CHARACTERIZATION OF IRON–SULFUR CLUSTERS OF NADH:UBIQUINONE OXIDOREDUCTASE (COMPLEX I) FROM BOVINE HEART MITOCHONDRIA

1993

RYOJI MASUI

DEPARTMENT OF BIOLOGY, FACULTY OF SCIENCE,
OSAKA UNIVERSITY

CHARACTERIZATION OF IRON–SULFUR CLUSTERS OF NADH:UBIQUINONE OXIDOREDUCTASE (COMPLEX I) FROM BOVINE HEART MITOCHONDRIA

1993

RYOJI MASUI

DEPARTMENT OF BIOLOGY, FACULTY OF SCIENCE,

OSAKA UNIVERSITY

GENERAL ACKNOWLEDGEMENTS

The present work has been carried out at Department of Biology, Faculty of Science, Osaka University under the guidance of Professor Hiroshi Matsubara. I am indebted to him for his invaluable advice and encouragement throughout this study. I am grateful to Dr. Sadao Wakabayashi for his guidance and valuable discussion on proceeding to this work. I also express my thanks to Drs. Yasuhiro Takahashi and Hirozo Oh-oka for their helpful advice.

I express appreciation to Mr. Tomomi Kubota for valuable suggestion and discussion, and the colleagues in this laboratory for their interest and encouragement.

CONTENTS

ABBREVIATIONS

Ac-, N-acetyl-

C-, carboxyl-

Cit-, citraconylated

Cm-, carboxymethylated

DTT, dithiothreitoll

EPR, electron paramagnetic resonance

FP, iron-sulfur flavoprotein fraction of complex I

HP, hydrophobic fraction of complex I

IP, iron-sulfur protein fraction of complex I

MES, 2-(*N*-morpholino)ehtanesulfonic acid

MOPS, 3-(*N*-morpholino)propanesulfonic acid

N-, amino-

NBS, *N*-bromosuccinimide

ODS, octadecylsilane

ORF, open reading frame

PCMPS, *p*-chloromercuriphenylsulfonic acid

PTH-, phenylthiohydantoin derivative

Chapter I

Structural Analysis of the Hydrophilic Subunits of Complex I

SUMMARY

Mitochondrial NADH:ubiquinone oxidoreductase (complex I) is the most complicated enzyme in the respiratory chain and is composed of at least 30 distinct polypeptides. Two hydrophilic subfractions of bovine heart complex I were systematically resolved into individual polypeptides by chromatography. Three polypeptides (51-kDa, 24-kDa and 9-kDa) were isolated from the flavoprotein fraction (FP) of complex I, and the complete amino acid sequence of the 9-kDa polypeptide was determined. The 9-kDa polypeptide is composed of 75 amino acids with a molecular weight of 8,437. This protein exhibits no obvious sequence similarity to other proteins. The iron-sulfur protein fraction (IP) of complex I was separated into eight polypeptides, 75-kDa, 49-kDa, 30-kDa, 20-kDa, 18-kDa, 15-kDa, 13-kDa-A and 13-kDa-B. The 20-kDa polypeptide was recognized as a novel component of IP for the first time. The N-terminal and several peptide sequences of the 20-kDa polypeptide were determined. Comparison of the sequences revealed significant sequence similarities of the 20-kDa polypeptide to the *psbG* gene products encoded in the chloroplast genome. The conserved sequence in these proteins was also found in the small subunit of the nickel-containing hydrogenases. These results suggest that complex I is related to other redox enzyme complexes.

INTRODUCTION

NADH:ubiquinone oxidoreductase (complex I) [EC 1.6.5.3] is the first enzyme in the mitochondrial respiratory chain. Complex I consists of at least 30 unlike polypeptides, a noncovalently bound FMN and several iron-sulfur centers involved in electron transfer between NADH and ubiquinone (*1–4*). This enzyme is so complicated that its detailed structure and mechanism of action are still uncertain.

Complex I can be resolved with chaotropic agents into three subfractions: the flavoprotein fraction (FP), the iron-sulfur protein fraction (IP) and the hydrophobic protein fraction (HP). FP and IP are water-soluble and HP is water-insoluble. FP is composed of the 51-kDa, 24-kDa and 9-kDa subunits. FP contains an FMN and two iron-sulfur centers, and retains NADH dehydrogenase activity. IP consists of six major protein components, the 75-kDa, 49-kDa, 30-kDa, 18-kDa, 15-kDa and 13-kDa subunits, and contains at least four iron-sulfur centers. HP contains the remaining hydrophobic polypeptides of complex I and one or two iron-sulfur centers.

Seven subunits of HP, known as ND1–ND6 and ND4L, are encoded in the mitochondrial genome (*5–7*). The chloroplast genome has been shown to contain seven open reading frames (ORFs) that code for protein sequences homologous to these subunits of complex I (*8–13*). These chloroplast ORFs are named *ndhA–ndhG* (or *ndh1–ndh6* and *ndh4L*). Recently, the 49-kDa, 30-kDa and 23-kDa subunits of mitochondrial complex I were also shown to exhibit sequence similarities to the ORFs encoded in the chloroplast genome (*14–16*). These ORFs were originally called ORF392, *frxB* and ORF158 in tobacco, for example, and it has been proposed that they should be renamed *ndhH*, *ndhI* and *ndhJ*, respectively (*16*). These findings suggest that these *ndh* gene products are the subunits of a complex I-like enzyme of chloroplasts. Furthermore, the sequences of the 51-kDa, 24-kDa and 75-kDa subunits of complex I have been found to be similar to those of subunits of the NAD-reducing hydrogenase from *Alcaligenes eutrophus* H16 (*17–19*). This observation suggests an evolutionary relationship between these enzymes. Many sequences of subunits of complex I from *Neurospora crassa* have also been published (*20–24*).

While some of the above protein sequences have provided significant information regarding the possible function of certain subunits, little is known about most of the nuclear-

encoded subunits of complex I. We recently reported the amino acid sequences of two 13-kDa polypeptides of complex I (*25*). In order to obtain further information on the structure of the subunits of complex I, I started the resolution of the component polypeptides of FP and IP. This section presents the subunit composition of each fraction and the sequence studies on the 9-kDa and 20-kDa polypeptides. The partial sequence of the 20-kDa polypeptide exhibits significant similarity to an ORF encoded in the chloroplast genome and also to a subunit of the hydrogenase. Based on these relationships, the structure and organization of complex I are discussed.

MATERIALS AND METHODS

Materials — FP and IP of complex I from bovine heart mitochondria were prepared as described in the literature (*26*).

Enzymes and Chemicals — Enzymes, chromatographic materials and chemicals were commercially purchased: arginylendopeptidase from Takara; Toyopearl HW-65F and DEAE-Toyopearl from Tosoh; Sephacryl S-200HR from Pharmacia Fine Chemicals; DEAE-cellulose (cellulofine A-500) from Seikagaku Kogyo Co., Ltd.; CM-cellulose (CM-52) from Whatmann Biochemicals; and other enzymes and reagents used in this experiment as listed previously (*25*).

Amino Acid Analysis — Amino acid analysis was performed with an amino acid analyzer (Irica model A-5500) after 6 N HCl hydrolysis for 24 h essentially according to Spackman *et al.* (*27*).

Sequence Determination — The digests were separated by HPLC in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile at 50°C on a Shodex ODSpak F-511A column. The flow rate was 0.8 ml/min. The amino (N-) terminal sequence was determined with a gas-phase protein sequencer (Applied Biosystems, model 473A) equipped with an on-line connected HPLC (model 120A) or manual Edman degradation. Procedures employed for the manual degradation were described previously (*25*).

Data Analysis — The polypeptide sequence was compared with those in the Swiss-Prot database using the GENETYX program (Software Development Co., Ltd.).

Nomenclature — K, R, T and V refer to the peptides derived on lysylendopeptidase, arginylendopeptidase, tryptic and staphylococcal V8 protease digestion, respectively.

SUPPLEMENTAL RESULTS

Separation of FP Component Polypeptides — FP was treated with trichloroacetic acid to destroy the iron-sulfur clusters, reduced with 2-mercaptoethanol and then carboxymethylated with iodoacetic acid (*28*). The carboxymethylated (Cm-) FP was insoluble in a normal aqueous solution and so was chromatographed on a Sephacryl S-200HR column (2 x 190 cm) in 50 mM Tris-HCl buffer (pH 7.5) containing 8 M urea. The Cm-FP was separated into three fractions (Fig. I-1S). The first, second and third fractions contained the 51-kDa, 24-kDa and 9-kDa polypeptides, respectively.

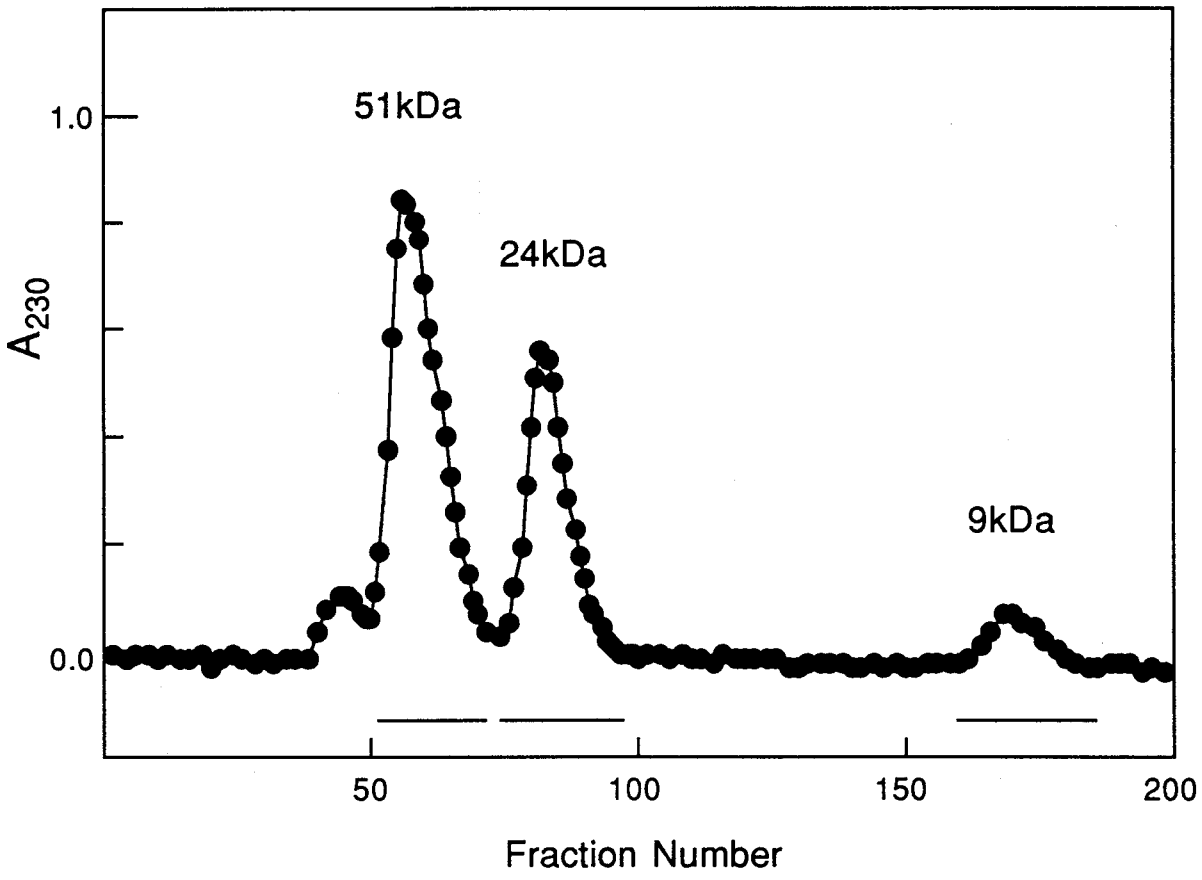

Fig. I-1S. Separation of Cm-FP on a Sephacryl S-200HR column. Fractions of 1 ml were collected at a flow rate of 10 ml/h after pre-elution of 250 ml.

