.

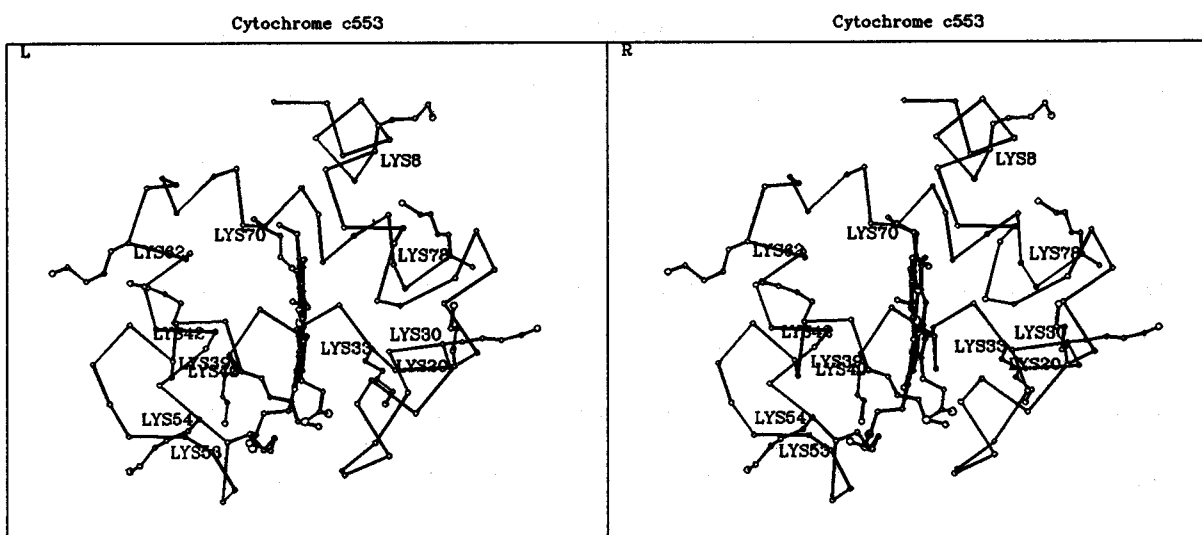
Cytochrome *c* transfer electrons from the pyrrole C region and lysine residues act as important residues to contact to electron acceptor. As shown in Figure (5-10(b)), cytochrome *c*553 also has lysine residues surrounding pyrrole C region. But Lys8 of cytochrome *c*553, which occupied the near position of the Lys13 of cytochrome *c* from horse heart, does not locate at the same position in the molecule. Ser9 of cytochrome *c*553 is located at that place, and water molecule bonded to O γ of Ser9 was found on the MAD electron density map. Lys8 in cytochrome *c*553 is located very near part of the molecule. Lysine residue in this position is not conserved on primary structures in all cytochromes *c* superfamily. But lysine residues are very important for activity of cytochrome *c* in the electron transfer system and overall location of lysine residues in cytochromes *c* molecules are similar to each other.

5-2-3. Structure and Redox Potential

As described in chapter 5-1-3, heme group of cytochrome *c*553 is ex-



a



b

Figure 5-10

Lysine residues in cytochromes c

(a) cytochrome c from albacore

(b) cytochrome c553

posed to its molecular surface at three region. These three solvent accessible surface areas of heme are summarized in Table (5-5). These areas and their size were calculated using the program ACCESS in the CCP4 computer program package. Radius of solvent probe was used 1.4 Å, which was assumed a water molecule. The heme group is mainly exposed to molecular surface at pyrrole C region(CMC and CBC of heme group). Stellwagen suggested the relationship between redox potential and exposed area of heme group (Stellwagen,1978). He tested six heme proteins³, and showed that reduction potential, E_0' , was inversely dependent on the exposure of the heme to an aqueous solvent. Figure (5-11) shows the dependence of reduction potential, E_0' , and the fraction of heme surface exposed and the fraction of apolarity of heme crevice. The fraction of heme surface exposed could be expressed as the following equation

$$E_0'(\text{mV}) = -15 \cdot A + 345,$$

where A is a percentage of exposure area of heme group. Using this relationship, calculated E_0' value for cytochrome c553, of which A is 7 %, becomes 240mV, although observed redox potential was about 0mV (Niki *et al.*, unpublished result). Cytochrome c553 has extremely low redox potential compare to other heme proteins. This result suggested that the exposure of heme moiety is not the only determinant for the magnitude of its redox

3. Stellwagen tested following six heme proteins,

Cytochrome c2, Cytochrome c, Cytochrome c550, Hemoglobin α -chain,
Hemoglobin β -chain, Myoglobin, Cytochrome b5

with their coordinates in the Protein Data Bank. (Bernstein *et al.*, 1977)

Heme+protein	Total accessible surface	4490.3
	Accessible apolar surface	2567.8
	Accessible heme surface	60.1
Heme	Total accessible surface	843.3
Heme crevice of apoprotein		
	Total accessible surface	639.9
	Accessible apolar surface	491.3
Tabulations		
	% Heme exposed in heme protein	7
	% Heme crevice apolar	76
	% Heme environment apolar	58

Accessible surface area of heme group

CHA	0.4
O2A	2.7
CMB	0.9
CAB	0.5
CBB	2.9
CMC	18.1
CBC	29.3
C3D	0.4
C4D	0.2
CAD	1.4
CGD	1.0
O2D	3.1

Table 5-5

Solvent accessible surface area
of cytochrome c553

The heme group is mainly exposed to
molecular surface at pyrroll C region

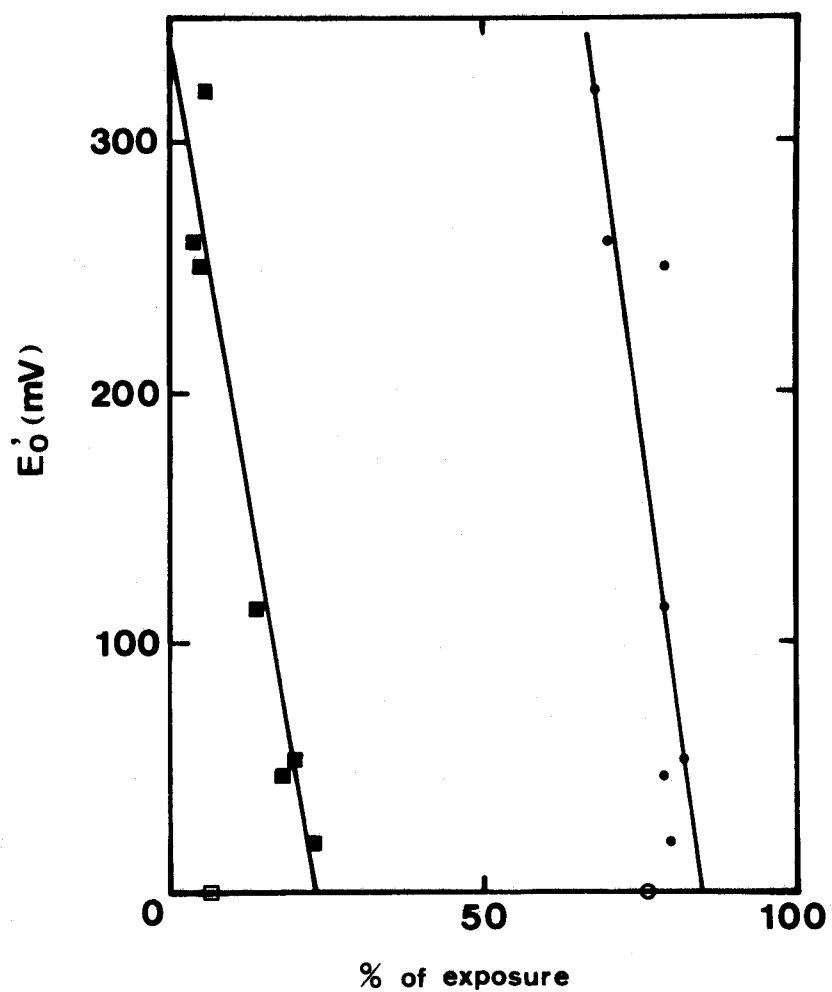


Figure 5-11

The dependence of reduction potential, E_0' ,
to the fraction of exposed surface of heme(● ○)
and the fraction of apolarity of the heme crevice(■ □)

The marks ○ and □ indicate the values of cytochrome c553

potential.

This irregular behavior on low redox potential of cytochrome c₅₅₃ must be depend on its structure. Considering hydrogen bonds to carbonyl oxygens of the two propionates. The O1A is bonded with N ϵ 2 of Gln32 and N ζ of Lys40, and the O2A is bonded to O γ of Tyr49 and Wat93 molecule. The O1D is bonded with N of Lys53 and N of Lys54 and the O2D is bonded to O γ of Tyr49 and N of Gly51 by hydrogen bonds (Table (5-2) and Figure (5-12)). Two propionates of heme group bond to many basic atom by hydrogen bonds. Furthermore, two side chains of tyrosine residues, Tyr44 and Tyr49, are closed together. The closest inter-residue distance is 3.65 Å, between O γ of Tyr44 and C ϵ 2 of Tyr49, that is comparable to their van der Waals distance, and it means that these two aromatic rings are contact each other. And dihedral angle between the best plane (Blow, 1960) of the aromatic ring of Tyr44 and that of Tyr49 is 89.8 degree. It seems that these residues are important on the electron transfer properties, but it must be necessary to study further to find the relationship between its structure and redox potential.

5-2-4. Alignment of Amino Acid Sequence

Alignment of amino acid sequence of cytochrome c₅₅₃ from sulfate-reducing bacterium was proposed by Dickerson *et al.* (Dickerson *et al.*, 1976), considering the amino acid sequence of cytochrome c₅₅₃ from *Desulfovibrio vulgaris* Hildenborough (Bruschi and Le Gall, 1972). Amino acid sequence of cytochrome c₅₅₃ from *Desulfovibrio vulgaris* Miyazaki was determined by Yagi and colleagues (Nakano *et al.*, 1983) and they proposed new

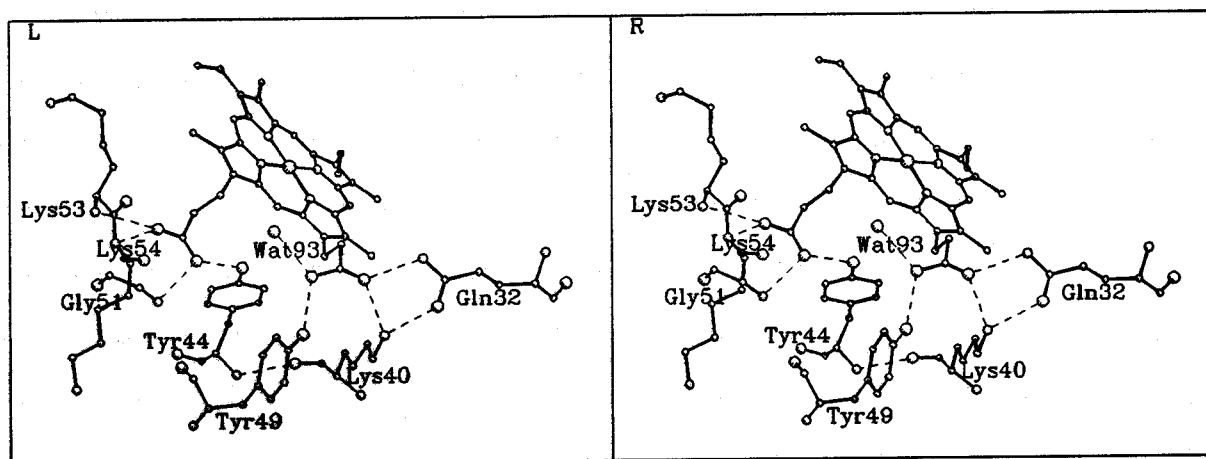


Figure 5-12

Heme group and its neighbor residues
which bond to heme by hydrogen bonds

alignment of amino acid sequence of cytochrome c₅₅₃ to that of other S-class cytochrome c, such as cytochrome c₅₅₁ from *Pseudomonas aeruginosa* and that from *Pseudomonas fluorescens*. Amino acid sequence of cytochrome c₅₅₃ of *Desulfovibrio vulgaris* Miyazaki is quite different from that of *Desulfovibrio vulgaris* Hildenborough, although amino acid sequences of cytochromes c₃ from both species are quite similar to each other. As shown in Figure (5-13), assuming that there was a mistake on the alignment of amino acid fragment during the determination of amino acid sequence, both amino acid sequences of cytochrome c₅₅₃ are quite similar to each other. The author propose new alignment of amino acid sequence based on the three-dimensional structure of cytochrome c₅₅₃ from *Desulfovibrio vulgaris* Miyazaki in Figure (5-14(a)). This alignment is essentially same as that proposed by Yagi *et al.* (Nakano *et al.*, 1983). There are two region where the three dimensional structure of cytochrome c₅₅₃ differs greatly from that of cytochrome c₅₅₁ from *Pseudomonas aeruginosa* as discussed in chapter 5-2-1. Alignment of amino acid sequence support this result. Although three dimensional structure of cytochrome c₅₅₃ is more similar to cytochrome c₅ from *Azotobacter vinelandii* than that of cytochrome c₅₅₁ from *Pseudomonas aeruginosa*, it seems that amino acid sequence of cytochrome c₅₅₃ is more similar to that of cytochrome c₅₅₁ than that of cytochrome c₅. And as shown in Figure (5-8(f)), in chapter 5-2-1, tertiary structure of cytochrome c₅₅₃ has "cytochrome c folding" and the upper part of its structure is similar to that of other cytochromes c, alignment of amino acid sequence of cytochrome c₅₅₃ to that of cytochrome c from albacore does not shows any similarity on its primary structure, see Figure (5-14(b)).

C553 ¹⁾	1	A	D	G	A	A	L	Y	K	S	10	C	V	G	C	H	G	A	D	G	S	K	Q	A	M	G	V	G	H	A	V	K	30	G	Q	K				
1CC5 ²⁾	5	G	G	G	A	R	S	G	D	D	V	V	A	K	Y	20	C	N	S	C	H	G	T	G	L	L	30	N	A	P	K	V	G	D	S					
351C ³⁾	1	E	D	P	E	V	L	F	K	N	K	G	C	V	A	10	C	H	A	I	D	T	K	20	M	V	G	P	A	Y	K	D	V	A	A	K				
									*		*	*	*	*																										
C553		A									40	F	K	K	L	K	G	Y	A	D	G	S	Y	G	G	E	K	K	A	V	M	T	N	60	L	V				
1CC5		40	A	A	W	K	T	R	A	D	A	K	G	G	L	D	G	L	L	A	Q	S	L	S	G	60	L	N	A	M	P	P	K	G	I	C				
351C			F	A	G	Q					40	A	Q	A	E	A	E	L	A	Q	R	I	K	N	G	S	Q	G	V	W	G	P	I	P	M	P	P	N	A	V
																																			*					
C553			K	R	Y	S	D	E	E	M	K	A	M	A	D	Y	M	S	K	L	70																			
1CC5		70	A	D	C	S	D	D	E	L	K	A	A	I	G	K	M	S	G	L	80																			
351C											70	S	D	D	E	A	Q	T	L	A	K	W	V	L	S	Q	K	80												

Figure 5-14

Alignment of amino acid sequence of cytochrome c

(a) S-type cytochrome c and cytochrome c₅₅₃

C5533)	1	A	D	G	A	A	L	Y	10	K	S	C	V	G	C	H	G	A	D	G	S	20	K	Q	A	M	G	V	G	H	A	V	K	G	Q	30	K									
3CYT4)	1	G	D	V	A	K	G	K	T	F	V	Q	K	C	A	Q	C	H	T	V	E	D	G	G	K	20	H	K	V	G	P	N		31												
									10																																					
C553		40	A	D	E	L	F	K	K	L	K	G	Y	A	D	G	S	50	Y	G	G	E	K	K	A	V	M	T	N	L	V	K	R	Y	S	60	D	E	E	M	K	A	M	A	D	70
3CYT	57	60	I	V	W	N	N	D	T	L	M	E	Y	L	E	N	P	K	K	Y	I	P	G	T	K	M	I	80	F	A	G	I	K	K	G	E	R	Q	D	L	V	A	90			
C553		79	Y	M	S	K	L																																							
3CYT			Y	L	K	S	A	T	S																																					
			100						103																																					

*) Underline shows helical conformations
 **) The mark * shows heme binding residues

Figure 5-14
 (continued)

(b) cytochrome c553 and cytochrome c from albacore

- 1) cytochrome c553 from *Desulfovibrio vulgaris* Miyazaki
- 2) cytochrome c5 from *Azotobacter vinelandii*
- 3) cytochrome c551 from *Pseudomonas aeruginosa*
- 4) cytochrome c from albacore

5-2-5. Evolution of Cytochrome *c* Superfamily

As discussed in chapter 5-2-1, although three-dimensional structure of cytochrome *c*₅₅₃ looks like "cytochrome *c* folding", it is quite different from that of mitochondrial cytochrome *c*. Dickerson classified cytochrome *c*₅₅₃ into S-class cytochrome *c* (Dickerson, 1980), but its three-dimensional structure is not so similar to that of cytochrome *c*₅₅₁ from *Pseudomonas aeruginosa* which is classified into S-class cytochrome *c*. Mathews classified cytochrome *c*₅₅₃ from *Desulfovibrio* into S₄-class as spectral behavior of cytochrome *c*₅₅₃ is quite different from other S-class cytochromes *c*(S₁-S₃) (Mathews, 1985). Three-dimensional structure of cytochrome *c*₅₅₃ determined by this thesis supported Mathews' classification. Folding pattern of cytochrome *c*₅₅₃ does not so similar to that of cytochrome *c*₅₅₁ from *Pseudomonas*, and it looks that folding pattern of cytochrome *c*₅₅₃ is more similar to that of cytochrome *c*₅ from *Azotobacter*. But primary structure of cytochrome *c*₅₅₃ is more similar to that of cytochrome *c*₅₅₁ from *Pseudomonas aeruginosa* than that of cytochrome *c*₅ from *Azotobacter vinelandii*. Considering these result, although *Desulfovibrio* is one of the ancient-type bacterium, *Desulfovibrio* is not so far from both *Pseudomonas* and *Azotobacter* in the phylogenetic tree of cytochrome *c* superfamily. Thus it is necessary to considering the position of *Desulfovibrio* on the phylogenetic tree of cytochrome *c* (Figure (5-15)). When Dickerson proposed his phylogenetic tree of cytochromes *c*, amino acid sequence of *Desulfovibrio*, although he used amino acid sequence of cytochrome *c*₅₅₃ from *Desulfovibrio vulgaris* Hildenborough (Bruschi and Le Gall, 1972), was quite different from other cytochromes *c*, and he proposed the branch of *Desulfovibrio* separated at the

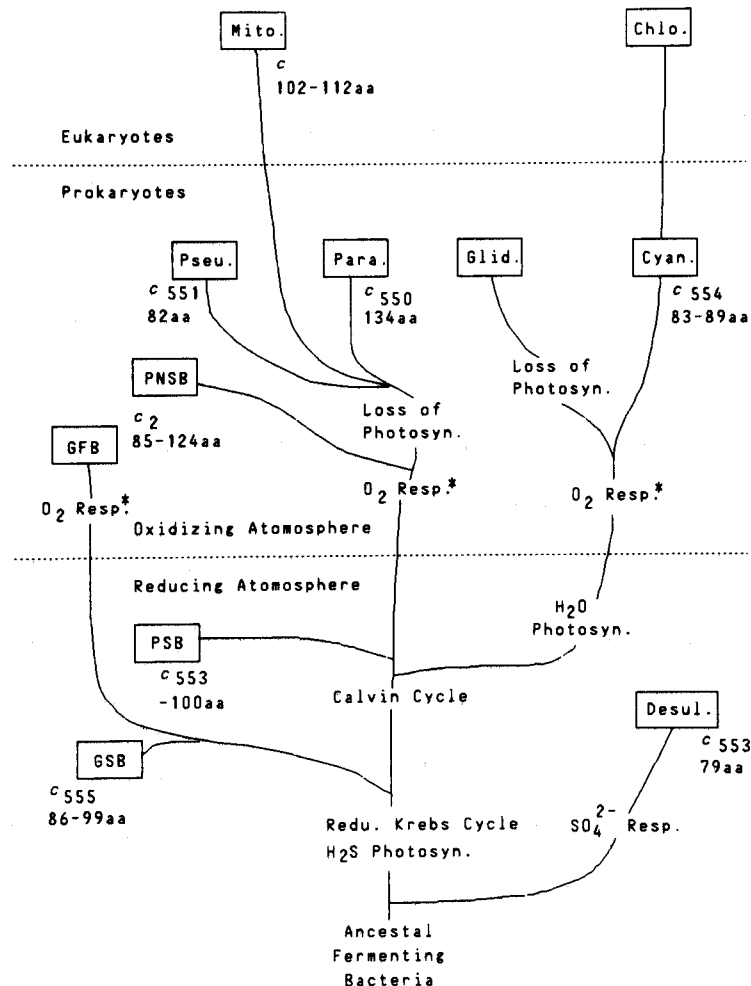


Figure 5-15

Phylogenetic tree of cytochrome *c* superfamily
(Dickerson, 1980)

Sulfate respiration is assumed to have arisen as a response to sulfate-releasing photosynthesis early on the primitive Earth, just as oxygen respiration arose as a consequence of oxygen-releasing photosynthesis at a later era. Oxygen respiration has evolved independently from photosynthesis at least three times: in the green, purple and blue-green photosynthetic bacteria.

GSB=green sulfur bacteria (Chlorobiaceae); GFB=green filamentous bacteria (Chloroflexaceae); PSB=purple sulfur bacteria (Chromatiaceae); PNSB=purple nonsulfur bacteria (Rhodospirillaceae); Pseu.=*Pseudomonas*; Mito.=eukaryotic mitochondria; Para.=*Paracoccus*; Glid.=gliding bacteria related to cyanobacteria; Cyan.=cyanobacteria or blue-green algae; Chlo.=eukaryotic chloroplasts; Desul.=*Desulfovibrio*

early stage of the evolution of cytochromes *c* superfamily.

Folding pattern of many cytochromes *c*, including L-, M- and S-class cytochromes *c*, seem that these have some insertion part into folding of cytochrome *c*₅₅₃. In this sense, it looks that cytochrome *c*₅₅₃ has one of the most primitive "cytochrome *c* folding" and other cytochrome *c* has some insertions into primitive folding. This result supports the hypothesis that the branch of *Desulfovibrio* had separated at the early stage of the evolution in the phylogenetic tree of cytochrome *c* superfamily proposed by Dickerson (Dickerson, 1980). But folding pattern of cytochrome *c*₅₅₃ is similar to that of cytochrome *c*₅ from *Azotobacter vinelandii*, and it seems that the location of *Desulfovibrio* is quite closed to that of *Azotobacter*. Comparison of primary structure of flavodoxin from *Desulfovibrio* and *Azotobacter* supported this result (Kobayashi and Fox, 1978).

5-3. Usage of Multi-wavelength Anomalous Phase determination for Protein Crystallography

Multi-wavelength anomalous phase determination technique is a novel method to apply protein crystallography. Only two unknown protein crystal structure has been determined by this method, one is blue-copper protein (Guss *et al.*, 1988) and the other is streptavidin (Hendrickson *et al.*, 1989). The former has an intrinsic heavy atom, copper, in native protein and the latter was use extrinsic anomalous scatterer, selenium atom was used in that case, as a part of coenzyme. Although the theory of multi-wavelength anomalous phase determination can be expressed by analogue of

isomorphous replacement method, there are many difficulty to apply to the determination of unknown structure of proteins. The most difficult problem is how high precision data can be obtained. Although isomorphous difference is usually greater than several ten electrons, anomalous difference is always less than ten electrons. Success of structure determination of cytochrome c553 crystal should be depend following points;

- 1) Very fast data measurement using synchrotron radiation, Weissenberg camera and imaging plate reduces radiation damage of protein crystal
- 2) Bijvoet pair data are diffract at a same time by Weissenberg geometry and fluctuation of incident beam does not affect to measure Bijvoet difference
- 3) High precision data can be detected by imaging plate system
- 4) Averaging of many symmetry-related reflections reduced non-systematic errors
- 5) Local scaling method could be succeeded to reduce systematic errors

Si(111) monochromator was used in this experiment. Resolution of wavelength dispersion ($\Delta\lambda/\lambda$) of this crystal is order of 10^{-3} and it means that when real part of anomalous scattering effect, $\Delta f'$, varies dramatically at absorption edge, apparent value of $\Delta f'$ is not so large. It must be necessary to use another monochromator of which wavelength dispersion is better than Si(111), such as Si(311) or double crystal monochromator, to get more useful data.

Many proteins in nature which have heavy atom(s) in native proteins and the multi-wavelength anomalous dispersion method will become powerful technique for protein crystallography. Absorption edges of elements of atomic

numbers in the range from 20(Ca) to 47(Ag) and from 50(Sn) to 92(U) are accessible in the range of 0.5 to 3.0 Å. As shown in Figure (5-16), although signal-to-noise ratio is not so good, native anomalous difference Patterson map showed iron-iron self vector even if $\Delta f''$ was 1.664 electrons at 1.04 Å. It means that it has an possibility to determine phases with proteins of which molecular weight is twice of that of cytochrome c553, about 20000, with one heavy atom with X-ray at K absorption edge. But it must be very difficult to determine phases in such conditions, optics must be improved for this use. The author determined the real part of anomalous dispersion term, $\Delta f'$, as a parameter of refinement of heavy atom parameter, that was treated as occupancy, in this study. Strictly speaking, it should be better to determine wavelength of absorption edge and magnitude of real and imaginary part of anomalous scattering term according to the measurements of fluorescence EXAFS of iron. Detection system of fluorescence from crystal must be necessary to construct for anomalous dispersion technique.