Separation of IP Component Polypeptides — After treatment with trichloroacetic acid, IP was carboxymethylated. The Cm-IP was also insoluble and chromatographed in the solution containing 8 M urea. Under these conditions some polypeptides aggregated and were eluted at the void volume of the column. Thus the sample was further citraconylated with citraconic anhydride (*29*). The introduction of negative charges to the polypeptides made them soluble in a slightly alkaline solution. Even under these conditions, however, some polypeptides still aggregated. Separation of citraconylated (Cit-) Cm-IP was most effectively achieved in a solution containing relatively high concentration of urea.

Thus the Cit-Cm-IP was separated into four fractions on a Toyopearl HW-65F column (2 x 190 cm) in 50 mM Tris-HCl buffer (pH 7.5) containing 8 M urea (Fig. I-2S). The first (I), second (II) and third (III) fractions predominantly contained the 75-kDa, 49-kDa and 30-kDa polypeptides, respectively. Each fraction was dialyzed, lyophilized and purified by rechromatography.

Fraction III was then applied to a DEAE-Toyopearl 650M column (1.2 x 26 cm), developed with a linear gradient of NaCl from 0 to 1.0 M in 50 mM Tris-HCl buffer (pH 8.0), and separated into two fractions (Fig. I-3S). Both the fractions obtained on polyacrylamide gel contained 30-kDa polypeptides, which were named the 30-kDa-A and 30-kDa-B polypeptides, respectively.

The fourth (IV) fraction was further separated into three fractions on a Sephacryl S-200HR column (2 x 190 cm) in 50 mM Tris-HCl buffer (pH 7.5) containing 8 M urea (Fig. I-4S). The first fraction (IV-1), containing the 30-kDa polypeptide, was combined with fraction III. The second fraction (IV-2), enriched with the 20-kDa and 18-kDa polypeptides, after an unsuccessful attempt by ion-exchange chromatography, was separated successfully by reverse-phase HPLC on a butyl column (Shodex RSpak D4-613) in 0.05% trimethylamine (pH 9.5) with a stepwise gradient of acetonitrile (Fig. I-5S). Thus the 20-kDa and 18-kDa polypeptides were purified.

The third fraction (IV-3), containing the 18-kDa, 15-kDa and 13-kDa polypeptides, was applied to a DEAE-cellulofine column (1.2 x 26 cm), developed with a linear gradient of 50 mM to 1.0 M ammonium bicarbonate (Fig. I-6S). Both the obtained fractions, IV3-1 and IV3-3, contained 13-kDa polypeptides, which were called the 13-kDa-A and 13-kDa-B polypeptides, respectively.

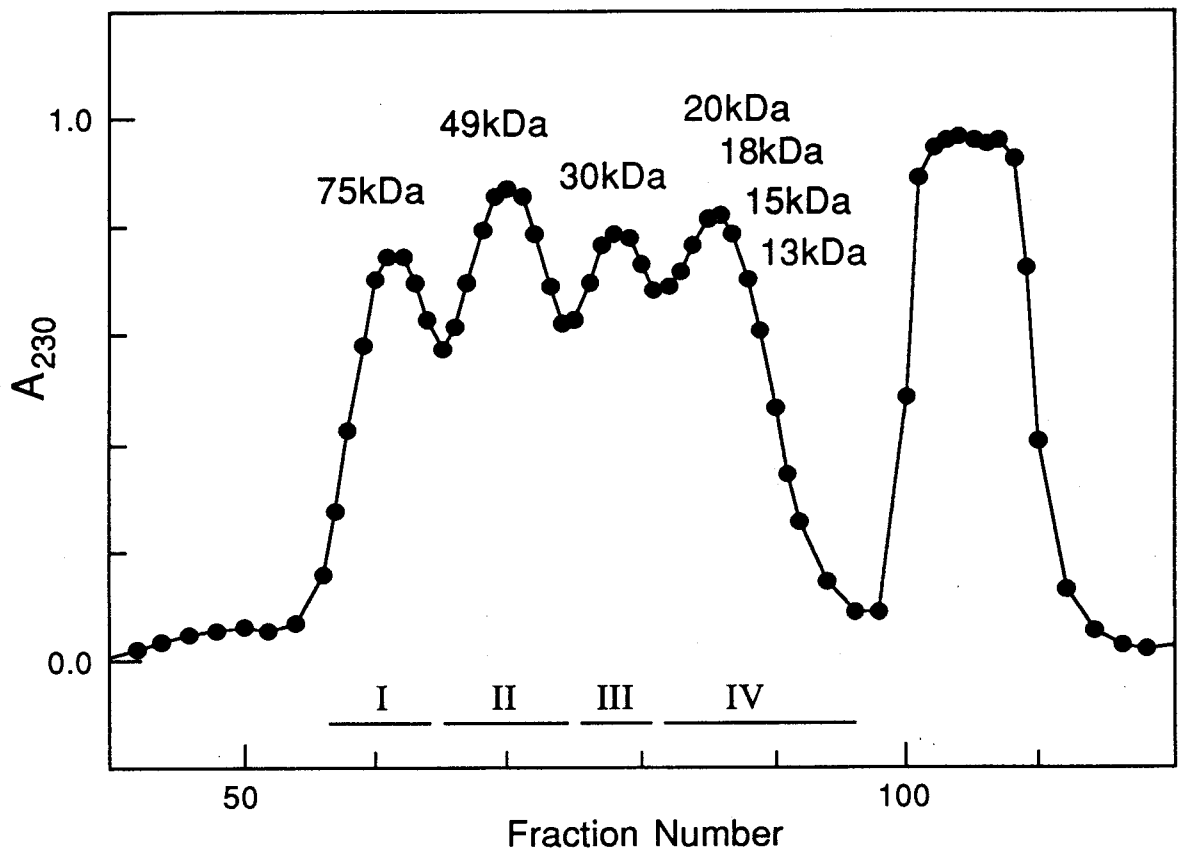

Fig. I-2S. Separation of Cit-Cm-IP on a Toyopearl HW-65F column. Fractions of 2.5 ml were collected at a flow rate of 12.5 ml/h after pre-elution of 300 ml.

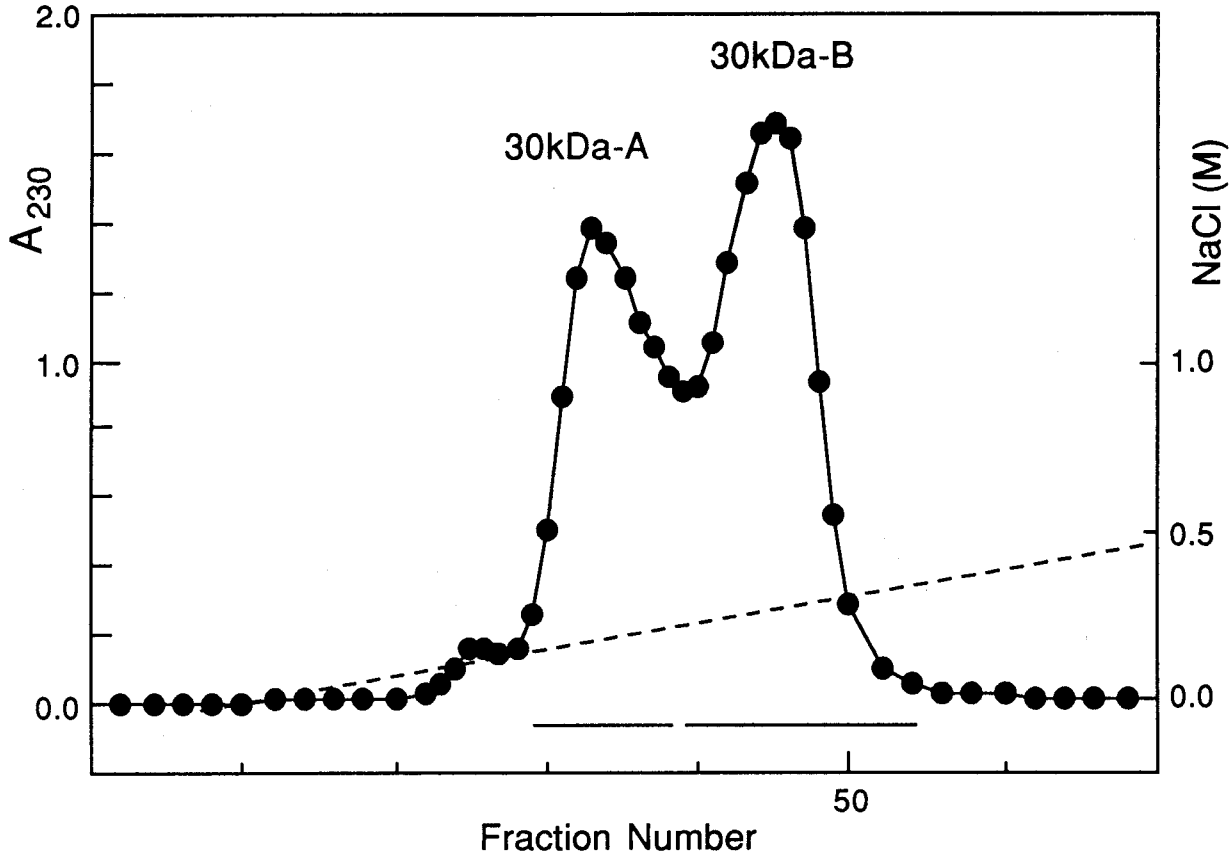

Fig. I-3S. Separation of Fraction III on a DEAE-Toyopearl 650M column. Fractions of 3 ml were collected at a fow rate of 15 ml/h.

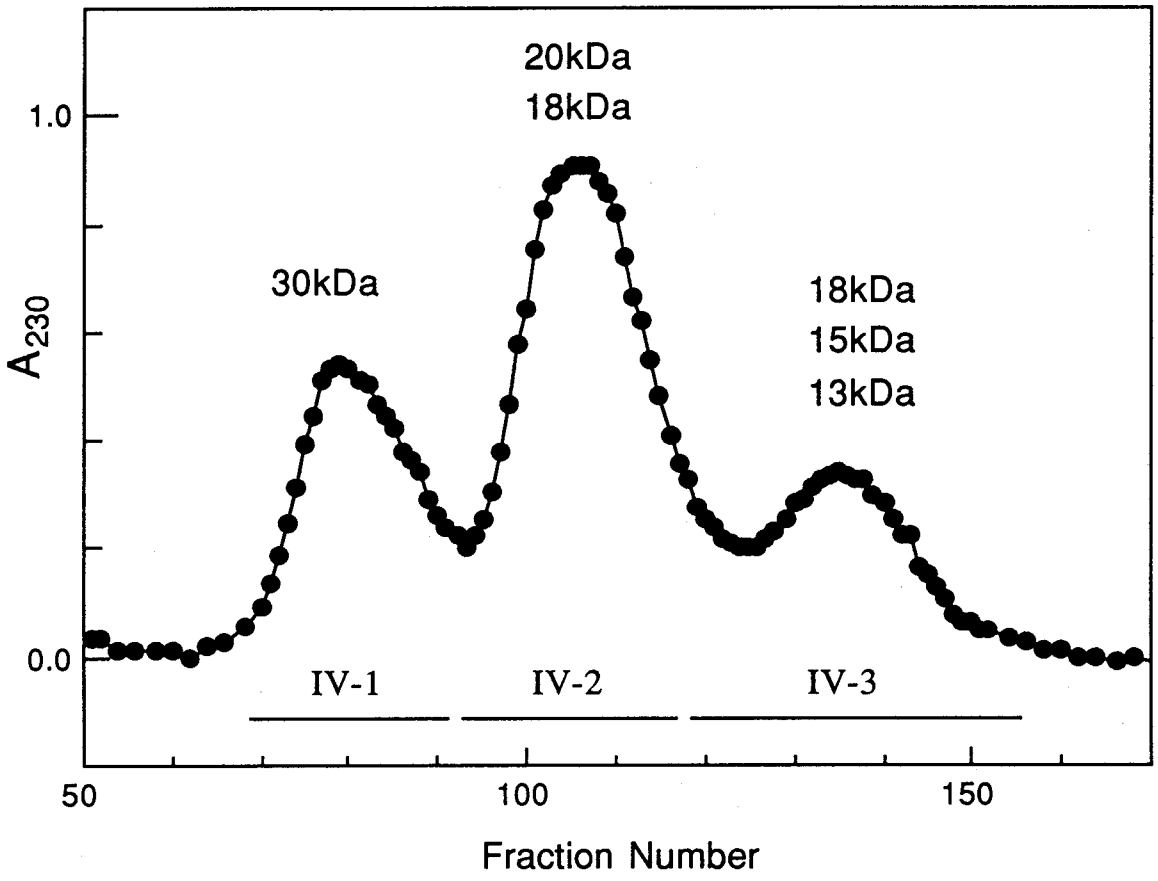

Fig. I-4S. Separation of Fraction IV on a Sephacryl S-200HR column. Fractions of 1 ml were collected at a flow rate of 10 ml/h after pre-elution of 250 ml.