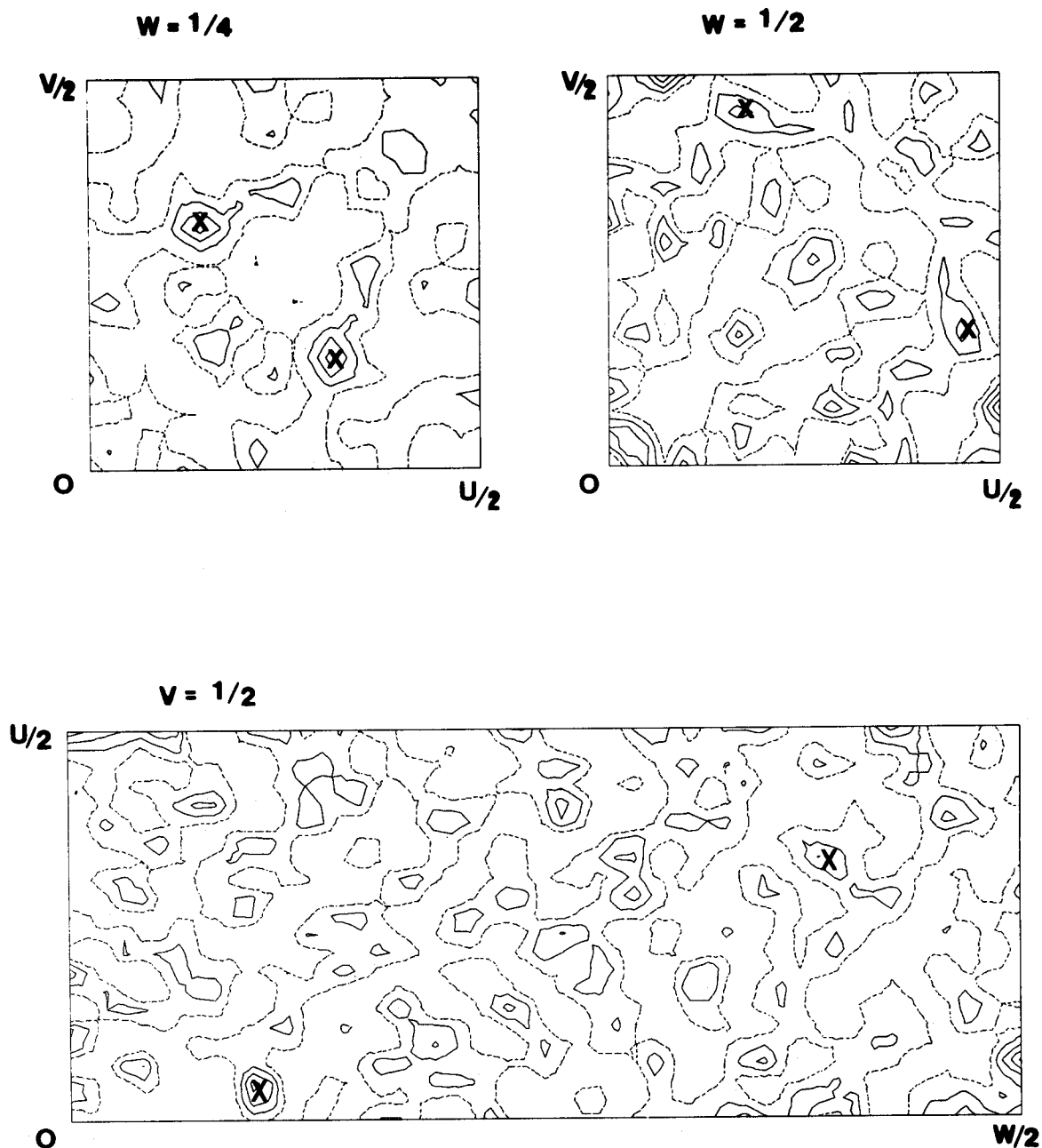


Figure 5-16

Native anomalous difference Patterson
of data collected at 1.04 Å
with coefficient of $|F_p(+)-F_p(-)|^2$

Dashed line shows zero level and these maps are contouring
of equal intervals in arbitrary unit

CONCLUDING REMARKS

Crystal of cytochrome c553 diffracts better than 1.5 Å resolution. And it is necessary to continue refining the structure to elucidate its solvent structure and side chain conformations much precisely.

The multi-wavelength anomalous dispersion technique is one of the powerful technique to determine the crystallographic phases without using any other derivatives. In this study, this technique was applied to relatively small protein with one anomalous scatterer, and modified technique of isomorphous replacement method was successfully applied to determine the crystallographic phases. According to many advantages of multi-wavelength anomalous dispersion method, such as, 1) does not necessary to make isomorphous derivatives, 2) it has no lack of isomorphism, it must be necessary to try determining structure of another protein which is larger than cytochrome c553 by multi-wavelength anomalous dispersion method.

Karle proposed a general algebraic analysis with no approximations for any number and type of anomalous scatterer (Karle, 1980), and Hendrickson proposed somewhat deviation of components that lends itself to direct analysis of the crystal structure at hand (Hendrickson, 1985). If $|^{\lambda}F(h)|$ is denoted the complete structure factors for reflection h at a particular wavelength λ , then the diffracted intensity, $I=K \cdot |F|^2$, is directly proportion to

$$\begin{aligned} |^{\lambda}F(\pm h)|^2 &= |^0F_T|^2 + a(\lambda) \cdot |^0F_A|^2 \\ &+ b(\lambda) \cdot |^0F_T| \cdot |^0F_A| \cdot \cos(^0\phi_T - ^0\phi_A) \\ &\pm c(\lambda) \cdot |^0F_T| \cdot |^0F_A| \cdot \sin(^0\phi_T - ^0\phi_A) \end{aligned}$$

Here, ${}^0F_T = |{}^0F_T| \exp(i \cdot {}^0\phi_T)$ is the normal scattering (f^0) contribution from all atoms in the structure and $|{}^0F_A|$ is the corresponding contribution just from the anomalous centers. These factors are ratios of anomalous to normal scattering components,

$$a(\lambda) = (f'^2 + f''^2) / f^0{}^2$$

$$b(\lambda) = 2 \cdot (f' / f^0)$$

$$c(\lambda) = 2 \cdot (f'' / f^0)$$

his new treatment will be applied to calculate the crystallographic phases of cytochrome c553 for check. In this treatment, it is necessary to determine the parameters, $a(\lambda)$, $b(\lambda)$, $c(\lambda)$. These parameters will be calculated from the measurement of fluorescence EXAFS of the crystal. The author wants to construct this measurement system.

And furthermore, another monochromators, such as Si(311) or double crystal monochromator, of which wavelength dispersion is better than Si(111) may be needed to measure the much precise data near absorption edge.

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Appendix

A. Imaging Plate system at Photon Factory and its software system

The imaging plate system at Photon Factory was designed and constructed by Amemiya *et al.* (Amemiya *et al.*, 1988). A conventional drum-type film densitometer, which has two drums with a common rotating axis, has been modified into a laser scanner for the image read-out. One drum is used to read out X-ray image on the imaging plate, and the other is used to imprint the image on normal photographic film. The imaging plate is attached to the outer surface of the drum by using Scotch tape. A 20mW He-Ne laser source and a focusing optical system are adapted in a shaded box which moves along the direction of the drum axis together with device to collect the photo-stimulable luminescence and two photomultiplier tubes. The outputs of the photomultiplier tubes are logarithmically amplified and digitized by 100KHz, 12-bit A/D converters. The digitized data are sent directly to disk memories via three buffer memories of a host computer, ECLIPSE MV/4000 made by Nippon Data General. The operation of the system is controlled by this computer. The digital image can be displayed on a color graphic display terminal immediately after the image is read out. The image on the graphic terminal is photocopied on instant film or 35mm roll film by hard copy machine. For more precise reproduction of the X-ray image, the digital data are reconverted by 100KHz, 12-bit D/A converter into a time-series of analogue signals which modify the intensity of a glow lamp to imprint the image on a photographic film attached on the other drum. The digital data

are finally stored on a magnetic tape (6250/1600bpi) for further data processing by another computers.

The following points are to be improved in comparison with the one which is commercially available.

- (a) The pixel sizes of $25\mu\text{m}^2$ and $50\mu\text{m}^2$ in addition to $100\mu\text{m}^2$ are available by making both a focus size and a scanning pitch of the laser beam changeable.
- (b) The output signals from a photomultiplier tube are digitized by a 12-bit A/D converter in place of a 8-bit A/D to improve the precision in X-ray intensity measurement.
- (c) In order to fully utilize a wide dynamic range ($1:10^5$) of the photo-stimulated luminescence, two photomultiplier tubes are used simultaneously to detect the intensities of the luminescence with different sensitivities. The second multiplier tube receives roughly 1% of the total amount of the luminescence and convert a higher intensity range where the first one is saturated.
- (d) Arbitrary size of the imaging plate are available simply by taping and the maximum size of the imaging plate is $200 \times 380 \text{mm}^2$.
- (e) In order to decrease the image distortion, a drum-type film densitometer is utilized which provides more precise scanning pitches than the flat-type one.
- (f) The non-uniformity of response is reduced as the incident angle of the laser beam is always constant by using a drum-type film densitometer. The phosphor can be stimulated more uniformly by the laser beam.
- (g) A high-resolution graphic display system (1280×1024 pixels, 8-bit

color depth, 19-inch) is also available in addition to a system for imprinting the image to get a visible image immediately.

Host computer, ECLIPSE MV/4000, has 4MB main memory, 354MB hard disk and magnetic tape unit (6250/1600bpi) as data storage system. The color raster graphic display, 1280 $\times$ 1024 pixels, 8-bit color depth (256 colors from 16 million color table can be displayed at a same time), hard copy machine for graphic display (Multi Color, Dunn Instruments) and X-Y plotter (7475A, YHP) are also available as image processing. Figure (A-1) shows a block diagram of the data handling components of the imaging plate system based on ECLIPSE MV/4000.

All software programs are coded using JIS Fortran (Fortran 77) and some subroutines are supported by Nippon Data General and runs under the AOS/VS operating system. Figure (A-2) shows a tree structure of the software system for the imaging plate system.

I. File Format

This imaging plate system can read any size of imaging plate. But five typical sizes and one arbitrary size are chosen in this program system. As it is necessary to make a file which has continuous region on the physical disk to get high speed for writing data on the disk. All this kind of files are only used to input the data read out by image reader.

Standard format file of this system has a header block at the beginning of the file. This header block is 512byte size and it contains some informations about data in the file (Table (A-1)). Almost all programs in this

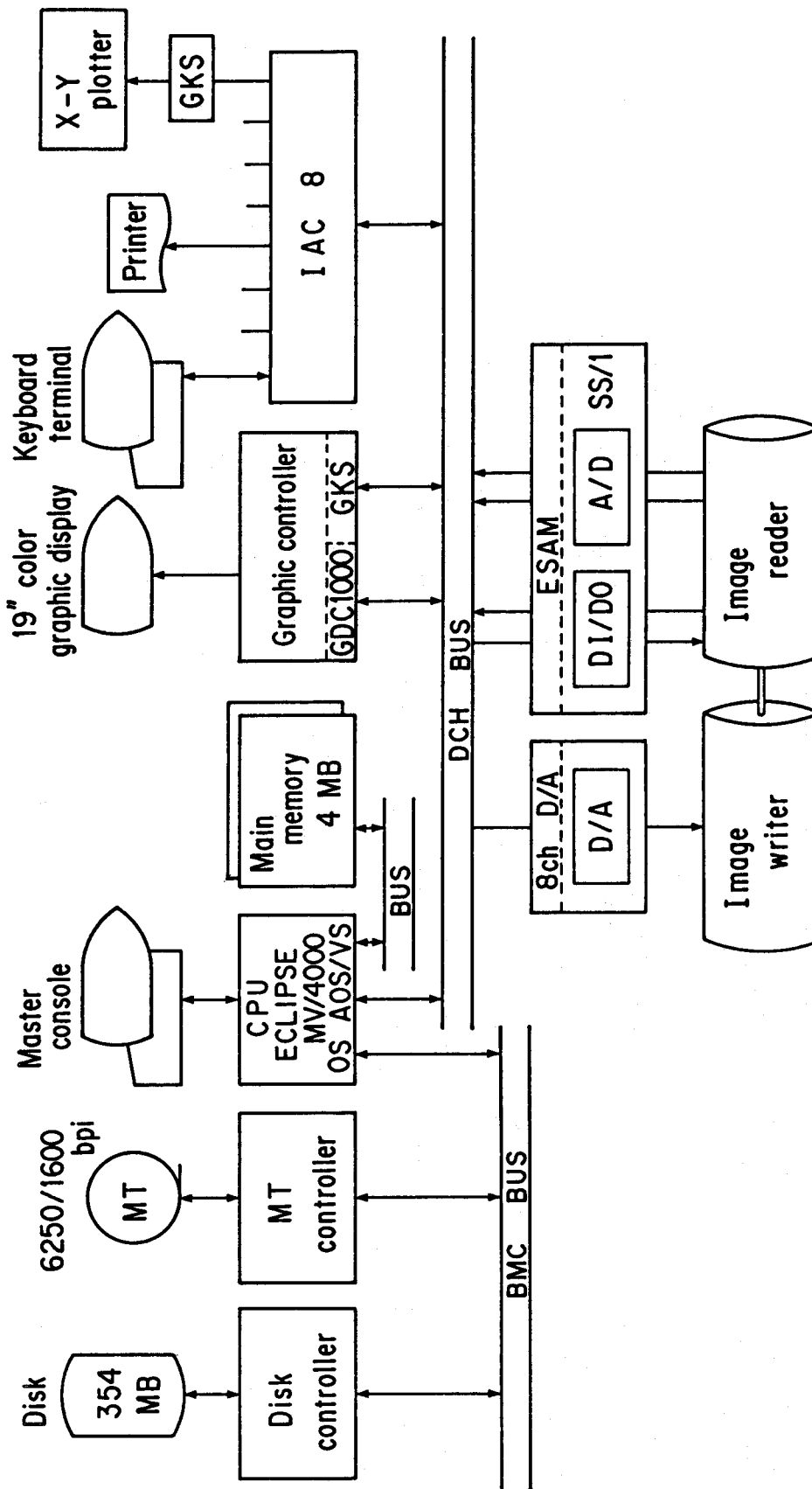


Figure A-1

Block diagram of the data handling components of the imaging plate system
based on ECLIPSE MV/4000