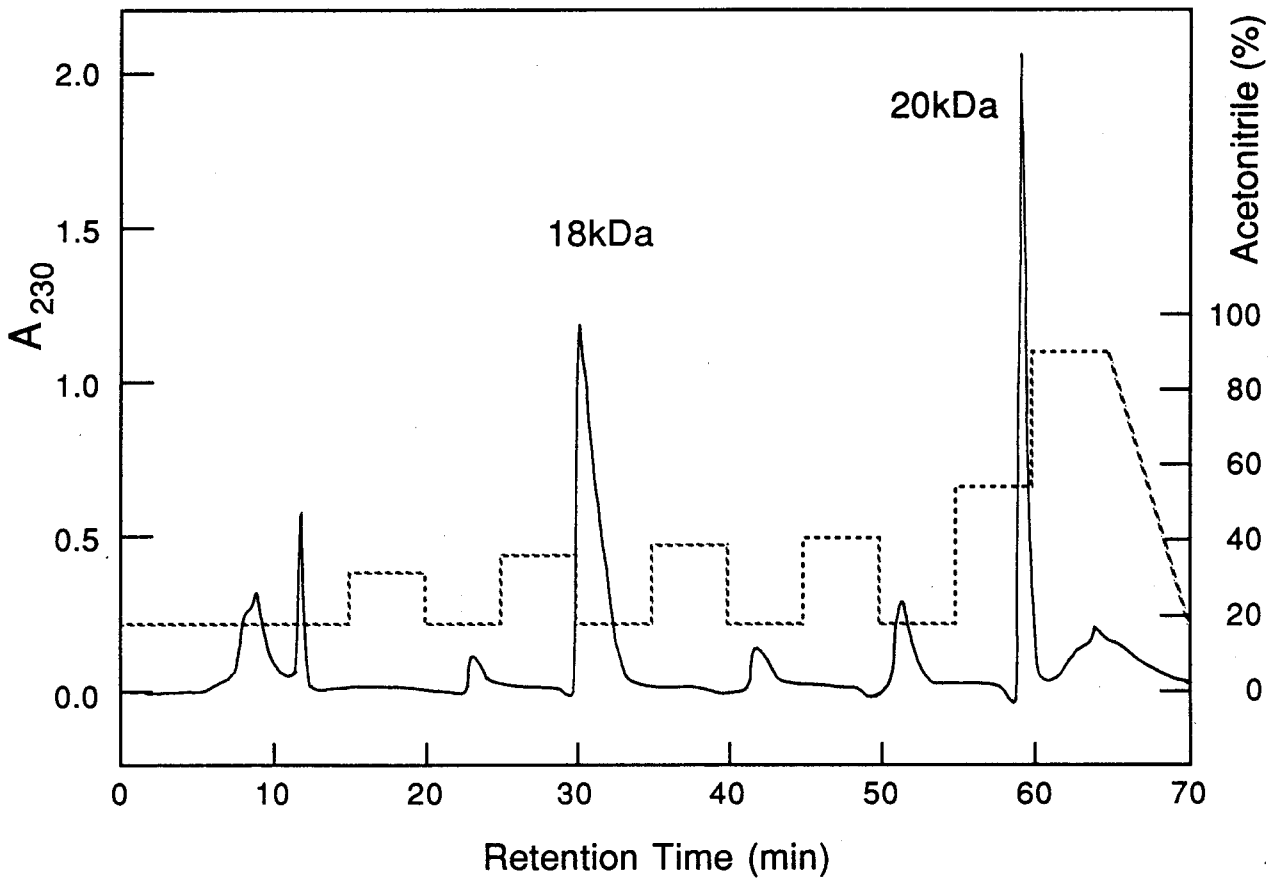

Fig. I-5S. Separation of Fraction IV-2 on a Shodex RSpak D4-613 column. Chromatography was carried out with a stepwise gradient system of acetonitrile, from 18% to 54%, in 0.05% triethylamine (pH 9.5). The flow rate was 0.5 ml/min.

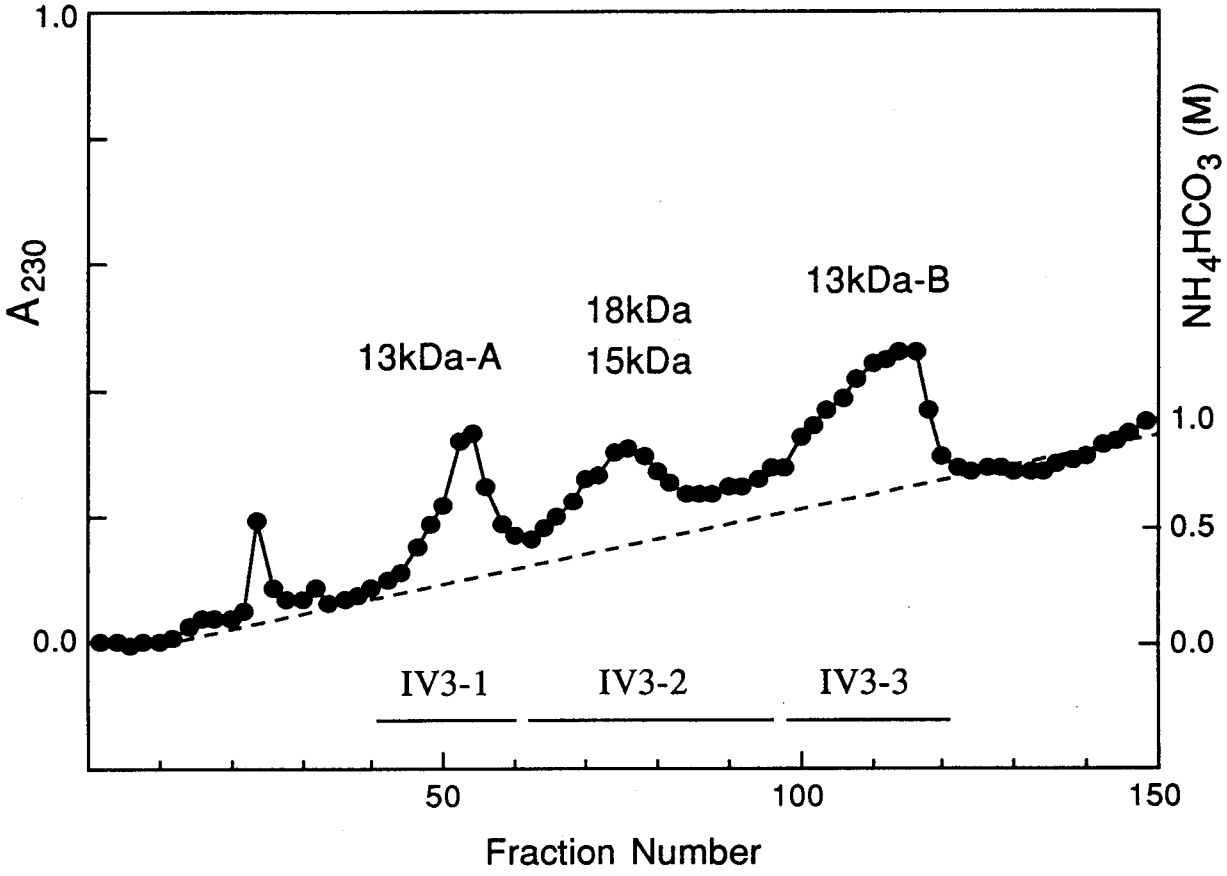

Fig. I-6S. Separation of Fraction IV-3 on a DEAE-cellulofine column. Fractions of 4 ml were collected at a flow rate of 20 ml/h.

The 18-kDa and 15-kDa polypeptides were eluted together (IV3-2) from the DEAE-cellulofine column, and separated on a CM-52 column, developed with a linear gradient of NaCl from 0 to 1.0 M in 25 mM ammonium acetate buffer (pH 5.3) containing 8 M urea after decitraconylation with 10% acetic acid (Fig. I-7S). But these proteins were recovered in low yields, even though they were recitraconylated upon recovery.

In order to minimize the complexity of the procedure, the fractions containing identical polypeptides were combined with each other. The purity of each polypeptide was assessed by SDS-PAGE (*30*) on a 15% gel containing 12% glycerol. The isolated polypeptides, other than the 20-kDa and 18-kDa polypeptides, were finally purified by reverse-phase HPLC for amino acid analysis.

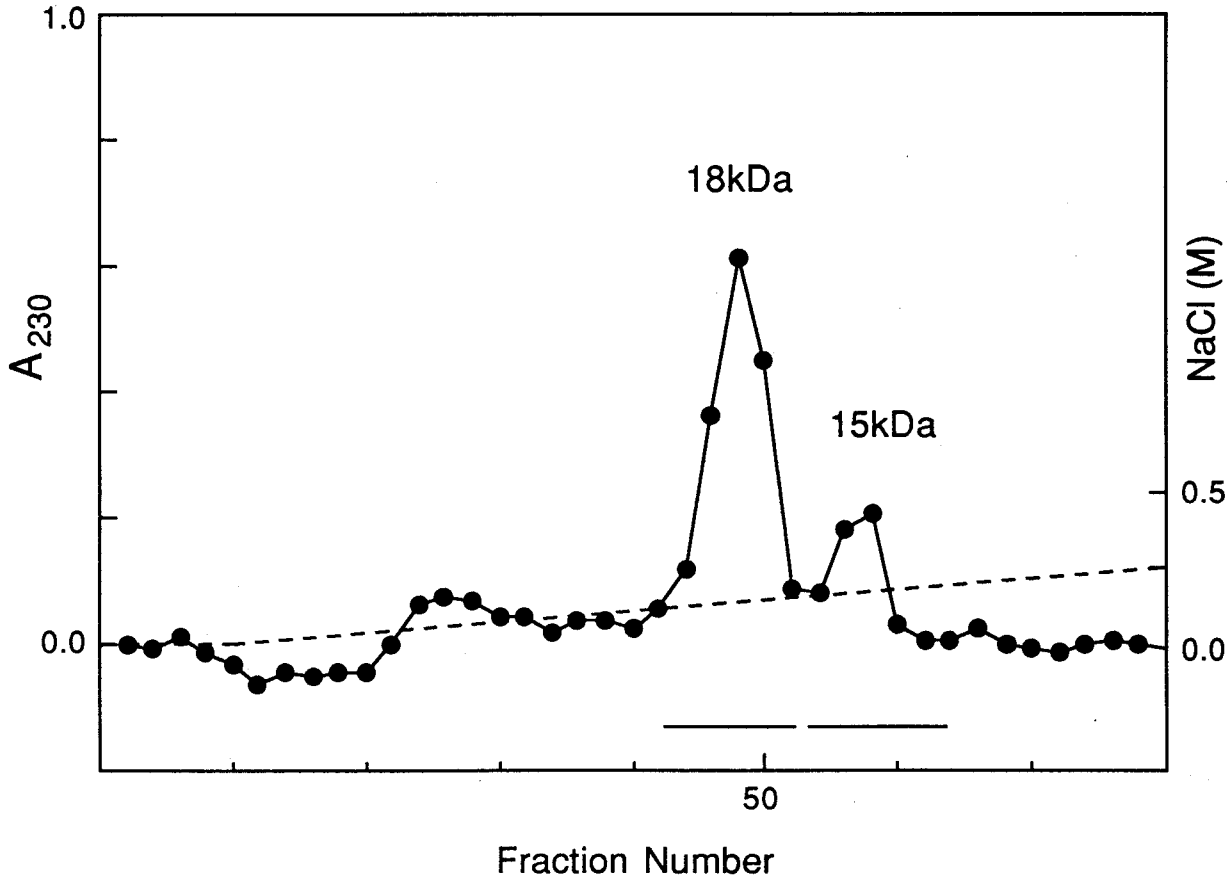

Fig. I-7S. Separation of Fraction IV3-2 on a CM-52 column. Fractions of 2.8 ml were collected at a flow rate of 14 ml/h.