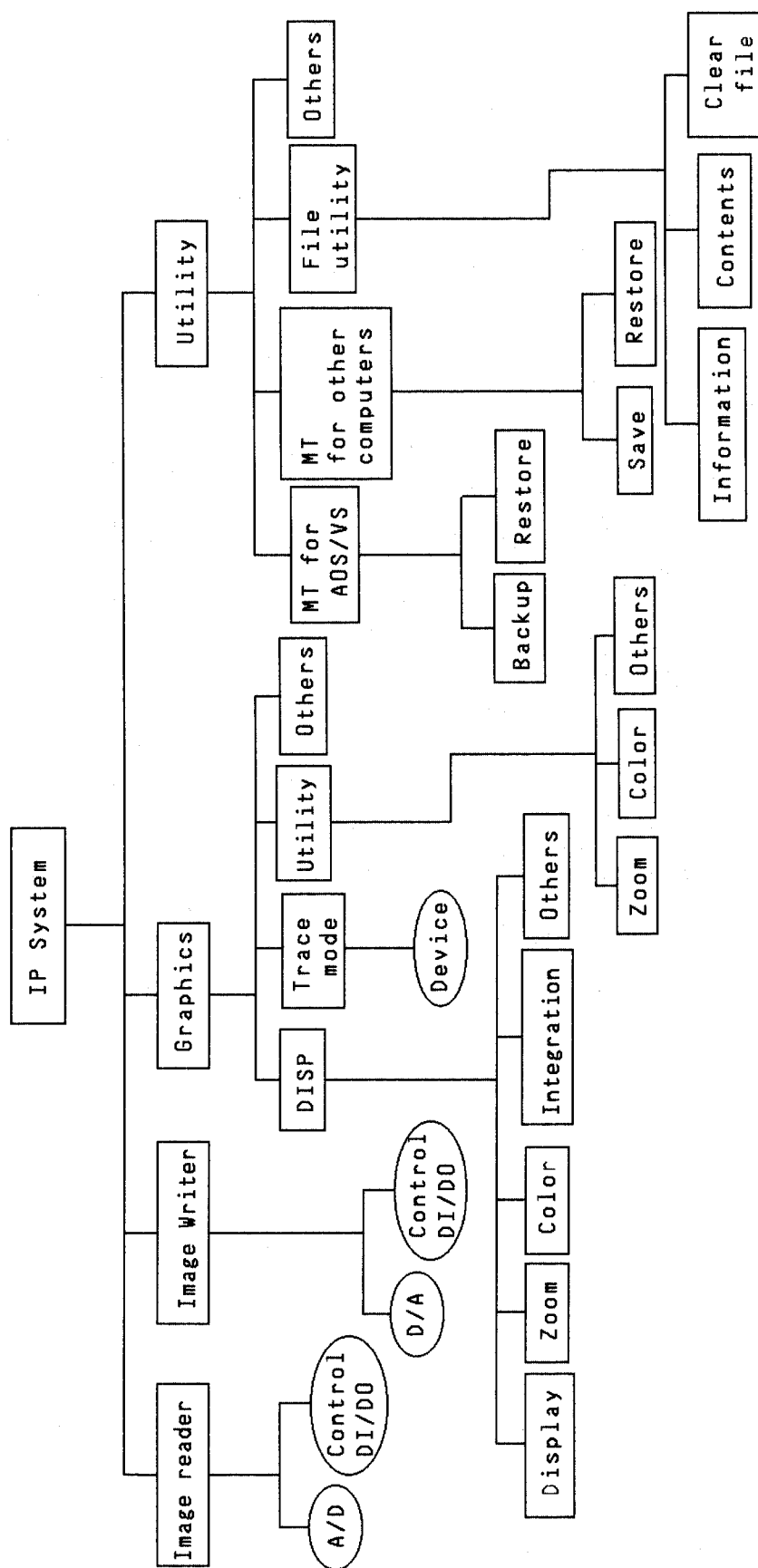


Figure A-2

Tree structure of the software system for the imaging plate system at Photon Factory

•MT format

1. AOS/VS DUMP_II format
2. ANSI standard (ASCII Code)
 - No Label, 6250bpi
 - Record length : 512 byte
 - Block size : 8192 byte

•Header record (Type 1) (512 byte)

1. HEAD	Character*4	'HDR1' ¹⁾
2. <i>null</i>	Character*4	(0000)h
3. SAMPLE	Character*64	Sample name
4. TITLE	Character*128	Title
5. DATE(3)	Integer*4	Date
6. TIME(3)	Integer*4	Time
7. IXPIX	Integer*4	Pixel number along X-direction
8. IYPIX	Integer*4	Pixel number along Y-direction
9. IPIXEL	Integer*4	Total pixel number
10. ISTEP	Integer*2	Raster step size ²⁾
11. ISPEED	Integer*2	Scanning speed ³⁾
12. IHV	Integer*2	High voltage for Main-PMT
13. IGAIN	Integer*2	A/D gain ⁴⁾ for Main-PMT
14. ISUB	Integer*2	Use Sub-PMT ⁵⁾
15. IHV2	Integer*2	High voltage for Sub-PMT
16. IGAIN2	Integer*2	A/D gain ⁴⁾ for Sub-PMT
17. <i>others</i>	282 byte	Keep for future use (must be 0)

1) Indicate IP-data file TYPE1.

2) = 0 ... 250 (line/inch)
 = 1 ... 500
 = 2 ... 1000

3) = 0 ... 2.5 (r.p.s)
 = 1 ... 5.0
 = 2 ... 10.0

4) = 0 ... ± 10 V
 = 1 ... ± 5
 = 2 ... ± 2.5
 = 3 ... ± 1.25

5) = 0 ... Only Main-PMT
 = 1 ... Use Main- and Sub-PMT

Table A-1

MT Format of Imaging Plate Data

system read header block at the beginning of the program and determine the default conditions for the data on the file.

II. Image Reader

The output of the photomultiplier tubes are logarithmically amplified and digitized by 100KHz, 12bit A/D converters. The digitized data are sent to disk memories via triple chaining buffer. A user must input only 1) sample name, 2) comment, 3) size of the imaging plate which is selected from menu. As the pixel size of the data is limited by the character of the imaging plate itself, only 100mm² pixel size can be read now. And even now, the data detected by sub-photomultiplier cannot read-out according to the limitation of sampling clock and only one photomultiplier mode can be used. As following operations; a) opening the shutter of the laser beam, b) operating the switch of high voltage power suppliers for photomultipliers, c) starting and stopping rotation of drum and d) reset and start of digital counter to count number of pixels are controlled by the host computer automatically using DI/DO (digital input and output) functions, users must be needed just mounting the imaging plate onto the drum. The SS/1 board on the computer and ESAM which is a software to control SS/1 board are used for both A/D and DI/DO functions.

III. Image Writer

The digital data on the computer are reconstructed by 100KHz, 12bit D/A converter into a time-series of analogue signals, which modify the intensity of a glow lamp, and it implements the image on a photographic film to get a precise reproduction of the X-ray image. D/A converter is used HSDA

board and 8ch D/A converter which is a software system to control HSDA board.

Present version of the software for the image writer reconstructs the image data as stored in the computer and does not do anything such as enhancement of image and so on.

IV. Graphics

19-inch raster-type color graphic display and X-Y plotter are connected with the host computer and the graphics system is used to support the interpretation of stored data.

Graphic system is roughly speaking composed of image display program and image scanning program. Image display program displays two-dimensional image data as two-dimensional graphics and intensities are expressed according to blackness or special color tables. Image scanning program displays intensities of one-direction scanning of data using line graph. Image display program is used the graphics package, GDC1000, which is a graphics system to support a raster graphics of AOS/VS system, and image scanning program is using the graphics package, GKS, graphic kernel system. AOS/VS operating system supports the GKS as a graphics package to control many kind of devices. 19-inch color raster graphics display, X-Y plotter and Kanji terminal can be used as output devices of this system.

Image display program is useful to recognize the images on the file which image reader has read-out. A users must input both the threshold value and the latitude to determine the color table at first. These two values are always determined at first considering the output monitor, which is a synchronous scope, connected with the output of the image reader. This

program also supports some display utilities, zooming, translation, changing color level and/or color table, integration of pixel data. These functions are selected from menu or by using the optical mouse. Image scanning mode is useful to recognize the one-dimensional data, such as powder diffraction or compton scattering and so on, on the imaging plate as as a two-dimensional detector. A user chooses data scanning direction, either drum scanning direction or drum rotating direction, and which lines to be sum up. And a user can select the output device, graphics display, X-Y plotter or Kanji terminal.

V. Utilities

This system has utilities for displaying or changing of file header block. And the utilities to save the data on the disk to the magnetic tape and restore the data on magnetic tape to the disk are also available using both AOS/VS backup format and no label ANSI standard format tape for other computers.

All these programs in the system are connected by using CLI (command line interpreter) and all of them can be executed by just only selected the item from menu-style dialogue command to be possible to use this system by anyone who does not familiar to both/either AOS/VS operating system and/or ECLIPSE MV/4000 hardware system.

B. Atomic Paramters of Cytochrome c553

COMPND	CYTOCHROME \$C=553= (OXIDIZED)										
SOURCE	(DESULFOVIBRIO VULGARIS) MIYAZAKI F										
CRYST	42.700	42.700	103.400	90.00	90.00	90.00	P	43	21	2	8
ORIGX1	1.000000	0.000000	0.000000			0.000000					
ORIGX2	0.000000	1.000000	0.000000			0.000000					
ORIGX3	0.000000	0.000000	1.000000			0.000000					
SCALE1	0.023419	0.000000	0.000000			0.000000					
SCALE2	0.000000	0.023419	0.000000			0.000000					
SCALE3	0.000000	0.000000	0.009671			0.000000					
ATOM	1	N	ALA	1	15.669	0.963	34.494	1.00	23.53		
ATOM	2	CA	ALA	1	15.919	-0.028	33.417	1.00	19.56		
ATOM	3	C	ALA	1	17.323	-0.575	33.455	1.00	7.27		
ATOM	4	O	ALA	1	18.374	0.073	33.290	1.00	18.81		
ATOM	5	CB	ALA	1	15.554	0.719	32.096	1.00	21.69		
ATOM	6	N	ASP	2	17.324	-1.836	33.815	1.00	11.41		
ATOM	7	CA	ASP	2	18.513	-2.628	34.018	1.00	12.07		
ATOM	8	C	ASP	2	18.843	-3.473	32.752	1.00	12.53		
ATOM	9	O	ASP	2	18.183	-4.489	32.605	1.00	13.93		
ATOM	10	CB	ASP	2	18.256	-3.582	35.202	1.00	19.29		
ATOM	11	CG	ASP	2	19.356	-4.410	35.738	1.00	31.41		
ATOM	12	OD1	ASP	2	19.430	-4.720	36.980	1.00	25.93		
ATOM	13	OD2	ASP	2	20.240	-4.813	34.956	1.00	22.76		
ATOM	14	N	GLY	3	19.855	-2.927	32.107	1.00	17.16		
ATOM	15	CA	GLY	3	20.243	-3.572	30.815	1.00	21.94		
ATOM	16	C	GLY	3	20.489	-5.034	30.929	1.00	25.11		
ATOM	17	O	GLY	3	20.052	-5.864	30.106	1.00	20.00		
ATOM	18	N	ALA	4	21.343	-5.439	31.880	1.00	15.65		
ATOM	19	CA	ALA	4	21.625	-6.829	32.119	1.00	14.44		
ATOM	20	C	ALA	4	20.413	-7.694	32.388	1.00	16.41		
ATOM	21	O	ALA	4	20.372	-8.858	31.925	1.00	17.83		
ATOM	22	CB	ALA	4	22.692	-6.812	33.224	1.00	17.89		
ATOM	23	N	ALA	5	19.385	-7.329	33.222	1.00	12.75		
ATOM	24	CA	ALA	5	18.241	-8.170	33.473	1.00	13.32		
ATOM	25	C	ALA	5	17.335	-8.264	32.159	1.00	13.16		
ATOM	26	O	ALA	5	16.826	-9.360	32.011	1.00	16.68		
ATOM	27	CB	ALA	5	17.366	-7.515	34.568	1.00	21.96		
ATOM	28	N	LEU	6	17.221	-7.074	31.573	1.00	16.47		
ATOM	29	CA	LEU	6	16.336	-7.144	30.312	1.00	14.79		
ATOM	30	C	LEU	6	17.010	-8.058	29.268	1.00	14.44		
ATOM	31	O	LEU	6	16.232	-8.784	28.606	1.00	14.77		
ATOM	32	CB	LEU	6	16.278	-5.683	29.892	1.00	15.76		
ATOM	33	CG	LEU	6	15.412	-4.781	30.777	1.00	17.07		
ATOM	34	CD1	LEU	6	15.660	-3.335	30.432	1.00	16.71		
ATOM	35	CD2	LEU	6	13.943	-5.186	30.625	1.00	22.75		
ATOM	36	N	TYR	7	18.297	-7.963	29.191	1.00	17.57		
ATOM	37	CA	TYR	7	19.016	-8.819	28.191	1.00	12.58		
ATOM	38	C	TYR	7	18.775	-10.271	28.329	1.00	20.19		
ATOM	39	O	TYR	7	19.006	-11.101	27.385	1.00	17.04		
ATOM	40	CB	TYR	7	20.463	-8.377	28.006	1.00	17.69		

ATOM	41	CG	TYR	7	21.165	-9.154	26.882	1.00	13.21
ATOM	42	CD1	TYR	7	21.993	-10.238	27.082	1.00	11.32
ATOM	43	CD2	TYR	7	20.993	-8.721	25.544	1.00	29.50
ATOM	44	CE1	TYR	7	22.680	-10.953	26.109	1.00	22.51
ATOM	45	CE2	TYR	7	21.697	-9.395	24.563	1.00	14.96
ATOM	46	CZ	TYR	7	22.448	-10.506	24.767	1.00	20.06
ATOM	47	OH	TYR	7	23.189	-11.163	23.812	1.00	20.09
ATOM	48	N	LYS	8	18.349	-10.827	29.529	1.00	14.11
ATOM	49	CA	LYS	8	18.153	-12.221	29.620	1.00	9.37
ATOM	50	C	LYS	8	17.102	-12.834	28.665	1.00	10.42
ATOM	51	O	LYS	8	17.186	-14.034	28.534	1.00	16.68
ATOM	52	CB	LYS	8	17.712	-12.582	31.112	1.00	16.54
ATOM	53	CG	LYS	8	18.842	-11.967	31.974	1.00	22.76
ATOM	54	CD	LYS	8	19.845	-13.046	32.324	1.00	31.43
ATOM	55	CE	LYS	8	20.629	-12.683	33.571	1.00	62.66
ATOM	56	NZ	LYS	8	21.698	-11.716	33.204	1.00	64.53
ATOM	57	N	SER	9	16.198	-11.985	28.193	1.00	9.11
ATOM	58	CA	SER	9	15.120	-12.432	27.293	1.00	13.20
ATOM	59	C	SER	9	15.773	-12.529	25.840	1.00	16.01
ATOM	60	O	SER	9	15.017	-13.139	25.053	1.00	20.50
ATOM	61	CB	SER	9	14.093	-11.316	27.203	1.00	13.74
ATOM	62	OG	SER	9	13.270	-11.387	28.401	1.00	26.24
ATOM	63	N	CYS	10	16.940	-12.073	25.692	1.00	20.89
ATOM	64	CA	CYS	10	17.613	-12.025	24.368	1.00	16.43
ATOM	65	C	CYS	10	18.584	-13.120	24.064	1.00	39.01
ATOM	66	O	CYS	10	18.941	-13.361	22.908	1.00	21.05
ATOM	67	CB	CYS	10	18.401	-10.690	24.357	1.00	12.51
ATOM	68	SG	CYS	10	17.403	-9.292	24.785	1.00	16.26
ATOM	69	N	VAL	11	19.103	-13.803	25.027	1.00	17.21
ATOM	70	CA	VAL	11	20.126	-14.809	25.039	1.00	17.82
ATOM	71	C	VAL	11	19.890	-16.048	24.218	1.00	16.34
ATOM	72	O	VAL	11	20.905	-16.607	23.757	1.00	27.82
ATOM	73	CB	VAL	11	20.283	-15.103	26.613	1.00	18.62
ATOM	74	CG1	VAL	11	20.655	-16.521	26.865	1.00	23.51
ATOM	75	CG2	VAL	11	21.228	-14.071	27.164	1.00	23.25
ATOM	76	N	GLY	12	18.698	-16.509	24.061	1.00	18.74
ATOM	77	CA	GLY	12	18.329	-17.705	23.308	1.00	28.61
ATOM	78	C	GLY	12	18.938	-17.500	21.873	1.00	21.02
ATOM	79	O	GLY	12	19.680	-18.385	21.475	1.00	27.55
ATOM	80	N	CYS	13	18.622	-16.343	21.356	1.00	23.06
ATOM	81	CA	CYS	13	19.130	-16.035	19.974	1.00	22.72
ATOM	82	C	CYS	13	20.453	-15.336	19.848	1.00	31.02
ATOM	83	O	CYS	13	21.206	-15.639	18.880	1.00	26.86
ATOM	84	CB	CYS	13	18.002	-15.267	19.285	1.00	23.72
ATOM	85	SG	CYS	13	16.398	-16.102	19.252	1.00	24.12
ATOM	86	N	HIS	14	20.809	-14.401	20.726	1.00	17.83
ATOM	87	CA	HIS	14	22.033	-13.610	20.639	1.00	12.25
ATOM	88	C	HIS	14	23.275	-14.106	21.360	1.00	17.12
ATOM	89	O	HIS	14	24.398	-13.579	21.238	1.00	20.88
ATOM	90	CB	HIS	14	21.648	-12.180	20.984	1.00	13.30
ATOM	91	CG	HIS	14	20.673	-11.588	20.008	1.00	14.19
ATOM	92	ND1	HIS	14	21.314	-11.139	18.825	1.00	23.80

ATOM	93	CE1	HIS	14	20.369	-10.625	18.111	1.00	15.10
ATOM	94	NE2	HIS	14	19.124	-10.731	18.573	1.00	14.03
ATOM	95	CD2	HIS	14	19.385	-11.392	19.837	1.00	15.65
ATOM	96	N	GLY	15	23.018	-15.121	22.199	1.00	25.55
ATOM	97	CA	GLY	15	24.082	-15.655	23.082	1.00	35.15
ATOM	98	C	GLY	15	24.300	-14.749	24.310	1.00	27.32
ATOM	99	O	GLY	15	24.048	-13.546	24.386	1.00	22.59
ATOM	100	N	ALA	16	24.888	-15.381	25.325	1.00	43.31
ATOM	101	CA	ALA	16	25.199	-14.686	26.595	1.00	22.18
ATOM	102	C	ALA	16	26.279	-13.683	26.390	1.00	24.77
ATOM	103	O	ALA	16	26.372	-12.670	27.109	1.00	24.70
ATOM	104	CB	ALA	16	25.638	-15.738	27.616	1.00	38.06
ATOM	105	N	ASP	17	27.130	-13.936	25.369	1.00	25.66
ATOM	106	CA	ASP	17	28.191	-12.965	25.081	1.00	21.67
ATOM	107	C	ASP	17	27.838	-12.056	23.867	1.00	24.07
ATOM	108	O	ASP	17	28.628	-11.158	23.537	1.00	31.01
ATOM	109	CB	ASP	17	29.591	-13.523	24.961	1.00	29.96
ATOM	110	CG	ASP	17	29.765	-14.525	23.838	1.00	28.82
ATOM	111	OD1	ASP	17	28.799	-15.038	23.263	1.00	35.69
ATOM	112	OD2	ASP	17	30.959	-14.810	23.614	1.00	47.96
ATOM	113	N	GLY	18	26.681	-12.286	23.315	1.00	27.34
ATOM	114	CA	GLY	18	26.135	-11.505	22.188	1.00	21.78
ATOM	115	C	GLY	18	26.895	-11.778	20.900	1.00	36.39
ATOM	116	O	GLY	18	26.673	-10.972	19.971	1.00	23.40
ATOM	117	N	SER	19	27.669	-12.833	20.861	1.00	27.09
ATOM	118	CA	SER	19	28.472	-13.051	19.623	1.00	37.70
ATOM	119	C	SER	19	27.810	-13.927	18.589	1.00	31.97
ATOM	120	O	SER	19	28.400	-14.245	17.523	1.00	31.34
ATOM	121	CB	SER	19	29.808	-13.656	20.068	1.00	33.40
ATOM	122	OG	SER	19	29.488	-14.996	20.440	1.00	28.40
ATOM	123	N	LYS	20	26.598	-14.345	18.837	1.00	24.11
ATOM	124	CA	LYS	20	25.882	-15.272	17.927	1.00	28.97
ATOM	125	C	LYS	20	25.179	-14.541	16.800	1.00	31.14
ATOM	126	O	LYS	20	24.678	-13.420	16.927	1.00	29.03
ATOM	127	CB	LYS	20	25.005	-16.118	18.790	1.00	27.86
ATOM	128	CG	LYS	20	24.290	-17.379	18.395	1.00	74.42
ATOM	129	CD	LYS	20	23.742	-18.049	19.662	1.00	59.70
ATOM	130	CE	LYS	20	22.744	-19.143	19.343	1.00	74.66
ATOM	131	NZ	LYS	20	22.215	-19.746	20.595	1.00	100.03
ATOM	132	N	GLN	21	25.225	-15.167	15.624	1.00	29.45
ATOM	133	CA	GLN	21	24.528	-14.595	14.431	1.00	24.26
ATOM	134	C	GLN	21	23.129	-15.177	14.595	1.00	18.58
ATOM	135	O	GLN	21	22.888	-16.375	14.299	1.00	27.06
ATOM	136	CB	GLN	21	25.206	-15.083	13.131	1.00	28.19
ATOM	137	CG	GLN	21	25.124	-14.033	12.050	1.00	32.30
ATOM	138	CD	GLN	21	25.356	-14.591	10.643	1.00	30.17
ATOM	139	OE1	GLN	21	25.176	-13.862	9.669	1.00	30.05
ATOM	140	NE2	GLN	21	25.576	-15.886	10.646	1.00	19.57
ATOM	141	N	ALA	22	22.239	-14.327	15.065	1.00	21.08
ATOM	142	CA	ALA	22	20.878	-14.781	15.439	1.00	23.89
ATOM	143	C	ALA	22	20.158	-15.419	14.251	1.00	29.50
ATOM	144	O	ALA	22	20.005	-14.763	13.215	1.00	23.33

ATOM	145	CB	ALA	22	20.074	-13.641	16.039	1.00	23.60
ATOM	146	N	MET	23	19.738	-16.638	14.410	1.00	19.61
ATOM	147	CA	MET	23	18.956	-17.411	13.433	1.00	20.92
ATOM	148	C	MET	23	19.714	-17.457	12.065	1.00	21.07
ATOM	149	O	MET	23	19.050	-17.484	11.031	1.00	32.83
ATOM	150	CB	MET	23	17.554	-16.850	13.284	1.00	20.97
ATOM	151	CG	MET	23	16.853	-16.752	14.632	1.00	30.91
ATOM	152	SD	MET	23	15.141	-16.216	14.414	1.00	29.90
ATOM	153	CE	MET	23	14.263	-17.728	14.704	1.00	36.20
ATOM	154	N	GLY	24	21.004	-17.393	12.154	1.00	24.60
ATOM	155	CA	GLY	24	21.979	-17.404	11.074	1.00	29.56
ATOM	156	C	GLY	24	21.866	-16.294	10.057	1.00	29.76
ATOM	157	O	GLY	24	22.196	-16.541	8.883	1.00	35.36
ATOM	158	N	VAL	25	21.440	-15.111	10.372	1.00	21.17
ATOM	159	CA	VAL	25	21.281	-13.927	9.512	1.00	20.21
ATOM	160	C	VAL	25	21.873	-12.727	10.241	1.00	50.87
ATOM	161	O	VAL	25	21.859	-12.698	11.487	1.00	21.77
ATOM	162	CB	VAL	25	19.752	-13.762	9.342	1.00	22.90
ATOM	163	CG1	VAL	25	19.256	-12.410	8.872	1.00	31.11
ATOM	164	CG2	VAL	25	19.204	-14.913	8.522	1.00	26.05
ATOM	165	N	GLY	26	22.359	-11.758	9.505	1.00	22.94
ATOM	166	CA	GLY	26	22.802	-10.489	10.069	1.00	20.20
ATOM	167	C	GLY	26	24.059	-10.424	10.880	1.00	20.41
ATOM	168	O	GLY	26	24.987	-11.223	10.714	1.00	24.68
ATOM	169	N	HIS	27	24.090	-9.447	11.802	1.00	20.15
ATOM	170	CA	HIS	27	25.295	-9.134	12.548	1.00	17.08
ATOM	171	C	HIS	27	25.100	-9.385	14.092	1.00	19.80
ATOM	172	O	HIS	27	23.977	-9.150	14.505	1.00	20.06
ATOM	173	CB	HIS	27	25.542	-7.643	12.315	1.00	14.84
ATOM	174	CG	HIS	27	25.482	-7.277	10.817	1.00	28.82
ATOM	175	ND1	HIS	27	26.615	-7.372	10.030	1.00	28.86
ATOM	176	CE1	HIS	27	26.228	-6.925	8.824	1.00	22.19
ATOM	177	NE2	HIS	27	24.943	-6.611	8.759	1.00	19.15
ATOM	178	CD2	HIS	27	24.454	-6.837	10.054	1.00	27.66
ATOM	179	N	ALA	28	26.129	-9.866	14.707	1.00	32.77
ATOM	180	CA	ALA	28	26.090	-10.150	16.180	1.00	15.59
ATOM	181	C	ALA	28	26.097	-8.830	16.893	1.00	33.48
ATOM	182	O	ALA	28	26.684	-7.901	16.339	1.00	21.69
ATOM	183	CB	ALA	28	27.310	-11.016	16.492	1.00	18.23
ATOM	184	N	VAL	29	25.506	-8.703	18.083	1.00	20.76
ATOM	185	CA	VAL	29	25.467	-7.361	18.733	1.00	19.62
ATOM	186	C	VAL	29	26.794	-6.980	19.384	1.00	13.08
ATOM	187	O	VAL	29	27.020	-5.799	19.603	1.00	21.13
ATOM	188	CB	VAL	29	24.247	-7.373	19.677	1.00	23.63
ATOM	189	CG1	VAL	29	22.969	-7.755	18.920	1.00	18.68
ATOM	190	CG2	VAL	29	24.497	-8.440	20.777	1.00	18.26
ATOM	191	N	LYS	30	27.556	-7.992	19.667	1.00	23.01
ATOM	192	CA	LYS	30	28.848	-7.878	20.341	1.00	26.61
ATOM	193	C	LYS	30	29.839	-7.107	19.482	1.00	30.94
ATOM	194	O	LYS	30	30.078	-7.447	18.313	1.00	52.69
ATOM	195	CB	LYS	30	29.431	-9.254	20.663	1.00	28.14
ATOM	196	CG	LYS	30	30.805	-9.255	21.339	1.00	29.95

ATOM	197	CD	LYS	30	31.387	-10.665	21.401	1.00	32.34
ATOM	198	CE	LYS	30	32.726	-10.664	22.175	1.00	28.50
ATOM	199	NZ	LYS	30	32.920	-12.061	22.659	1.00	94.77
ATOM	200	N	GLY	31	30.322	-6.040	20.062	1.00	26.03
ATOM	201	CA	GLY	31	31.363	-5.181	19.498	1.00	33.42
ATOM	202	C	GLY	31	30.841	-3.840	19.026	1.00	36.56
ATOM	203	O	GLY	31	31.646	-2.950	18.703	1.00	52.68
ATOM	204	N	GLN	32	29.533	-3.717	18.981	1.00	26.87
ATOM	205	CA	GLN	32	28.870	-2.506	18.485	1.00	21.32
ATOM	206	C	GLN	32	28.754	-1.445	19.560	1.00	41.90
ATOM	207	O	GLN	32	28.745	-1.735	20.761	1.00	37.65
ATOM	208	CB	GLN	32	27.539	-2.865	17.801	1.00	28.78
ATOM	209	CG	GLN	32	27.636	-3.882	16.654	1.00	30.30
ATOM	210	CD	GLN	32	26.328	-3.940	15.889	1.00	14.83
ATOM	211	OE1	GLN	32	25.788	-2.897	15.519	1.00	24.33
ATOM	212	NE2	GLN	32	25.702	-5.087	15.670	1.00	21.86
ATOM	213	N	LYS	33	28.669	-0.215	19.136	1.00	27.08
ATOM	214	CA	LYS	33	28.560	0.990	19.942	1.00	29.49
ATOM	215	C	LYS	33	27.125	1.133	20.418	1.00	40.09
ATOM	216	O	LYS	33	26.189	0.938	19.638	1.00	27.52
ATOM	217	CB	LYS	33	28.981	2.234	19.157	1.00	38.70
ATOM	218	CG	LYS	33	30.481	2.252	18.910	1.00	39.05
ATOM	219	CD	LYS	33	30.980	3.613	18.446	1.00	64.26
ATOM	220	CE	LYS	33	32.416	3.538	17.957	1.00	108.72
ATOM	221	NZ	LYS	33	32.745	4.733	17.116	1.00	104.93
ATOM	222	N	ALA	34	26.921	1.527	21.664	1.00	23.76
ATOM	223	CA	ALA	34	25.562	1.667	22.173	1.00	26.24
ATOM	224	CB	ALA	34	25.628	2.257	23.579	1.00	29.56
ATOM	225	C	ALA	34	24.672	2.506	21.275	1.00	16.86
ATOM	226	O	ALA	34	23.488	2.213	21.138	1.00	20.35
ATOM	227	N	ASP	35	25.141	3.669	20.884	1.00	21.25
ATOM	228	CA	ASP	35	24.339	4.567	20.027	1.00	23.50
ATOM	229	CB	ASP	35	25.253	5.749	19.617	1.00	24.78
ATOM	230	CG	ASP	35	25.254	6.874	20.637	1.00	114.95
ATOM	231	OD1	ASP	35	24.507	6.826	21.631	1.00	82.07
ATOM	232	OD2	ASP	35	26.033	7.827	20.394	1.00	91.56
ATOM	233	C	ASP	35	23.854	3.766	18.777	1.00	15.56
ATOM	234	O	ASP	35	22.666	3.930	18.419	1.00	23.34
ATOM	235	N	GLU	36	24.788	3.001	18.278	1.00	28.75
ATOM	236	CA	GLU	36	24.494	2.201	17.058	1.00	35.03
ATOM	237	CB	GLU	36	25.751	1.614	16.482	1.00	25.68
ATOM	238	CG	GLU	36	25.872	0.172	16.038	1.00	106.65
ATOM	239	CD	GLU	36	27.004	-0.164	15.108	1.00	118.71
ATOM	240	OE1	GLU	36	27.011	0.064	13.907	1.00	81.37
ATOM	241	OE2	GLU	36	27.952	-0.727	15.698	1.00	91.68
ATOM	242	C	GLU	36	23.460	1.143	17.326	1.00	34.60
ATOM	243	O	GLU	36	22.476	1.039	16.605	1.00	20.81
ATOM	244	N	LEU	37	23.599	0.370	18.386	1.00	18.97
ATOM	245	CA	LEU	37	22.635	-0.660	18.786	1.00	24.73
ATOM	246	CB	LEU	37	23.334	-1.466	19.893	1.00	18.32
ATOM	247	CG	LEU	37	24.519	-2.317	19.524	1.00	16.73
ATOM	248	CD1	LEU	37	25.275	-2.741	20.805	1.00	32.07