Sequence Studies on the 9-kDa Polypeptide — The N-terminal sequence of the Cm-9-kDa polypeptide was determined up to the 11th residue. The C-terminal sequence was analyzed by carboxypeptidase A digestion. Histidine (0.9 mol/mol) was released during a 4 h incubation.

The Cm-9-kDa polypeptide (40 nmol) was digested with arginylendopeptidase (25 mg) in 50 mM Tris-HCl buffer (pH 8.0) at 37°C for 3 h. The digest was separated on HPLC into four peptides, R-1 to R-4 (Fig. I-8S). Their amino acid compositions are listed in Table I-1S. Peptide R-1 (18 nmol) was further digested with lysylendopeptidase (6 mg) in 50 mM Tris-HCl buffer (pH 9.0) at 30°C for 5 h. Seven peptides (R1-K-1 to R1-K-7) were obtained on HPLC (Fig. I-9S, Table I-2S). But their yields were very low and peptides corresponding to residues 1–9 and 61–62 could not be recovered. Non-specific digestion was observed for peptide R1-K-6, which resulted from cleavage on the C-terminal side of a tyrosine residue (residue 49). The amino acid sequence of about 90% of the protein was determined by sequencing each peptide.

In order to determine the sequence of the remaining parts, we digested the Cm-9-kDa polypeptide (30 nmol) with staphylococcal protease (8 mg) in 40 mM sodium phosphate buffer (pH 7.4) at 37°C for 3 h. Four peptides (V-1 to V-4) were obtained (Fig. I-10S, Table I-3S). The amino acid compositions of peptides V-3 and V-4 were very similar to each other except for the content of methionine. This might be accounted for by modification or destruction of the side chain of a methionine residue. The sequencing of these peptides established the complete amino acid sequence of the 9-kDa polypeptide. A summary of these studies is presented in Fig. I-2.

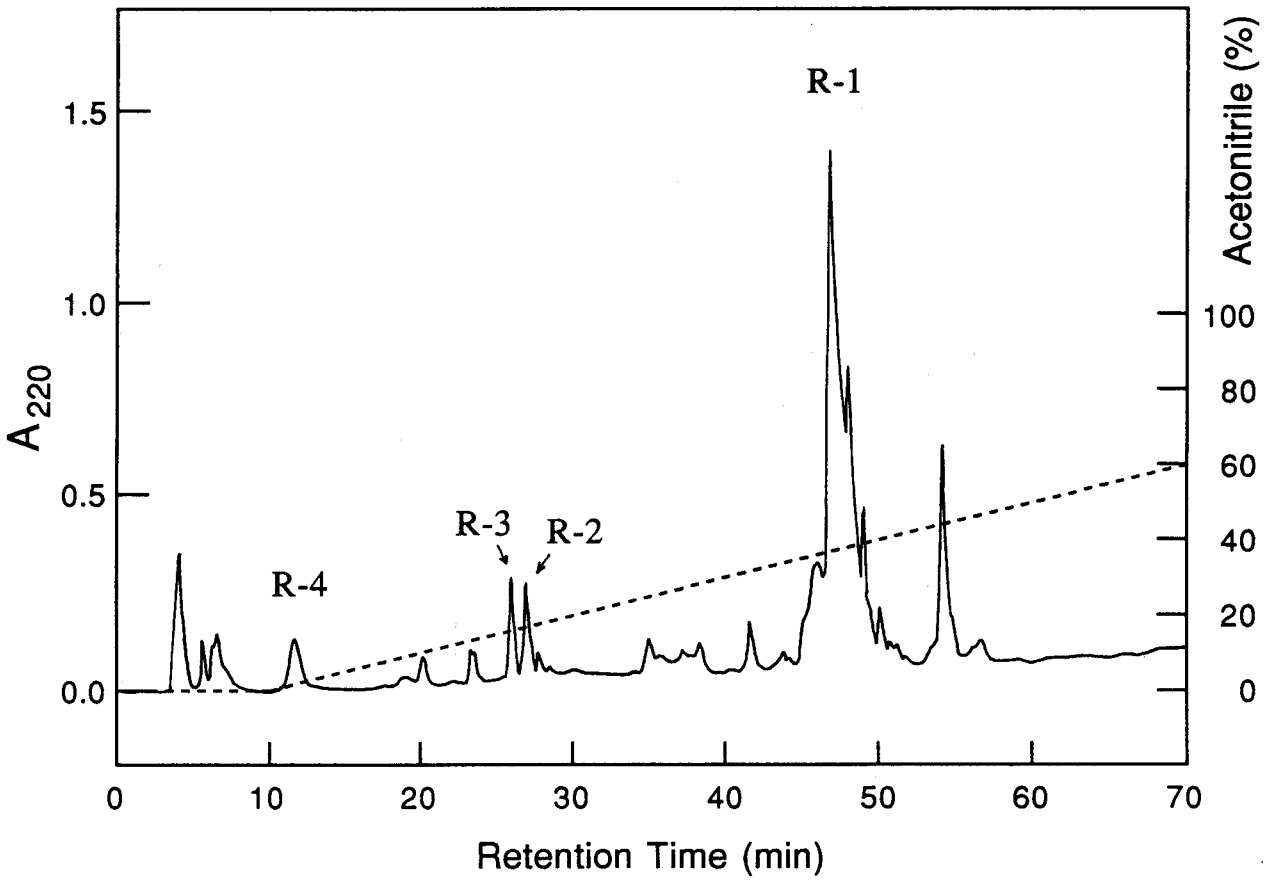

Fig. I-8S. Separation of an arginylendopeptidase digest of the Cm-9-kDa polypeptide on a Shodex ODSpak F-511A column.

Table I-1S. Amino acid compositions of arginylendopeptidase peptides of the Cm-9-kDa polypeptide.

	R-1	R-2	R-3	R-4
Cmc				
Asx	9.3 (9)	0.3		
Thr	5.0 (5)			
Ser	5.3 (6)	2.7 (3)	1.7 (2)	0.9 (1)
Glx	5.5 (5)	2.4 (2)	1.1 (1)	1.1 (1)
Pro	8.3 (8)	2.9 (3)	1.9 (2)	1.0 (1)
Gly	1.9 (2)	1.3 (1)	1.0 (1)	
Ala	3.6 (3)			
Val	1.3 (1)			
Met		0.9 (1)	0.9 (1)	
Ile				
Leu	6.3 (6)			
Tyr	2.8 (3)			
Phe	2.7 (3)			
Lys	7.5 (8)	0.4		
His	1.8 (2)	1.0 (1)		1.0 (1)
Arg	1.1 (1)	1.9 (2)	0.9 (1)	1.0 (1)
Trp				
Total	62	13	8	5
Position	1–62	63–75	63–70	71–75
Yield(%)	63	13	45	48

Cmc; S-carboxymethylcysteine.

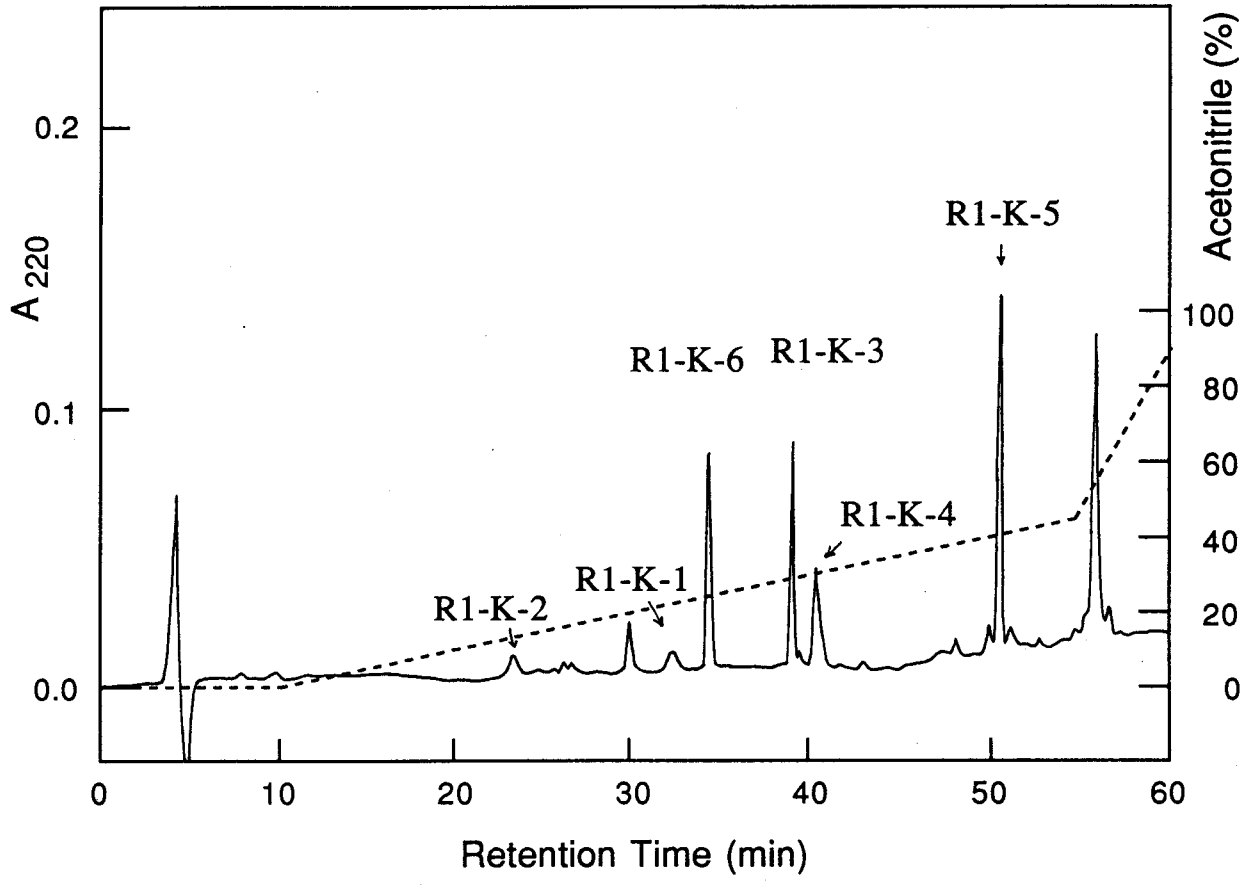

Fig. I-9S. Separation of a lysylendopeptidase digest of peptide R-1 of the Cm-9-kDa polypeptide on a Shodex ODSpak F-511A column.

Table I-2S. Amino acid compositions of lysylendopeptidase peptides of peptide R-1.

	R1-K-1	R1-K-2	R1-K-3	R1-K-4	R1-K-5	R1-K-6
Cmc						
Asx	1.1 (1)		2.2 (2)	1.9 (2)	4.9 (5)	2.4 (2)
Thr		0.3	2.6 (3)	2.5 (3)	1.9 (2)	1.1 (1)
Ser		1.0 (1)	1.1 (1)	1.0 (1)	2.2 (2)	1.1 (1)
Glx		1.4 (1)	1.3 (1)	1.3 (1)	0.9 (1)	1.3 (1)
Pro	2.8 (3)	1.7 (2)	2.9 (3)	2.5 (3)		
Gly	1.0 (1)		0.4	0.4	0.4	
Ala			1.6 (2)	1.7 (2)		
Val			1.4 (1)	1.2 (1)		
Met						
Ile						
Leu	0.8 (1)				4.6 (5)	1.0 (1)
Tyr			0.9 (1)	0.8 (1)	1.9 (2)	1.7 (2)
Phe			0.7 (1)	0.8 (1)	0.7 (1)	
Lys	1.0 (1)	0.9 (1)	2.1 (2)	1.1 (1)	1.2 (1)	
His					1.7 (2)	1.8 (2)
Arg						
Trp						
Total	7	5	17	16	21	10
Position	10–16	18–22	23–39	24–39	40–60	40–49
Yield(%)	7	8	4	4	2	3

Cmc; S-carboxymethylcysteine.