ATOM	249	CD2	LEU	37	24.050	-3.598	18.828	1.00	18.93
ATOM	250	C	LEU	37	21.313	-0.020	19.085	1.00	13.62
ATOM	251	O	LEU	37	20.243	-0.572	18.778	1.00	18.94
ATOM	252	N	PHE	38	21.263	1.165	19.724	1.00	16.32
ATOM	253	CA	PHE	38	20.001	1.808	20.045	1.00	17.83
ATOM	254	CB	PHE	38	20.234	2.917	21.081	1.00	17.23
ATOM	255	CG	PHE	38	18.972	3.640	21.424	1.00	23.40
ATOM	256	CD1	PHE	38	17.874	2.946	21.896	1.00	28.19
ATOM	257	CE1	PHE	38	16.682	3.595	22.173	1.00	32.71
ATOM	258	CZ	PHE	38	16.580	4.970	21.988	1.00	45.81
ATOM	259	CE2	PHE	38	17.686	5.695	21.520	1.00	50.17
ATOM	260	CD2	PHE	38	18.876	5.014	21.241	1.00	40.33
ATOM	261	C	PHE	38	19.211	2.240	18.804	1.00	15.92
ATOM	262	O	PHE	38	18.002	1.992	18.715	1.00	18.46
ATOM	263	N	LYS	39	19.960	2.784	17.854	1.00	32.70
ATOM	264	CA	LYS	39	19.338	3.200	16.579	1.00	19.13
ATOM	265	CB	LYS	39	20.419	3.775	15.660	1.00	23.10
ATOM	266	CG	LYS	39	20.365	5.278	15.410	1.00	57.99
ATOM	267	CD	LYS	39	20.777	5.567	13.970	1.00	73.23
ATOM	268	CE	LYS	39	21.773	6.698	13.841	1.00	102.75
ATOM	269	NZ	LYS	39	22.137	6.928	12.413	1.00	99.02
ATOM	270	C	LYS	39	18.711	2.014	15.856	1.00	20.80
ATOM	271	O	LYS	39	17.601	2.128	15.281	1.00	23.54
ATOM	272	N	LYS	40	19.433	0.901	15.926	1.00	16.94
ATOM	273	CA	LYS	40	18.948	-0.345	15.289	1.00	17.33
ATOM	274	CB	LYS	40	19.980	-1.424	15.172	1.00	21.37
ATOM	275	CG	LYS	40	21.126	-1.065	14.203	1.00	14.59
ATOM	276	CD	LYS	40	22.292	-1.969	14.262	1.00	14.71
ATOM	277	CE	LYS	40	23.315	-1.604	13.173	1.00	16.76
ATOM	278	NZ	LYS	40	24.411	-2.586	13.174	1.00	17.75
ATOM	279	C	LYS	40	17.675	-0.829	15.948	1.00	39.03
ATOM	280	O	LYS	40	16.712	-1.180	15.246	1.00	19.82
ATOM	281	N	LEU	41	17.629	-0.834	17.288	1.00	19.61
ATOM	282	CA	LEU	41	16.401	-1.269	17.981	1.00	17.46
ATOM	283	CB	LEU	41	16.684	-1.300	19.529	1.00	17.39
ATOM	284	CG	LEU	41	17.704	-2.284	19.989	1.00	18.49
ATOM	285	CD1	LEU	41	18.095	-2.046	21.471	1.00	22.18
ATOM	286	CD2	LEU	41	17.243	-3.732	19.809	1.00	19.21
ATOM	287	C	LEU	41	15.200	-0.429	17.620	1.00	7.85
ATOM	288	O	LEU	41	14.107	-0.954	17.356	1.00	17.45
ATOM	289	N	LYS	42	15.399	0.858	17.610	1.00	13.10
ATOM	290	CA	LYS	42	14.359	1.828	17.247	1.00	23.58
ATOM	291	CB	LYS	42	14.769	3.268	17.436	1.00	24.02
ATOM	292	CG	LYS	42	15.036	3.625	18.914	1.00	34.89
ATOM	293	CD	LYS	42	14.195	4.827	19.323	1.00	43.46
ATOM	294	CE	LYS	42	12.715	4.460	19.289	1.00	52.82
ATOM	295	NZ	LYS	42	11.906	5.710	19.363	1.00	62.13
ATOM	296	C	LYS	42	14.001	1.574	15.760	1.00	19.87
ATOM	297	O	LYS	42	12.810	1.609	15.510	1.00	20.69
ATOM	298	N	GLY	43	14.985	1.210	14.971	1.00	30.27
ATOM	299	CA	GLY	43	14.718	0.891	13.551	1.00	20.59
ATOM	300	C	GLY	43	13.765	-0.264	13.370	1.00	19.43

ATOM	301	O	GLY	43	12.938	-0.327	12.455	1.00	35.24
ATOM	302	N	TYR	44	13.938	-1.285	14.183	1.00	21.44
ATOM	303	CA	TYR	44	13.099	-2.474	14.157	1.00	19.10
ATOM	304	CB	TYR	44	13.653	-3.587	15.035	1.00	16.58
ATOM	305	CG	TYR	44	14.780	-4.407	14.472	1.00	19.32
ATOM	306	CD1	TYR	44	14.584	-5.138	13.268	1.00	22.17
ATOM	307	CE1	TYR	44	15.600	-5.918	12.798	1.00	18.13
ATOM	308	CZ	TYR	44	16.881	-5.896	13.311	1.00	16.17
ATOM	309	OH	TYR	44	17.892	-6.660	12.850	1.00	18.89
ATOM	310	CE2	TYR	44	17.117	-5.166	14.499	1.00	21.30
ATOM	311	CD2	TYR	44	16.055	-4.406	14.966	1.00	12.49
ATOM	312	C	TYR	44	11.665	-2.081	14.501	1.00	31.72
ATOM	313	O	TYR	44	10.699	-2.714	14.041	1.00	34.88
ATOM	314	N	ALA	45	11.567	-1.046	15.317	1.00	40.74
ATOM	315	CA	ALA	45	10.296	-0.485	15.785	1.00	56.96
ATOM	316	CB	ALA	45	10.442	0.371	17.029	1.00	46.36
ATOM	317	C	ALA	45	9.563	0.238	14.660	1.00	31.51
ATOM	318	O	ALA	45	8.395	-0.171	14.499	1.00	53.74
ATOM	319	N	ASP	46	10.162	1.165	13.929	1.00	45.22
ATOM	320	CA	ASP	46	9.520	1.851	12.803	1.00	41.37
ATOM	321	CB	ASP	46	10.183	3.139	12.307	1.00	44.23
ATOM	322	CG	ASP	46	11.665	3.195	12.621	1.00	38.68
ATOM	323	OD1	ASP	46	12.204	4.238	13.027	1.00	71.33
ATOM	324	OD2	ASP	46	12.262	2.106	12.503	1.00	77.86
ATOM	325	C	ASP	46	9.386	0.919	11.586	1.00	45.54
ATOM	326	O	ASP	46	8.501	1.146	10.755	1.00	96.85
ATOM	327	N	GLY	47	10.265	-0.047	11.501	1.00	51.07
ATOM	328	CA	GLY	47	10.333	-1.015	10.402	1.00	44.64
ATOM	329	C	GLY	47	11.464	-0.602	9.446	1.00	49.78
ATOM	330	O	GLY	47	11.818	-1.309	8.489	1.00	64.96
ATOM	331	N	SER	48	12.033	0.546	9.735	1.00	43.30
ATOM	332	CA	SER	48	13.118	1.202	8.997	1.00	27.69
ATOM	333	CB	SER	48	13.185	2.642	9.495	1.00	32.40
ATOM	334	OG	SER	48	14.276	2.977	10.319	1.00	44.67
ATOM	335	C	SER	48	14.471	0.522	9.002	1.00	46.26
ATOM	336	O	SER	48	15.372	0.933	8.231	1.00	32.60
ATOM	337	N	TYR	49	14.684	-0.472	9.847	1.00	28.07
ATOM	338	CA	TYR	49	15.963	-1.188	9.900	1.00	28.48
ATOM	339	CB	TYR	49	16.808	-0.797	11.168	1.00	17.61
ATOM	340	CG	TYR	49	18.094	-1.594	11.204	1.00	14.72
ATOM	341	CD1	TYR	49	19.232	-1.122	10.518	1.00	15.49
ATOM	342	CE1	TYR	49	20.405	-1.830	10.475	1.00	11.49
ATOM	343	CZ	TYR	49	20.601	-3.059	11.136	1.00	8.76
ATOM	344	OH	TYR	49	21.776	-3.687	11.123	1.00	17.30
ATOM	345	CE2	TYR	49	19.458	-3.514	11.861	1.00	16.46
ATOM	346	CD2	TYR	49	18.277	-2.778	11.900	1.00	15.15
ATOM	347	C	TYR	49	15.726	-2.676	9.814	1.00	20.43
ATOM	349	N	GLY	50	16.553	-3.475	9.138	1.00	16.74
ATOM	350	CA	GLY	50	16.403	-4.890	9.160	1.00	17.82
ATOM	351	C	GLY	50	16.633	-5.676	7.880	1.00	19.03
ATOM	352	O	GLY	50	16.432	-5.147	6.771	1.00	34.85
ATOM	353	N	GLY	51	16.977	-6.885	8.119	1.00	17.79

ATOM	354	CA	GLY	51	17.317	-7.891	7.072	1.00	26.60
ATOM	355	C	GLY	51	16.117	-8.737	6.759	1.00	10.29
ATOM	356	O	GLY	51	14.919	-8.351	6.963	1.00	18.32
ATOM	357	N	GLU	52	16.348	-9.963	6.309	1.00	13.03
ATOM	358	CA	GLU	52	15.320	-10.888	5.914	1.00	17.91
ATOM	359	C	GLU	52	14.407	-11.413	7.008	1.00	28.37
ATOM	360	O	GLU	52	13.301	-11.871	6.730	1.00	19.20
ATOM	361	CB	GLU	52	15.891	-12.055	5.115	1.00	23.93
ATOM	362	CG	GLU	52	17.087	-12.705	5.791	1.00	48.07
ATOM	363	CD	GLU	52	17.928	-13.568	4.889	1.00	57.60
ATOM	364	OE1	GLU	52	17.453	-14.595	4.431	1.00	50.50
ATOM	365	OE2	GLU	52	19.076	-13.069	4.767	1.00	35.66
ATOM	366	N	LYS	53	14.931	-11.353	8.235	1.00	22.11
ATOM	367	CA	LYS	53	14.096	-11.842	9.383	1.00	21.68
ATOM	368	C	LYS	53	13.758	-10.687	10.309	1.00	33.49
ATOM	369	O	LYS	53	13.504	-10.951	11.503	1.00	26.91
ATOM	370	CB	LYS	53	14.894	-12.845	10.171	1.00	19.86
ATOM	371	CG	LYS	53	15.528	-14.001	9.430	1.00	30.93
ATOM	372	CD	LYS	53	15.358	-15.315	10.154	1.00	34.04
ATOM	373	CE	LYS	53	14.832	-16.421	9.262	1.00	63.23
ATOM	374	NZ	LYS	53	13.343	-16.417	9.286	1.00	55.73
ATOM	375	N	LYS	54	13.738	-9.470	9.842	1.00	18.73
ATOM	376	CA	LYS	54	13.455	-8.255	10.554	1.00	18.10
ATOM	377	C	LYS	54	12.212	-8.364	11.422	1.00	30.51
ATOM	378	O	LYS	54	12.224	-7.867	12.570	1.00	20.81
ATOM	379	CB	LYS	54	13.504	-6.983	9.761	1.00	25.11
ATOM	380	CG	LYS	54	12.350	-6.790	8.781	1.00	30.54
ATOM	381	CD	LYS	54	12.352	-5.342	8.290	1.00	31.82
ATOM	382	CE	LYS	54	11.781	-5.234	6.888	1.00	64.13
ATOM	383	NZ	LYS	54	11.815	-3.809	6.475	1.00	58.42
ATOM	384	N	ALA	55	11.165	-8.991	10.980	1.00	22.40
ATOM	385	CA	ALA	55	9.885	-9.136	11.667	1.00	34.21
ATOM	386	C	ALA	55	10.058	-9.821	13.031	1.00	20.33
ATOM	387	O	ALA	55	9.239	-9.477	13.905	1.00	23.99
ATOM	388	CB	ALA	55	8.806	-9.838	10.859	1.00	25.63
ATOM	389	N	VAL	56	10.971	-10.735	13.143	1.00	21.69
ATOM	390	CA	VAL	56	11.197	-11.427	14.432	1.00	24.30
ATOM	391	C	VAL	56	11.556	-10.400	15.508	1.00	44.52
ATOM	392	O	VAL	56	11.086	-10.448	16.657	1.00	22.85
ATOM	393	CB	VAL	56	12.234	-12.522	14.208	1.00	19.52
ATOM	394	CG1	VAL	56	12.991	-12.961	15.455	1.00	26.49
ATOM	395	CG2	VAL	56	11.571	-13.719	13.544	1.00	27.99
ATOM	396	N	MET	57	12.345	-9.427	15.113	1.00	16.88
ATOM	397	CA	MET	57	12.808	-8.365	16.052	1.00	16.86
ATOM	398	C	MET	57	11.728	-7.332	16.257	1.00	29.35
ATOM	399	O	MET	57	11.533	-6.787	17.372	1.00	20.70
ATOM	400	CB	MET	57	14.148	-7.772	15.710	1.00	8.99
ATOM	401	CG	MET	57	15.309	-8.666	15.822	1.00	7.79
ATOM	402	SD	MET	57	15.529	-8.739	17.731	1.00	17.60
ATOM	403	CE	MET	57	15.666	-7.078	18.113	1.00	14.84
ATOM	404	N	THR	58	10.989	-6.949	15.222	1.00	20.65
ATOM	405	CA	THR	58	9.902	-5.996	15.312	1.00	17.09