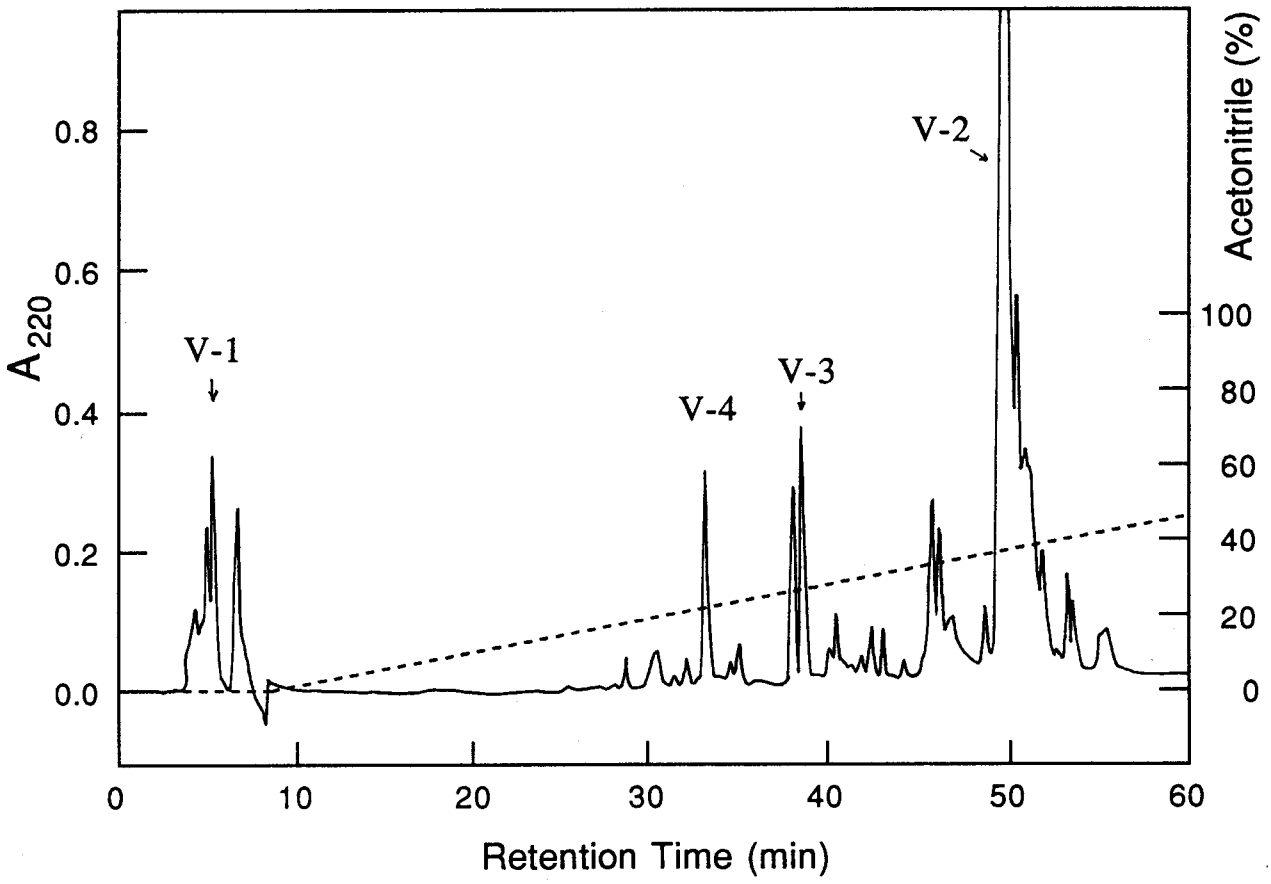

Fig. I-10S. Separation of a staphylococcal protease digest of the Cm-9-kDa polypeptide on a Shodex ODSpak F-511A column.

Table I-3S. Amino acid compositions of staphylococcal V8 protease peptides of the Cm-9-kDa polypeptide.

	V-1	V-2	V-3	V-4
Cmc				
Asx	1.1 (1)	8.1 (8)	0.1	0.1
Thr	0.1	4.4 (5)		
Ser	1.5 (2)	3.3 (3)	3.5 (4)	3.6 (4)
Glx	2.2 (2)	3.5 (3)	2.3 (2)	2.2 (2)
Pro		7.7 (8)	2.9 (3)	2.9 (3)
Gly	1.2 (1)	1.4 (1)	1.1 (1)	1.2 (1)
Ala	1.3 (1)	2.3 (2)		
Val	0.4	1.4 (1)		
Met			0.9 (1)	0.2
Ile				
Leu		4.9 (5)	1.0 (1)	1.0 (1)
Tyr		2.6 (3)		
Phe		1.8 (2)	0.8 (1)	0.8 (1)
Lys	1.0 (1)	6.1 (6)	1.0 (1)	1.1 (1)
His	0.3	1.8 (2)	0.9 (1)	0.9 (1)
Arg		0.3	2.9 (3)	3.0 (3)
Trp				
Total	8	49	18	17
Position	1–8	9–57	58–75	58–75
Yield(%)	63	70	40	27

Cmc; S-carboxymethylcysteine.

Sequence Studies on the IP 20-kDa Polypeptide — The N-terminal sequence of the Cm-20-kDa polypeptide was determined up to the 28th residue (Fig. I-12S). The Cit-Cm-20-kDa polypeptide (30 nmol) was digested with trypsin (6 mg) in 50 mM ammonium bicarbonate at 37°C for 4 h. After decitraconylation, the resultant peptides (T-1 to T-9) were separated (Fig. I-11S) and sequenced (Fig. I-12S).

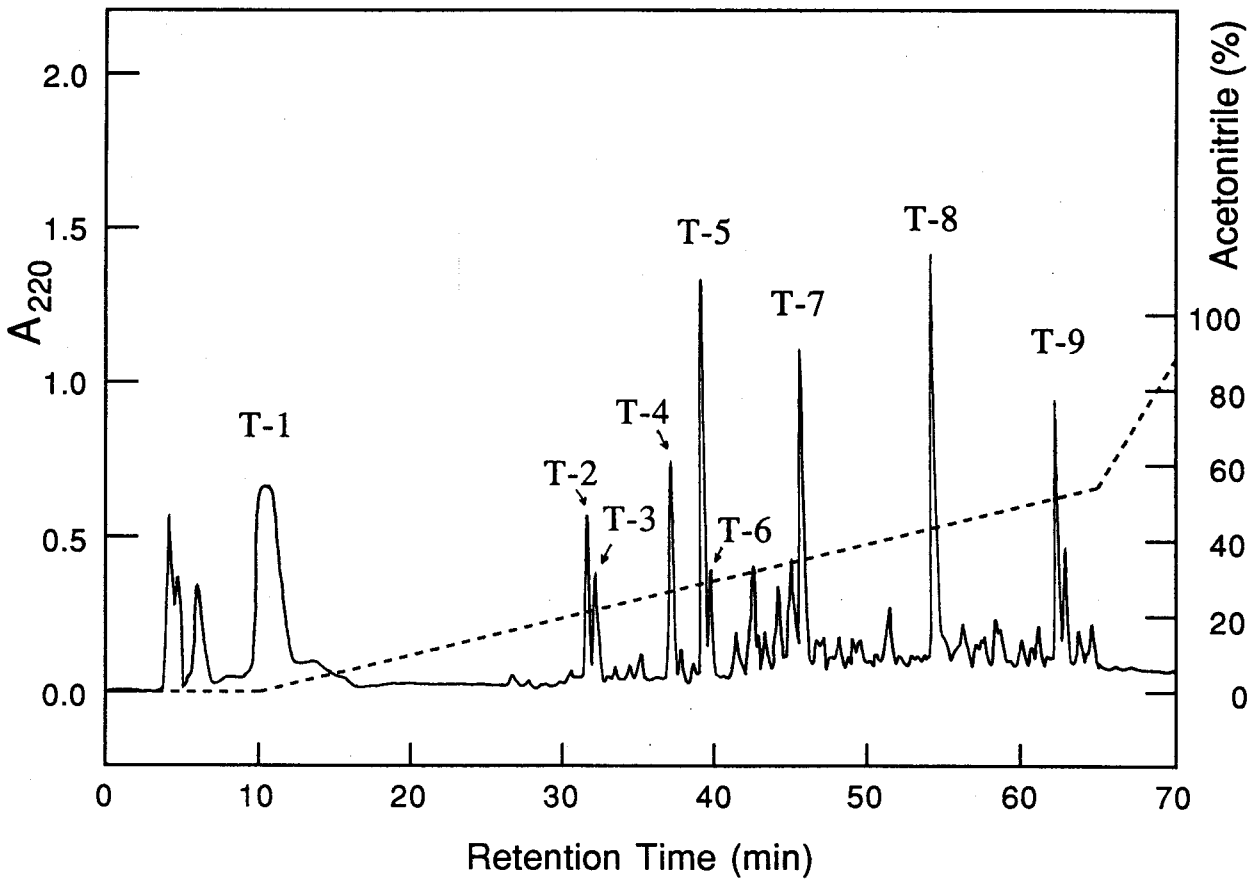

Fig. I-11S. Separation of a tryptic digest of the Cit-Cm-20-kDa polypeptide on a Shodex ODSpak F-511A column.

N-terminus	PSSTQPAVSQARAVVPKPAALPSSRGEY--
T-1	ASPR
T-2	YDMDR
T-3	PSSTQPAVSQAR
T-4	KVYDQMPEPR
T-5	IWYR
T-6	AVVPKPAALPSSR
T-7	YVVSMGSCANGGGYYHYSYSVVR
T-8	GEYVVAKLDDLI--
T-9	IVPVDIYVPGCPP--

Fig. I-12S. Partial amino acid sequences of the 20-kDa polypeptide.

RESULTS AND DISCUSSION

Subunit Composition of FP — Three polypeptides, 51-kDa, 24-kDa and 9-kDa, were isolated from FP of bovine heart complex I. Fig. I-1a shows the electrophoretic patterns of the purified polypeptides on polyacrylamide gel. Table I-1 presents the amino acid composition of the isolated polypeptides. The compositions of the 51-kDa and 24-kDa subunits derived from the cDNA sequences are also listed in the table and agree well with those obtained in this study or published earlier (*31*).

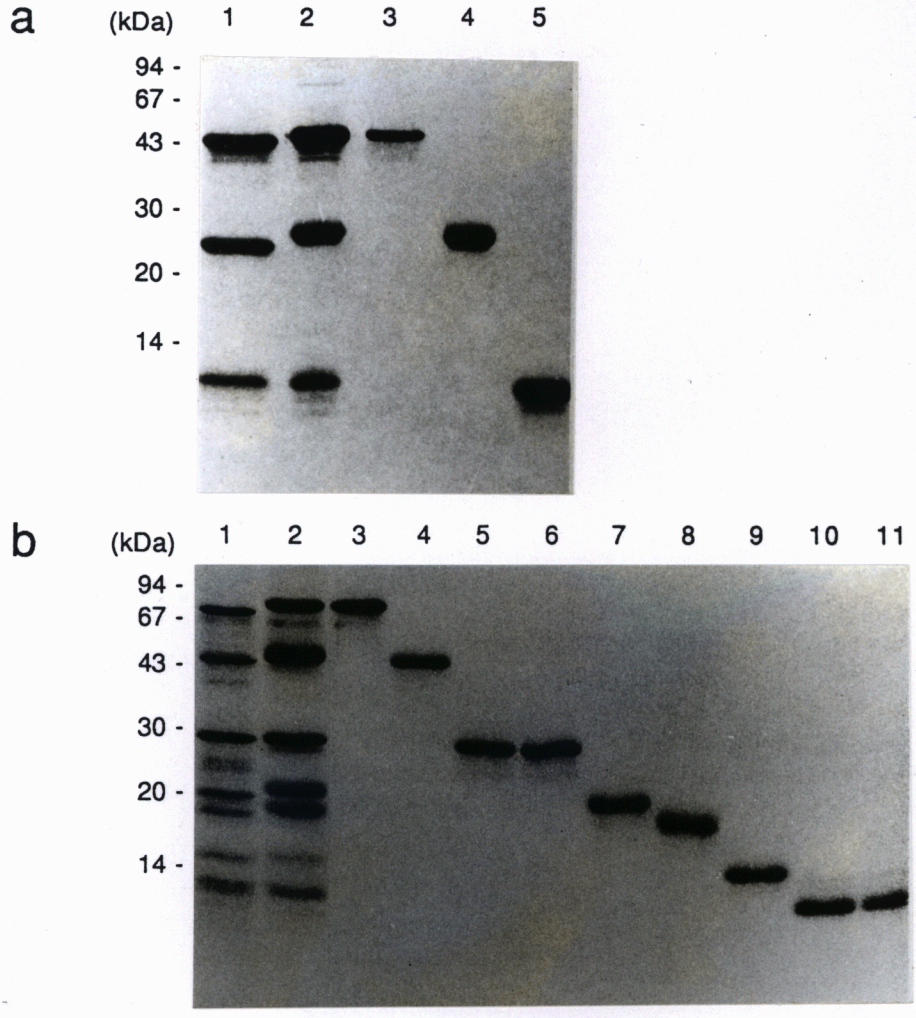

Fig. I-1. SDS-PAGE of isolated polypeptides from FP (a) and IP (b). a: The samples applied were as follows: lane 1, FP; lane 2, Cm-FP; lane 3, 51-kDa; lane 4, 24-kDa; lane 5, 9-kDa. Each polypeptide in lanes 3 to 5 was carboxymetylated. b: The samples applied were as follows: lane 1, IP; lane 2, Cit-Cm-IP; lane 3, 75-kDa; lane 4, 49-kDa; lane 5, 30-kDa-A; lane 6, 30-kDa-B; lane 7, 20-kDa; lane 8, 18-kDa; lane 9, 15-kDa; lane 10, 13-kDa-A; lane 11, 13-kDa-B. Each polypeptide in lanes 3 to 11 was carboxymethylated and citraconylated.