ATOM	406	C	THR	58	8.852	-6.410	16.391	1.00	18.08
ATOM	407	O	THR	58	8.422	-5.533	17.182	1.00	21.07
ATOM	408	CB	THR	58	9.139	-5.797	13.928	1.00	22.97
ATOM	409	OG1	THR	58	10.116	-5.005	13.183	1.00	26.60
ATOM	410	CG2	THR	58	7.871	-4.979	14.139	1.00	23.70
ATOM	411	N	ASN	59	8.495	-7.643	16.393	1.00	16.81
ATOM	412	CA	ASN	59	7.473	-8.219	17.277	1.00	17.22
ATOM	413	C	ASN	59	7.965	-8.060	18.772	1.00	22.47
ATOM	414	O	ASN	59	7.049	-7.881	19.601	1.00	20.94
ATOM	415	CB	ASN	59	7.226	-9.642	16.948	1.00	15.95
ATOM	416	CG	ASN	59	6.289	-10.387	17.865	1.00	26.82
ATOM	417	OD1	ASN	59	6.795	-10.859	18.915	1.00	23.21
ATOM	418	ND2	ASN	59	5.044	-10.591	17.543	1.00	18.33
ATOM	419	N	LEU	60	9.257	-8.174	18.901	1.00	20.94
ATOM	420	CA	LEU	60	9.860	-8.024	20.261	1.00	19.42
ATOM	421	C	LEU	60	10.060	-6.625	20.765	1.00	29.98
ATOM	422	O	LEU	60	9.576	-6.336	21.887	1.00	22.66
ATOM	423	CB	LEU	60	11.196	-8.752	20.229	1.00	18.07
ATOM	424	CG	LEU	60	11.132	-10.245	20.030	1.00	29.40
ATOM	425	CD1	LEU	60	12.543	-10.825	20.064	1.00	30.18
ATOM	426	CD2	LEU	60	10.183	-10.831	21.070	1.00	31.87
ATOM	427	N	VAL	61	10.817	-5.818	20.058	1.00	22.39
ATOM	428	CA	VAL	61	11.136	-4.463	20.452	1.00	23.96
ATOM	429	C	VAL	61	9.922	-3.598	20.706	1.00	20.00
ATOM	430	O	VAL	61	10.091	-2.530	21.369	1.00	20.99
ATOM	431	CB	VAL	61	12.023	-3.701	19.424	1.00	29.03
ATOM	432	CG1	VAL	61	13.288	-4.372	19.037	1.00	21.16
ATOM	433	CG2	VAL	61	11.188	-3.256	18.234	1.00	28.50
ATOM	434	N	LYS	62	8.743	-3.882	20.205	1.00	14.46
ATOM	435	CA	LYS	62	7.610	-3.015	20.403	1.00	19.32
ATOM	436	C	LYS	62	7.104	-3.187	21.896	1.00	12.24
ATOM	437	O	LYS	62	6.249	-2.368	22.201	1.00	19.36
ATOM	438	CB	LYS	62	6.470	-3.459	19.459	1.00	23.37
ATOM	439	CG	LYS	62	6.737	-2.723	18.106	1.00	39.58
ATOM	440	CD	LYS	62	5.638	-3.080	17.121	1.00	33.74
ATOM	441	CE	LYS	62	4.261	-2.780	17.683	1.00	116.98
ATOM	442	NZ	LYS	62	3.185	-3.417	16.883	1.00	79.49
ATOM	443	N	ARG	63	7.682	-4.239	22.450	1.00	16.31
ATOM	444	CA	ARG	63	7.190	-4.489	23.899	1.00	16.98
ATOM	445	C	ARG	63	8.022	-3.741	24.898	1.00	27.14
ATOM	446	O	ARG	63	7.796	-3.885	26.132	1.00	21.60
ATOM	447	CB	ARG	63	7.343	-5.977	24.076	1.00	13.71
ATOM	448	CG	ARG	63	6.398	-6.842	23.246	1.00	17.25
ATOM	449	CD	ARG	63	6.941	-8.211	23.174	1.00	25.37
ATOM	450	NE	ARG	63	6.249	-9.092	22.242	1.00	16.38
ATOM	451	CZ	ARG	63	5.039	-9.541	22.164	1.00	17.63
ATOM	452	NH1	ARG	63	3.983	-9.304	22.968	1.00	13.60
ATOM	453	NH2	ARG	63	4.597	-10.250	21.047	1.00	18.05
ATOM	454	N	TYR	64	8.979	-2.986	24.473	1.00	17.46
ATOM	455	CA	TYR	64	9.946	-2.224	25.231	1.00	17.91
ATOM	456	C	TYR	64	9.892	-0.740	25.072	1.00	25.89
ATOM	457	O	TYR	64	9.522	-0.146	24.042	1.00	21.87

ATOM	458	CB	TYR	64	11.383	-2.739	25.087	1.00	20.93
ATOM	459	CG	TYR	64	11.588	-4.160	25.489	1.00	22.48
ATOM	460	CD1	TYR	64	12.068	-4.476	26.795	1.00	17.52
ATOM	461	CD2	TYR	64	11.276	-5.227	24.655	1.00	18.01
ATOM	462	CE1	TYR	64	12.249	-5.795	27.122	1.00	11.66
ATOM	463	CE2	TYR	64	11.426	-6.572	24.986	1.00	15.21
ATOM	464	CZ	TYR	64	11.896	-6.863	26.322	1.00	20.64
ATOM	465	OH	TYR	64	12.031	-8.138	26.685	1.00	19.60
ATOM	466	N	SER	65	10.305	-0.038	26.158	1.00	18.24
ATOM	467	CA	SER	65	10.302	1.410	26.098	1.00	15.19
ATOM	468	C	SER	65	11.646	1.860	25.564	1.00	7.79
ATOM	469	O	SER	65	12.669	1.133	25.546	1.00	17.41
ATOM	470	CB	SER	65	10.038	2.072	27.466	1.00	21.94
ATOM	471	OG	SER	65	11.213	1.803	28.240	1.00	21.28
ATOM	472	N	ASP	66	11.671	3.126	25.153	1.00	16.84
ATOM	473	CA	ASP	66	12.914	3.713	24.678	1.00	19.95
ATOM	474	C	ASP	66	14.006	3.583	25.735	1.00	25.79
ATOM	475	O	ASP	66	15.139	3.274	25.386	1.00	18.09
ATOM	476	CB	ASP	66	12.806	5.138	24.183	1.00	27.02
ATOM	477	CG	ASP	66	12.143	5.301	22.824	1.00	36.59
ATOM	478	OD1	ASP	66	11.371	4.482	22.333	1.00	27.49
ATOM	479	OD2	ASP	66	12.415	6.379	22.269	1.00	43.72
ATOM	480	N	GLU	67	13.700	3.894	27.029	1.00	19.39
ATOM	481	CA	GLU	67	14.789	3.781	28.015	1.00	21.46
ATOM	482	C	GLU	67	15.241	2.350	28.221	1.00	9.83
ATOM	483	O	GLU	67	16.427	2.120	28.478	1.00	13.77
ATOM	484	CB	GLU	67	14.275	4.453	29.325	1.00	24.18
ATOM	485	CG	GLU	67	13.902	5.948	29.209	1.00	16.90
ATOM	486	CD	GLU	67	12.574	6.214	28.598	1.00	21.99
ATOM	487	OE1	GLU	67	11.659	5.446	28.329	1.00	32.90
ATOM	488	OE2	GLU	67	12.444	7.425	28.282	1.00	52.26
ATOM	489	N	GLU	68	14.367	1.383	28.117	1.00	13.20
ATOM	490	CA	GLU	68	14.639	-0.034	28.195	1.00	10.94
ATOM	491	C	GLU	68	15.620	-0.424	27.047	1.00	15.65
ATOM	492	O	GLU	68	16.684	-1.015	27.142	1.00	14.00
ATOM	493	CB	GLU	68	13.465	-0.948	28.246	1.00	16.97
ATOM	494	CG	GLU	68	12.736	-0.783	29.640	1.00	14.62
ATOM	495	CD	GLU	68	11.473	-1.532	29.680	1.00	27.02
ATOM	496	OE1	GLU	68	11.112	-2.074	30.725	1.00	22.09
ATOM	497	OE2	GLU	68	10.739	-1.533	28.664	1.00	18.45
ATOM	498	N	MET	69	15.228	0.120	25.852	1.00	18.89
ATOM	499	CA	MET	69	16.174	-0.159	24.699	1.00	17.19
ATOM	500	C	MET	69	17.555	0.366	24.856	1.00	8.25
ATOM	501	O	MET	69	18.642	-0.258	24.659	1.00	19.50
ATOM	502	CB	MET	69	15.483	0.415	23.413	1.00	12.14
ATOM	503	CG	MET	69	14.248	-0.369	23.135	1.00	11.91
ATOM	504	SD	MET	69	13.661	0.374	21.511	1.00	30.10
ATOM	505	CE	MET	69	12.400	-0.843	21.263	1.00	32.61
ATOM	506	N	LYS	70	17.698	1.612	25.369	1.00	16.93
ATOM	507	CA	LYS	70	18.872	2.403	25.651	1.00	18.44
ATOM	508	C	LYS	70	19.747	1.622	26.638	1.00	16.24
ATOM	509	O	LYS	70	20.925	1.349	26.482	1.00	16.38

ATOM	510	CB	LYS	70	18.578	3.782	26.173	1.00	21.15
ATOM	511	CG	LYS	70	19.668	4.822	26.029	1.00	41.91
ATOM	512	CD	LYS	70	21.072	4.291	25.828	1.00	80.78
ATOM	513	CE	LYS	70	21.429	4.094	24.371	1.00	86.91
ATOM	515	N	ALA	71	18.995	1.095	27.654	1.00	17.56
ATOM	516	CA	ALA	71	19.741	0.258	28.632	1.00	16.15
ATOM	517	C	ALA	71	20.284	-1.024	28.079	1.00	12.30
ATOM	518	O	ALA	71	21.391	-1.481	28.448	1.00	17.80
ATOM	519	CB	ALA	71	18.844	0.010	29.880	1.00	17.17
ATOM	520	N	MET	72	19.484	-1.725	27.237	1.00	12.96
ATOM	521	CA	MET	72	19.958	-2.988	26.590	1.00	18.84
ATOM	522	C	MET	72	21.149	-2.623	25.660	1.00	17.37
ATOM	523	O	MET	72	22.096	-3.421	25.624	1.00	18.30
ATOM	524	CB	MET	72	18.800	-3.661	25.890	1.00	16.46
ATOM	525	CG	MET	72	17.909	-4.369	26.858	1.00	16.08
ATOM	526	SD	MET	72	16.588	-5.357	26.159	1.00	21.27
ATOM	527	CE	MET	72	15.581	-4.111	25.420	1.00	20.06
ATOM	528	N	ALA	73	21.032	-1.508	24.964	1.00	21.95
ATOM	529	CA	ALA	73	22.114	-1.096	24.032	1.00	20.03
ATOM	530	C	ALA	73	23.429	-0.877	24.754	1.00	16.05
ATOM	531	O	ALA	73	24.499	-1.374	24.434	1.00	19.55
ATOM	532	CB	ALA	73	21.655	0.160	23.298	1.00	21.16
ATOM	533	N	ASP	74	23.226	-0.129	25.897	1.00	22.79
ATOM	534	CA	ASP	74	24.433	0.062	26.755	1.00	19.94
ATOM	535	C	ASP	74	25.062	-1.241	27.121	1.00	10.93
ATOM	536	O	ASP	74	26.285	-1.416	27.039	1.00	18.32
ATOM	537	CB	ASP	74	24.086	1.060	27.880	1.00	31.87
ATOM	538	CG	ASP	74	24.374	2.454	27.316	1.00	90.18
ATOM	539	OD1	ASP	74	25.549	2.843	27.215	1.00	87.93
ATOM	540	OD2	ASP	74	23.386	3.099	26.915	1.00	74.31
ATOM	541	N	TYR	75	24.320	-2.188	27.689	1.00	17.97
ATOM	542	CA	TYR	75	24.725	-3.509	28.143	1.00	16.32
ATOM	543	C	TYR	75	25.416	-4.300	27.026	1.00	21.85
ATOM	544	O	TYR	75	26.513	-4.819	27.151	1.00	20.14
ATOM	545	CB	TYR	75	23.539	-4.300	28.747	1.00	21.70
ATOM	546	CG	TYR	75	23.940	-5.657	29.245	1.00	17.27
ATOM	547	CD1	TYR	75	23.694	-6.815	28.513	1.00	22.09
ATOM	548	CD2	TYR	75	24.705	-5.851	30.432	1.00	17.13
ATOM	549	CE1	TYR	75	24.074	-8.071	28.893	1.00	23.40
ATOM	550	CE2	TYR	75	25.123	-7.118	30.800	1.00	17.54
ATOM	551	CZ	TYR	75	24.824	-8.243	30.083	1.00	17.82
ATOM	552	OH	TYR	75	25.141	-9.505	30.445	1.00	23.77
ATOM	553	N	MET	76	24.725	-4.346	25.880	1.00	23.41
ATOM	554	CA	MET	76	25.272	-5.112	24.741	1.00	23.60
ATOM	555	C	MET	76	26.631	-4.526	24.325	1.00	14.91
ATOM	556	O	MET	76	27.531	-5.326	24.024	1.00	23.72
ATOM	557	CB	MET	76	24.190	-5.178	23.663	1.00	19.07
ATOM	558	CG	MET	76	23.080	-6.147	23.943	1.00	15.35
ATOM	559	SD	MET	76	22.006	-6.189	22.407	1.00	21.02
ATOM	560	CE	MET	76	21.313	-4.604	22.371	1.00	16.60
ATOM	561	N	SER	77	26.778	-3.242	24.356	1.00	15.16
ATOM	562	CA	SER	77	27.954	-2.463	24.017	1.00	20.88