Table I-1 also gives the yield of each polypeptide obtained from one sample of FP. Previous studies showed that the 51-kDa, 24-kDa and 9-kDa subunits seemed to be present in equimolar amounts (*31*). In this study the 51-kDa and 24-kDa polypeptides were obtained in comparable amounts, whereas the yield of the 9-kDa polypeptide was considerably lower than those of the others. This might have resulted from dissociation of the 9-kDa polypeptide from FP during purification and/or incomplete precipitation of this small protein by trichloroacetic acid.

Table I-1. Amino acid compositions of isolated polypeptides of FP.

	51kDa	24kDa	9kDa
Asx	35.0 (35)	20.8 (20)	9.0 (9)
Thr	22.2 (23)	13.4 (14)	4.8 (5)
Ser	21.7 (22)	6.4 (6)	8.0 (9)
Glx	51.1 (46)	25.5 (24)	7.6 (7)
Pro	22.0 (22)	20.7 (21)	11.0 (11)
Gly	51.8 (52)	17.8 (18)	3.1 (3)
Ala	43.2 (39)	17.3 (16)	3.8 (3)
Cys	11.3 (11)	4.9 (5)	0.0
Val	31.9 (29)	17.0 (15)	1.3 (1)
Met	4.9 (8)	3.0 (5)	0.8 (1)
Ile	24.1 (27)	13.2 (14)	0.0
Leu	29.9 (29)	17.1 (17)	6.0 (6)
Tyr	10.4 (10)	7.9 (8)	3.0 (3)
Phe	16.0 (18)	6.0 (7)	2.8 (3)
Lys	22.7 (25)	13.8 (15)	7.3 (8)
His	8.9 (9)	3.0 (3)	2.9 (3)
Arg	29.2 (29)	8.0 (8)	3.3 (3)
Trp	n.d. (8)	n.d. (1)	n.d. (0)
Total	(444)	(217)	(75)
Yield (nmol)	179	217	98

The composition was calculated so that the total number of amino acid residues agreed with that of the sequence. The yield of each polypeptide from one sample of FP was determined on the basis of the amino acid analysis. Cysteine was determined as S-carboxymethylcysteine. Values in parentheses were deduced from the sequences (*18*, *19*). n.d.; not determined.

Protein Structure of the 9-kDa Polypeptide — We determined the complete amino acid sequence of the 9-kDa polypeptide of FP, as summarized in Fig. I-2. The total number of amino acid residues was 75 and the molecular weight was calculated to be 8,437, which was close to the value of 9,000 estimated by SDS-PAGE. The amino acid composition calculated from the sequence was in good agreement with that obtained by direct analysis of the protein. Topological studies suggested that the 9-kDa subunit of FP is exposed to the matrix side of the mitochondrial inner membrane (*32*). The hydropathy plot and prediction of the secondary structure indicate that the 9-kDa subunit contains no obvious membrane-spanning domain.

FP contains an FMN and at least two EPR-visible iron-sulfur centers. Comparison of the sequences has shown that none of the 51-kDa, 24-kDa and 9-kDa subunits contain sequences related to FMN-binding proteins (*18, 19*); however, it is most probable that the FMN-binding site is located in the 51-kDa subunit since this subunit binds NADH, from which electrons are likely to be directly transferred to FMN (*4*).

The 4Fe-4S center designated N-3 is assigned to the 51-kDa subunit, whereas the 2Fe-2S center assigned to the 24-kDa or 9-kDa subunit (*33*). The mammalian 24-kDa subunit contains five conserved cysteine residues but has no sequence motif characteristic of various iron-sulfur proteins (*18, 34–36*). The 9-kDa subunit contains no cysteine residue and exhibits no sequence similarity to any known iron-sulfur protein. Thus the 2Fe-2S center is probably located in the 24-kDa subunit.

Pilkington *et al.* have recently shown that the sequences of the 51-kDa and 24-kDa subunits exhibit an extensive similarity to that of the α subunit of the NAD-reducing hydrogenase from the bacterium *Alcaligenes eutrophus* and that the N-terminal region of the 75-kDa subunit of IP is also similar to that of the γ subunit of the hydrogenase (*19*). These similarities suggest an evolutionary relationship between the FP of complex I and the NAD-linked hydrogenase. However, the *A. eutrophus* NAD-linked hydrogenase does not contain a counterpart of the 9-kDa subunit of FP (*37*) and comparison of the sequences revealed no homologue of the 9-kDa subunit in the operon for this hydrogenase (*38*). Moreover, no subunit corresponding to the 9-kDa subunit was detected in a FP-type fraction of the NADH:quinone oxidoreductase from *Paracoccus denitrificans* (*39*). Generally, mammalian respiratory complexes contain additional polypeptides as compared with their bacterial counterparts.

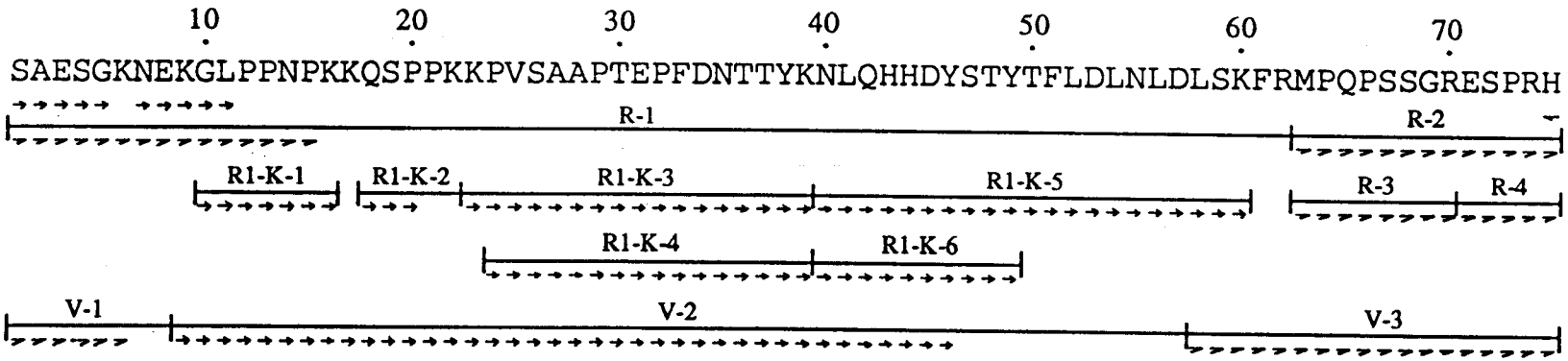

Fig. I-2. Summary of sequence studies on the 9-kDa polypeptide. Arrows, ⇀ , → , and ↼ , show determination by manual Edman degradation, with a gas-phase protein sequencer, and by carboxypeptidase digestion, respectively. Dashed arrows indicate ambiguous identification.

These results indicate that the 9-kDa subunit has been identified as a constituent only in the mammalian complex I. A search in database did not reveal any homologous proteins to the 9-kDa subunit. Further studies will be required to elucidate the function of this subunit.

Subunit Composition of IP — From the IP of bovine heart complex I, nine polypeptides, that is, 75-kDa, 49-kDa, two 30-kDa, 20-kDa, 18-kDa, 15-kDa and two 13-kDa polypeptides, were isolated. Fig. I-1b shows the electrophoretic patterns of the isolated polypeptides. Most proteins showed slightly lower electrophoretic mobilities upon carboxymethylation and citraconylation. Previous studies have shown that the major protein components of IP are the 75-kDa, 49-kDa, 30-kDa, 18-kDa, 15-kDa and 13-kDa polypeptides (*1–4*), and suggested the multiplicity of the 13-kDa band (*40*). Table I-2 gives the amino acid composition of each polypeptide. The compositions deduced from the cDNA sequences agreed well with the values obtained in this study. The two 13-kDa polypeptides showed dissimilar compositions. Our previous study showed that the primary structures of the 13-kDa-A and 13-kDa-B polypeptides differ from each other (*25*).

IP is rather heterogeneous and may contain variable amounts of FP subunits (*3, 4*). The IP preparation used for this study contained a relatively minor amount of the 24-kDa polypeptide of FP, as can be seen in Fig. I-1b (lane 1). The Cm-24-kDa polypeptide gave a band corresponding to an apparent molecular weight of about 30,000 (Fig. I-1a). Comparison of amino acid compositions revealed that those of the 30-kDa-A and 24-kDa polypeptides resembled each other. In fact the partial amino acid sequence of the 30-kDa-A was identical to that of the 24-kDa subunit (data not shown), suggesting that the 30-kDa-A protein corresponds to the contaminating 24-kDa protein and that IP probably contains only one subunit with a molecular weight around 30,000.

The cDNA sequence of the 30-kDa subunit has recently been determined (*15*) and the calculated composition was in good agreement with that of the 30-kDa-B polypeptide. We have reported the partial amino acid sequence of the 30-kDa-B band separated from IP (*25*). Most of the peptide sequences of the 30-kDa-B polypeptide could be aligned with the cDNA sequence, except for one peptide (CIYCGFCQEACPVDAIVE). This peptide sequence contained the cysteine-rich motif that is common in a 4Fe-4S cluster-containing protein. More recently, Dupuis *et al.* have reported the cDNA sequence of the 23-kDa subunit of complex I, which is homologous to the *frxB* gene product encoded in the chloroplast DNA (*16*).

Table I-2. Amino acid compositions of isolated polypeptides of IP.

	75kDa	49kDa	30kDa-A	30kDa-B	20kDa	18kDa	15kDa	13kDa-A	13kDa-B
Asx	71.5 (68)	41.0 (39)	20.8 (20)	23.5 (24)	11.3	20.1	6.3	9.5 (9)	5.3 (5)
Thr	43.9 (45)	20.0 (19)	13.3 (14)	10.4 (10)	5.0	15.3	4.1	6.9 (7)	5.7 (6)
Ser	37.3 (40)	18.4 (19)	6.1 (6)	12.0 (13)	12.1	9.8	6.8	4.4 (4)	3.2 (3)
Glx	74.8 (73)	47.4 (45)	26.7 (24)	29.4 (29)	11.7	17.9	23.1	13.3 (12)	20.4 (20)
Pro	32.0 (33)	26.8 (28)	20.9 (21)	17.3 (17)	14.3	7.4	8.2	3.4 (3)	8.2 (7)
Gly	51.9 (50)	31.3 (31)	18.2 (18)	8.2 (7)	10.5	5.7	5.8	12.7 (14)	6.4 (6)
Ala	68.6 (65)	31.6 (28)	16.4 (16)	17.8 (18)	20.8	12.7	8.0	4.4 (3)	8.6 (8)
Cys	16.5 (18)	6.6 (6)	4.8 (5)	2.6 (2)	5.2	0.4	4.5	3.1 (3)	1.0 (1)
Val	60.2 (58)	32.7 (30)	15.1 (15)	15.6 (19)	18.6	12.3	4.0	10.4 (10)	6.9 (7)
Met	21.2 (23)	17.1 (19)	4.2 (5)	1.4 (1)	7.8	3.1	2.2	0.3 (0)	2.0 (2)
Ile	42.8 (43)	23.0 (24)	12.3 (14)	8.6 (10)	7.0	6.2	9.3	4.0 (4)	7.4 (8)
Leu	65.4 (63)	38.6 (36)	17.4 (17)	18.6 (19)	12.4	7.4	7.5	4.4 (4)	14.7 (15)
Tyr	13.4 (15)	17.4 (20)	7.6 (8)	10.0 (10)	10.4	2.8	1.4	3.4 (4)	3.1 (3)
Phe	16.7 (18)	14.8 (16)	7.0 (7)	14.9 (15)	3.4	5.6	5.1	2.8 (3)	0.4 (0)
Lys	31.6 (36)	18.2 (21)	14.1 (15)	10.9 (11)	8.0	15.9	11.4	5.1 (5)	12.1 (13)
His	9.4 (11)	10.4 (13)	3.1 (3)	4.9 (5)	2.0	1.2	5.1	3.4 (4)	2.0 (2)
Arg	38.5 (38)	27.2 (28)	9.1 (8)	18.0 (17)	15.5	10.6	12.4	6.8 (7)	4.2 (4)
Trp	n.d. (7)	n.d. (8)	n.d. (1)	n.d. (3)	n.d.	n.d.	n.d.	n.d. (0)	n.d. (4)
Total	(704)	(430)	(217)	(228)	176	154	124	(96)	(114)
Yield (nmol)	80	123	23	53	25	40	9	46	38

The total number of amino acid residues was determined based on the apparent molecular weight. The yield of each polypeptide from one sample of IP was determined on the basis of the amino acid analysis. The composition of 30kDa-A was calculated according to that of FP 24kDa (*18*). Cysteine was determined as S-carboxymethylcysteine. Values in parentheses were deduced from the sequences (*14*, *15*, *17*, *25*). n.d.; not determined.

Surprisingly, the cysteine-rich peptide obtained from the 30-kDa-B band had the same sequence as residues 116 to 134 of the 23-kDa subunit. This means that the 30-kDa-B band contained the 23-kDa protein. The 23-kDa subunit is likely to be a component of HP. However, the *frxB* protein, which is homologous to the 23-kDa subunit, has been obtained from a high-salt extract of the thylakoid membrane (*41*). It is possible that upon resolution of complex I with chaotropic salts a considerable amount of the 23-kDa polypeptide dissociates from HP and becomes incorporated into IP. It is further possible that this polypeptide migrates together with the 30-kDa polypeptide of IP after reduction and alkylation of the cysteine residues, as in the case of the 24-kDa polypeptide. That the 30-kDa subunit has no sequence for harboring an iron-sulfur cluster is now clear from the data of Pilkington *et al.* (*15*).

IP also contains variable amounts of several polypeptides with apparent molecular weights in the region of 20,000 (*4*). In this experiment we obtained a relatively large amount of one of these polypeptides. The amino acid composition of this polypeptide was quite different from those of the 18-kDa, 23-kDa and 24-kDa subunits. HP contains several polypeptides including the ND6 gene product in this apparent size range. The composition of the ND6 gene product was also different from that of the 20-kDa polypeptide. It is at present unclear whether this protein fraction contains multiple polypeptides or not. However, the N-terminal sequence analysis suggests that this fraction contains mainly a single polypeptide (Fig. I-12S). Therefore, this 20-kDa polypeptide may be a novel component of IP or an unidentified subunit of HP.

The stoichiometry of the component polypeptides was difficult to determine in this experiment because parts of the proteins were lost at some steps of the resolution procedure. For example, trichloroacetic acid could only incompletely precipitate small proteins, and lyophilization decreased the solubilities of some proteins. In addition, the relative amounts of some polypeptides varied from one preparation of IP to another. However, the yields of the polypeptides could roughly reflect their stoichiometric relationships.

Table I-2 gives the yield of each polypeptide isolated from one sample of IP. The 30-kDa-B, 20-kDa, 18-kDa, 13-kDa-A and 13-kDa-B polypeptides were obtained in comparable amounts. Previous studies showed that the 49-kDa, 30-kDa and 13-kDa subunits seemed to be present in a 1:1:1 molar ratio (*42*). The yields of the 75-kDa and 49-kDa polypeptides

were higher than those of other polypeptides, whereas that of the 15-kDa polypeptide was lower. Both the 75-kDa and 49-kDa polypeptides could be isolated only by gel filtration chromatography, and the high yields of these proteins might be due to this simple purification method. In contrast, several resolution steps and the decreased solubility due to decitraconylation appear to be at least partly responsible for the low yield of the 15-kDa polypeptide.

The 20-kDa polypeptide was isolated from every sample of IP by reverse-phase HPLC and its yield was comparable with those of other component polypeptides. This suggests that the 20-kDa polypeptide may be present as a major component of IP. This expectation would be supported by the observation that the sequence of this polypeptide is similar to the ORF cotranscribed with two *ndh* genes in the chloroplast DNA. The relationship between these proteins will be discussed later.

Minor amounts of other proteins smaller than 13-kDa are also present in IP, as can be seen in Fig. I-1b (lane 1). We isolated the 10-kDa polypeptide, whose amino acid composition seemed to be different from that of the 9-kDa subunit of FP (data not shown). However, its yield was considerably lower than those of other major polypeptides. Probably this polypeptide is a minor component, although other respiratory complexes contain small polypeptides as true constituents.

In conclusion, these results indicated that major protein components of IP were eight polypeptides, that is, 75-kDa, 49-kDa, 30-kDa, 20-kDa, 18-kDa, 15-kDa, 13-kDa-A and 13-kDa-B polypeptides.

Protein Structure of the 20-kDa Polypeptide — Iron-sulfur proteins contain essential cysteine or histidine residues that participate in cluster formation. In addition to the subunits that have been sequenced, the 20-kDa and 15-kDa polypeptides contain enough cysteines and histidines to be candidates for iron-sulfur proteins. In order to obtain structural information, we determined the partial amino acid sequence of the 20-kDa polypeptide (Fig. I-12S). The N-terminal sequence of this protein was not related to any other reported sequences; however, a comparison of several peptide sequences with protein sequences in database revealed a significant similarity to the *psbG* gene product encoded in the chloroplast DNA (*10*, *12*, *13*, *43*, *44*) and cyanobacterial genomic DNA (*45*, *46*). Fig. I-3 shows an alignment of eight peptide sequences (except for T-5) of the 20-kDa polypeptide with the PsbG sequences from

several species. A *psbG*-like ORF in the mitochondrial genome of the ciliate *Paramecium* (*47*) is also aligned in Fig. I-3.

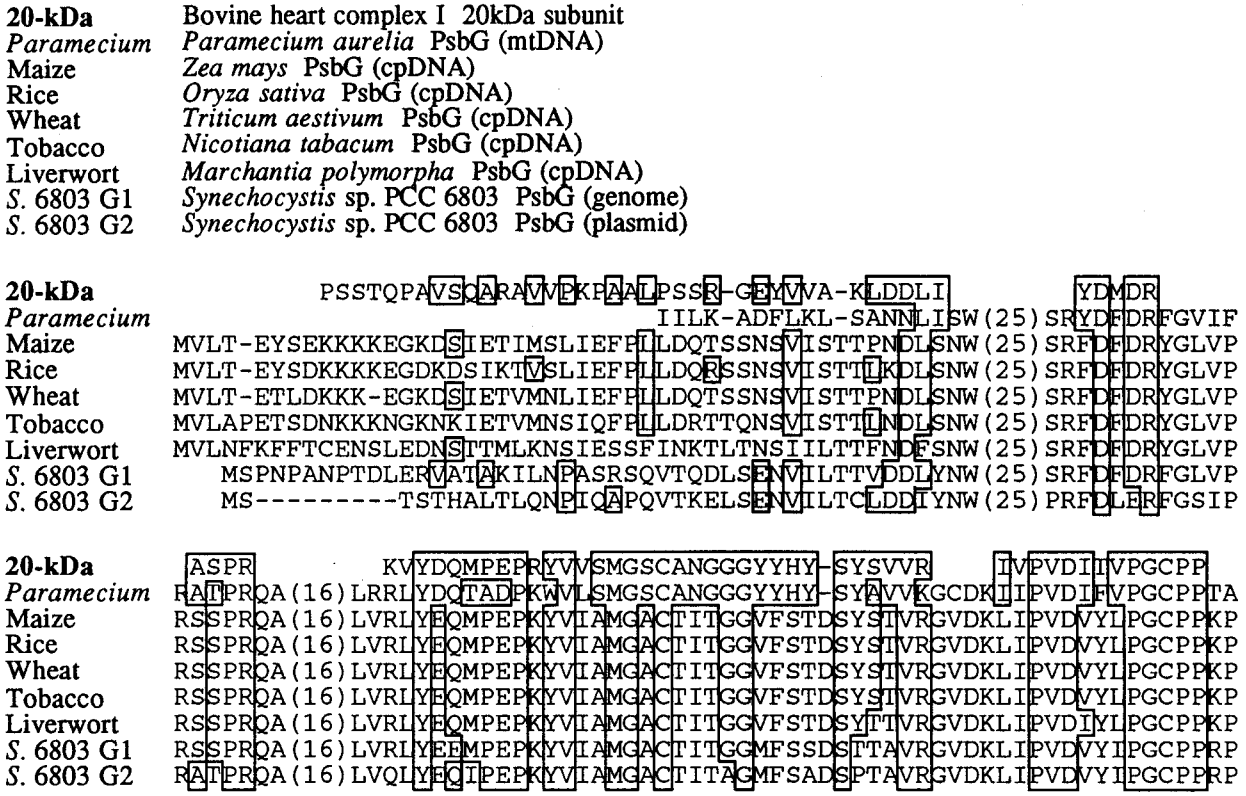

Fig. I-3. Alignment of peptide sequences of the 20-kDa polypeptide with the sequences of the *psbG* gene products. Peptides T-3, -6, -8, -2, -1, -4, -7, and -9 from the 20-kDa polypeptide are aligned in that order. Identical amino acids are boxed. The figures in parentheses are the numbers of amino acids in these regions. Dashes (–) show insertions to improve alignment. mtDNA; mitochondrial DNA, cpDNA; chloroplast DNA. PsbG2 is the product of second *psbG* gene located on a megaplasmid in that cyanobacterium (*46*).

The intensive similarity is limited to short stretches of the primary structures. However, since these sequences are located at the center of highly conserved regions in all the PsbG proteins, they could be functionally significant. The 20-kDa sequence appears more similar to that of the *Paramecium* protein thatn the others. These results suggest that the 20-kDa polypeptide of complex I shows overall similarity to the *psbG* gene product. It should be pointed out, however, that the size of the 20-kDa protein is smaller than those of the PsbG proteins and its N-terminal sequence is not related to them. Also, in PsbG proteins the terminal regions are not well conserved (*46*). In particular, the *Paramecium* protein is shortened at the N- and C-termini, and its size (156 residues) is significantly smaller than the others (243–248 residues) (*47*).

The *psbG* gene is located between *ndhC* and *ndhJ* in the chloroplast DNA and cyanobacterial genome DNA, and has been proposed to encode a component of photosystem II (*43*). However, the similarity to the 20-kDa polypeptide suggests that the PsbG protein might be a component of a putative complex I-like assembly in the chloroplast and cyanobacteria, which agrees with the more recent view of Nixon *et al.* (*44*). Therefore, it is reasonable that the *psbG* gene should be renamed *ndhK*, according to the proposal of Dupuis *et al.*(*16*). Inconsistency of the amount of this protein in IP preparations may be explained by its loose binding to the subcomplex since the PsbG polypeptide has been suggested to be a peripheral protein (*9*).

Another *psbG*-like ORF has recently been found in an operon coding for components of formate hydrogenlyase from *Escherichia coli* (*48, 49*). Some of gene products in this operon share the sequence similarity with the subunits of complex I and the *ndh* gene products. Among them *hycG* gene product is related to those of *psbG* (Fig. I-4).

In order to obtain clues to the function of the 20-kDa and PsbG proteins, we searched for protein sequences similar to the well conserved region among these proteins in databases and currently available sequences, and detected a limited but significant similarity to the small subunits of nickel-containing hydrogenases from eubacteria and archaebacteria (*38, 50–58*). All known nickel-containing hydrogenases have at least two unlike subunits that seem to comprise the catalytic core of the hydrogenase. Similarity to the 20-kDa and PsbG proteins was detected in the central segment of the small subunits. Fig. I-4 presents the related regions of the small subunits from several bacteria. Two cysteine residues and several

other residues in these segments appear to be conserved in all the sequences currently available. Like complex I, the hydrogenase system contains multiple iron-sulfur centers, and at least one cluster is presumed to be located in the small subunit (*38*). These conserved cysteine residues may be involved in the formation of an iron-sulfur cluster within each enzyme complex.