ATOM	563	C	SER	77	29.147	-2.832	24.892	1.00	32.99
ATOM	564	O	SER	77	30.301	-2.688	24.437	1.00	27.83
ATOM	565	CB	SER	77	27.632	-1.008	23.973	1.00	22.59
ATOM	566	OG	SER	77	27.934	-0.357	25.188	1.00	41.41
ATOM	567	N	LYS	78	28.854	-3.401	26.064	1.00	30.31
ATOM	568	CA	LYS	78	29.907	-3.843	26.984	1.00	27.15
ATOM	569	C	LYS	78	30.188	-5.311	26.897	1.00	30.91
ATOM	570	O	LYS	78	31.135	-5.812	27.532	1.00	30.33
ATOM	571	CB	LYS	78	29.571	-3.404	28.418	1.00	36.23
ATOM	572	CG	LYS	78	29.607	-1.874	28.525	1.00	37.56
ATOM	573	CD	LYS	78	28.909	-1.373	29.780	1.00	62.12
ATOM	574	CE	LYS	78	28.954	0.143	29.866	1.00	38.12
ATOM	575	NZ	LYS	78	27.633	0.666	30.308	1.00	67.07
ATOM	576	N	LEU	79	29.433	-6.073	26.124	1.00	22.86
ATOM	577	CA	LEU	79	29.716	-7.492	25.958	1.00	18.50
ATOM	578	C	LEU	79	31.061	-7.721	25.259	1.00	39.59
ATOM	579	O	LEU	79	31.622	-8.784	25.592	1.00	37.51
ATOM	580	CB	LEU	79	28.528	-8.170	25.296	1.00	36.02
ATOM	581	CG	LEU	79	27.234	-8.291	26.077	1.00	38.32
ATOM	582	CD1	LEU	79	26.234	-8.993	25.153	1.00	25.28
ATOM	583	CD2	LEU	79	27.409	-9.141	27.343	1.00	26.53
ATOM	584	OXT	LEU	79	31.500	-6.855	24.487	1.00	32.12
ATOM	585	FE	HEM	80	17.581	-9.819	18.175	1.00	16.80
ATOM	586	CHA	HEM	80	18.823	-8.651	15.271	1.00	16.62
ATOM	587	CHB	HEM	80	18.888	-7.144	19.848	1.00	14.44
ATOM	588	CHC	HEM	80	15.892	-10.800	20.992	1.00	15.01
ATOM	589	CHD	HEM	80	16.667	-12.821	16.636	1.00	20.82
ATOM	590	N A	HEM	80	18.601	-8.233	17.715	1.00	17.89
ATOM	591	C1A	HEM	80	19.086	-7.817	16.385	1.00	11.64
ATOM	592	C2A	HEM	80	19.873	-6.652	16.422	1.00	15.00
ATOM	593	C3A	HEM	80	19.898	-6.192	17.731	1.00	17.63
ATOM	594	C4A	HEM	80	19.132	-7.165	18.426	1.00	13.12
ATOM	595	CMA	HEM	80	20.602	-4.960	18.272	1.00	16.58
ATOM	596	CAA	HEM	80	20.617	-6.021	15.227	1.00	15.34
ATOM	597	CBA	HEM	80	22.038	-6.497	14.957	1.00	16.49
ATOM	598	CGA	HEM	80	22.619	-5.691	13.753	1.00	14.06
ATOM	599	O1A	HEM	80	23.631	-5.009	13.910	1.00	16.72
ATOM	600	O2A	HEM	80	21.752	-5.780	12.818	1.00	20.26
ATOM	601	N B	HEM	80	17.414	-9.095	20.110	1.00	6.35
ATOM	602	C1B	HEM	80	18.020	-7.974	20.438	1.00	12.73
ATOM	603	C2B	HEM	80	17.644	-7.811	21.910	1.00	12.77
ATOM	604	C3B	HEM	80	16.777	-8.757	22.183	1.00	14.25
ATOM	605	C4B	HEM	80	16.611	-9.678	21.083	1.00	14.66
ATOM	606	CMB	HEM	80	18.179	-6.609	22.678	1.00	17.50
ATOM	607	CAB	HEM	80	16.056	-9.004	23.575	1.00	17.21
ATOM	608	CBB	HEM	80	15.181	-7.987	23.925	1.00	19.16
ATOM	609	N C	HEM	80	16.514	-11.388	18.618	1.00	19.47
ATOM	610	C1C	HEM	80	15.969	-11.713	19.914	1.00	16.31
ATOM	611	C2C	HEM	80	15.413	-13.010	19.950	1.00	19.16
ATOM	612	C3C	HEM	80	15.585	-13.579	18.734	1.00	19.75
ATOM	613	C4C	HEM	80	16.310	-12.595	17.990	1.00	12.49
ATOM	614	CMC	HEM	80	14.771	-13.617	21.247	1.00	17.12

ATOM	615	CAC	HEM	80	15.254	-15.029	18.329	1.00	17.89
ATOM	616	CBC	HEM	80	13.995	-15.467	18.258	1.00	16.91
ATOM	617	N D	HEM	80	17.731	-10.577	16.276	1.00	7.16
ATOM	618	C1D	HEM	80	17.199	-11.777	15.961	1.00	13.60
ATOM	619	C2D	HEM	80	17.420	-11.859	14.447	1.00	13.16
ATOM	620	C3D	HEM	80	17.951	-10.718	14.110	1.00	15.80
ATOM	621	C4D	HEM	80	18.215	-9.854	15.228	1.00	28.85
ATOM	622	CMD	HEM	80	16.877	-13.048	13.622	1.00	14.68
ATOM	623	CAD	HEM	80	18.261	-10.184	12.610	1.00	12.58
ATOM	624	CBD	HEM	80	16.920	-9.723	12.022	1.00	13.32
ATOM	625	CGD	HEM	80	17.132	-8.993	10.655	1.00	16.98
ATOM	626	O1D	HEM	80	16.800	-9.764	9.713	1.00	22.19
ATOM	627	O2D	HEM	80	17.513	-7.832	10.654	1.00	16.77
ATOM	628	O	WAT	81	21.185	-0.619	33.042	1.00	32.58
ATOM	629	O	WAT	82	13.222	1.294	35.169	1.00	24.01
ATOM	630	O	WAT	83	12.233	-2.707	33.177	1.00	19.76
ATOM	631	O	WAT	84	10.654	-10.092	8.338	1.00	31.78
ATOM	632	O	WAT	85	19.101	-10.450	5.173	1.00	26.05
ATOM	633	O	WAT	86	3.278	-5.160	21.384	1.00	40.18
ATOM	634	O	WAT	87	4.444	-7.045	19.155	1.00	23.24
ATOM	635	O	WAT	88	13.351	-8.597	28.873	1.00	19.19
ATOM	636	O	WAT	89	28.727	-7.919	10.754	1.00	34.32
ATOM	637	O	WAT	90	33.699	-13.010	19.924	1.00	36.39
ATOM	638	O	WAT	91	27.763	4.829	21.882	1.00	43.95
ATOM	639	O	WAT	92	18.173	3.455	30.316	1.00	24.39
ATOM	640	O	WAT	93	21.286	-8.246	11.952	1.00	26.00
ATOM	641	O	WAT	94	9.608	-12.889	17.365	1.00	34.80
ATOM	642	O	WAT	95	20.097	-18.047	16.983	1.00	31.88
ATOM	643	O	WAT	96	18.867	2.747	33.126	1.00	23.77
ATOM	644	O	WAT	97	24.163	-11.383	18.607	1.00	24.72
ATOM	645	O	WAT	98	15.753	-10.562	34.027	1.00	27.73
ATOM	646	O	WAT	99	15.993	-15.698	24.496	1.00	36.67
ATOM	647	O	WAT	100	9.005	4.682	25.102	1.00	35.01
ATOM	648	O	WAT	101	7.830	-0.687	29.268	1.00	37.82
ATOM	649	O	WAT	102	22.483	-10.467	31.345	1.00	38.95
ATOM	650	O	WAT	103	11.822	0.479	32.879	1.00	27.37
ATOM	651	O	WAT	104	5.092	-3.230	27.124	1.00	36.57
ATOM	652	O	WAT	105	8.910	-1.957	32.511	1.00	29.05
ATOM	653	O	WAT	106	20.924	3.764	29.722	1.00	36.97
ATOM	654	O	WAT	107	24.823	-18.771	25.060	1.00	51.29
ATOM	655	O	WAT	108	12.911	-11.305	23.734	1.00	31.08
ATOM	656	O	WAT	109	29.610	2.182	23.142	1.00	39.93
ATOM	657	O	WAT	110	29.890	-5.394	22.706	1.00	32.26
ATOM	658	O	WAT	111	22.415	-11.273	15.858	1.00	29.69
ATOM	659	O	WAT	112	20.191	2.293	11.974	1.00	52.69
ATOM	660	O	WAT	113	15.749	-8.137	38.277	1.00	49.22
ATOM	661	O	WAT	114	21.050	-11.025	13.633	1.00	27.43
ATOM	662	O	WAT	115	10.995	-9.928	24.829	1.00	32.51
ATOM	663	O	WAT	116	9.910	6.337	27.553	1.00	42.08
ATOM	664	O	WAT	117	6.626	-0.141	27.066	1.00	42.13
ATOM	665	O	WAT	118	16.439	6.048	29.359	1.00	43.33
ATOM	666	O	WAT	119	9.391	2.196	22.553	1.00	47.26

ATOM	667	O	WAT	120	24.833	-11.803	29.288	1.00	36.70
ATOM	668	O	WAT	121	22.825	-13.167	30.121	1.00	49.20
ATOM	669	O	WAT	122	27.794	-6.247	29.382	1.00	46.25
ATOM	670	O	WAT	123	14.836	-3.532	6.121	1.00	42.19
ATOM	671	O	WAT	124	12.735	-8.188	31.677	1.00	34.17
ATOM	672	O	WAT	125	16.138	4.176	13.977	1.00	41.10
ATOM	673	O	WAT	126	18.524	-6.817	38.638	1.00	43.91
ATOM	674	O	WAT	127	33.448	-7.188	22.713	1.00	47.37
ATOM	675	O	WAT	128	33.310	-14.451	21.791	1.00	44.92
ATOM	676	O	WAT	129	18.772	-15.748	29.993	1.00	37.62
ATOM	677	O	WAT	130	5.332	-0.003	21.206	1.00	66.42
ATOM	678	O	WAT	131	28.713	-9.693	30.358	1.00	43.30
ATOM	679	O	WAT	132	14.193	-10.563	31.711	1.00	65.35
ATOM	681	O	WAT	134	6.524	0.935	18.929	1.00	62.24
ATOM	682	O	WAT	135	6.708	-0.571	31.549	1.00	50.99
ATOM	683	O	WAT	136	8.778	7.150	18.757	1.00	66.91
ATOM	684	O	WAT	137	19.346	-17.049	33.467	1.00	77.15
ATOM	685	O	WAT	138	7.213	2.709	17.230	1.00	60.94
ATOM	686	O	WAT	139	20.281	-22.137	17.117	1.00	72.37
ATOM	687	O	WAT	140	27.162	-17.609	15.266	1.00	52.56
ATOM	688	O	WAT	141	28.769	-7.352	14.873	1.00	45.14
ATOM	689	O	WAT	142	17.077	-10.725	36.734	1.00	45.41
ATOM	690	O	WAT	143	10.215	-16.354	10.876	1.00	64.95

LIST OF PUBLICATIONS

- (1) Crystallographic study of Cytochrome *c553* from *Desulfovibrio vulgaris* Miyazaki

Atsushi Nakagawa, Etsuro Nagashima, Yoshiki Higuchi, Masami Kusunoki, Yoshiki Matsuura, Noritake Yasuoka, Yukiteru Katsube, Hideaki Chihara, and Tatsuhiko Yagi (1986) *J.Biochem. (Tokyo)*, **99**, 605-606

- (2) Design and performance of an Imaging Plate system for X-ray diffraction study

Yoshiyuki Amemiya, Tadashi Matsushita, Atsushi Nakagawa, Yoshinori Satow, Junji Miyahara and Jun-ichi Chikawa, (1988) *Nucl.Instrum.Methods*, **A266**, 645-653

- (3) Structure determination of Cytochrome *c553* by the multi-wavelength anomalous dispersion method

(*in preparation*)