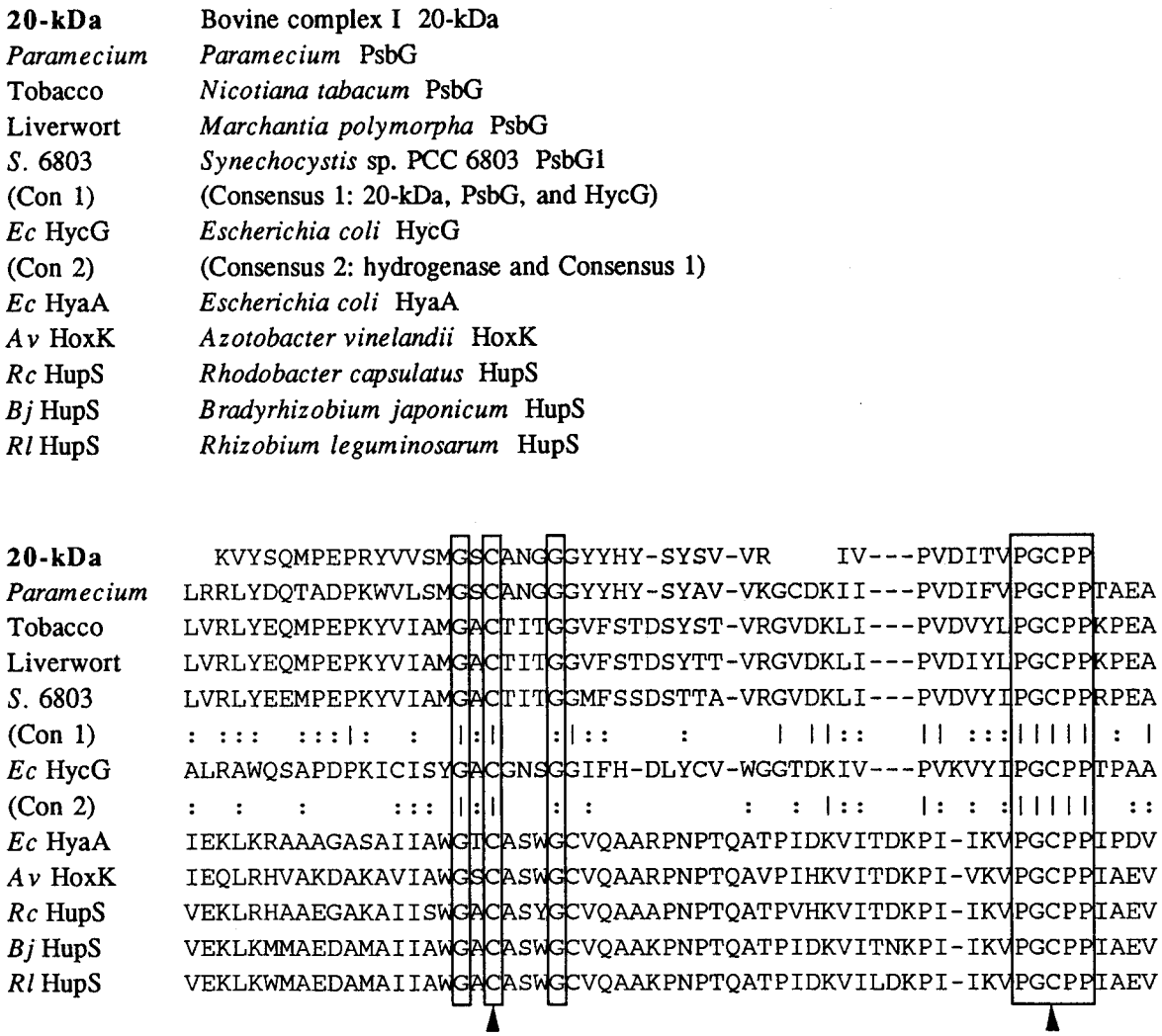

Fig. I-4. Sequence similarity to the 20-kDa/PsbG proteins to the small subunits of nickel-containing hydrogenases. Consensus 1 indicates the location at which an amino acid residue identical with the HycG protein occurs in all the 20-kDa/PsbG proteins. Consensus 2 indicates the location at which an identical residue with Consensus 1 occurs in most of the sequences of the small subunits. Vertical bars (|) show identical amino acids. Colons (:) show conservative substitutions. Dashes (–) show insertions to improve alignment. Sequences from hydrogenases were obtained from the references (*50–54*).

In addition, it should be noted that the large subunits of the nickel-containing hydrogenases also showed a significant similarity to the 49-kDa subunit of complex I and the *ndhH* gene products. Therefore, it seems that both the core subunit of the nickel-containing hydrogenases are related to the subunits of complex I. In this respect it should be noted that the *Paramecium* mitochondrial genome contains the two ORFs homologous to the *ndhH* and *psbG* genes of the chloroplast DNA, respectively. The results presented in this chapter suggest that these genes may encode the functional 49-kDa and 20-kDa subunits of the *Paramecium* complex I although these have never been identified in other mitochondrial genomes. These relationships imply that the 49-kDa and 20-kDa subunits constitute a structural unit in mitochondrial complex I, which may play a fuctional role as redox carriers.

Acknowledgments

I would like to thank Dr. Y. Hatefi (Research Institute of Scripps Clinic) for preparing the complex I and its subfractions. I also thank Drs. S. Wakabayashi (Himeji Institute of Technology) and T. Yagi (Research Institute of Scripps Clinic) for the helpful suggestions and advice.

REFERENCES

1. Hatefi, Y., Haavik, A. G., & Griffiths, D. E. (1962) *J. Biol. Chem.* **237**,1676–1680
2. Hatefi, Y. (1985) *Ann. Rev. Biochem.* **54**, 1015–1069
3. Hatefi, Y., Ragan, C. I., & Galante, Y. M. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., ed.) Vol. 4, pp. 1–70, Plenum Press, New York
4. Ragan, C. I. (1986) *Curr. Topics Bioenerg.* **15**, 1–36
5. Chomyn, A., Mariottini, P., Cleeter, M. W. J., Ragan, C.I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. F., & Attardi, G. (1985) *Nature* **314**, 592–597
6. Chomyn, A., Cleeter, M. W. J., Ragan, C. I., Riley, M., Doolittle, R. F., & Attardi, G. (1986) *Science* **234**, 614–618
7. Gibb, G. M. & Ragan, C. I. (1990) *Biochem. J.* **265**, 903–906
8. Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H., & Ozeki, H. (1986) *Nature* **322**, 571–574
9. Ohyama, K., Fukuzawa, H., Kohchi, T., Sano, T., Sano, S., Shirai, H., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H., & Ozeki, H. (1986) *J. Mol. Biol.* **203**, 281–298
10. Umesono, K., Inokuchi, H., Shiki, Y., Takeuchi, M., Chang, Z., Fukuzawa, H., Kohchi, T., Shirai, H., Ohyama, K., & Ozeki, H. (1986) *J. Mol. Biol.* **203**, 299–331
11. Kohchi, T., Shirai, H., Fukuzawa, H., Sano, T., Komano, T., Umesono, K., Inokuchi, H., Ozeki, H., & Ohyama, K. (1988) *J. Mol. Biol.* **203**, 353–372
12. Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B. Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H., & Sugiura, M. (1986) *EMBO J.* **5**, 2043–2049
13. Hiratsuka, J., Shimada, H., Whitter, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C. R., Meng, B. Y., Li, Y. Q., Kanno, A., Hishizawa, Y., Hirai, A., Shinozaki, K., & Sugiura, M. (1989) *Mol. Gen. Genet.* **217**, 185–194
14. Fearnley, I. M., Runswick, M. J., & Walker, J. E. (1989) *EMBO J.* **8**, 665–672
15. Pilkington, S. J., Skehel, J.M., & Walker, J. E. (1991) *Biochemistry* **30**, 1901–1908
16. Dupuis, A., Skehel, J. M., & Walker, J. E. (1991) *Biochemistry* **30**, 2954–2960
17. Runswick, M. J., Gennis, R. B., Fearnley, I. M., & Walker, J. E. (1989) *Biochemistry*

28, 9452–9459

18. Pilkington, S.J. & Walker, J.E. (1989) *Biochemistry* **28**, 3257–3265
19. Pilkington, S. J., Skehel, J. M., Gennis, R. B., & Walker, J. E. (1991) *Biochemistry* **30**, 2166–2175
20. Videira, A., Tropschüng, M., Wachter, E., Schnerder, H., & Werner, S. (1990) *J. Biol. Chem.* **265**, 13060–13065
21. Videira, A., Tropschüng, M., & Werner, S. (1990) *Biochem. Biophys. Res. Commun.* **166**, *280–285*
22. Videira, A., Tropschüng, M., & Werner, S. (1990) *Biochem. Biophys. Res. Commun.* **171**, 1168–1174
23. Preis, D., van der Pas, J. C., Nehls, U., Röhlen, D. -A., Sackmann, U., Jahnke, U., & Weiss, H. (1990) *Curr. Genet.* **18**, 59–64
24. Röhlen, D. -A., Hoffmann, J., van der Pas, J. C., Nehls, U., Preis, D., Sackmann, U., & Weiss, H. (1991) *FEBS Lett.* **278**, 75–78
25. Masui, R., Wakabayashi, S., Matsubara, H., & Hatefi, Y. (1991) *J. Biochem.* **109**, 534–543
26. Galante, Y. M. & Hatefi, Y. (1978) *Methods Enzymol.* **53**, 15–21
27. Spackman, D. H., Moore, S., & Stein, W. H. (1958) *Anal. Chem.* **30**, 1190–1206
28. Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
29. Dixon, H. B. F. & Perham, R. N. (1968) *Biochem. J.* **109**, 312–314
30. Laemmli, U. K. (1970) *Nature* **227**, 680–685
31. Galanti, Y. M. & Hatefi, Y. (1979) *Arch. Biochem. Biophys.* **192**, 559–568
32. Han, A. -L., Yagi, T., & Hatefi, Y. (1989) *Arch. Biochem. Biophys.* **267**, 490–496
33. Ragan, C. I., Galanti, Y. M., Hatefi, Y., & Ohnishi, T. (1982) *Biochemistry* **21**, 590–594
34. Nishikimi, M., Hosokawa, Y., Toda, H., Suzuki, H., & Ozawa, T. (1988) *Biochem. Biophys. Res. Commun.* **157**, 914–920
35. Chomyn, A. & Tsai, S. S. -A. (1989) *Curr. Genet.* **16**, 117–126
36. Toda, H., Hosokawa, Y., Nishikimi, M., Suzuki, H., Kato, K., & Ozawa, T. (1989) *Int. J. Biochem.* **21**, 1161–1168
37. Schneider, K. & Schlegel, H. G. (1976) *Biochim. Biophys. Acta* **452**, 66–80
38. Tran-Betcke, A., Warnecke, U., Böcker, C., Zaborosch, C., & Friedrich, B. (1990) *J. Bacteriol.* **172**. 2920–2929
39. George, C. L. & Ferguson, S. J. (1984) *Eur. J. Biochem.* **143**, 567–573
40. Heron, C., Smith, S., & Ragan, C. I. (1979) *Biochem. J.* **181**, 435–443

41. Wu, M., Nie, Z. Q., & Yang, J. (1989) *Plant Cell* **1**, 551–557
42. Ragan, C. I., Galante, Y. M., & Hatefi, Y. (1982) *Biochemistry* **21**, 2518–2524
43. Steimetz, A. A., Castroviejo, M., Sayre, R. T., & Bogorad, L. (1986) *J. Biol. Chem.* **261**, 2485–2488
44. Nixon, P. J., Gounaris, K., Coomber, S. A., Hunter, C. N., Dyer, T. A., & Barber, J. (1989) *J. Biol. Chem.* **264**, 14129–14135
45. Steinmüller, K., Ley, A. C., Steinmetz, A. A., Sayre, R. T., & Bogorad, L. (1989) *Mol. Gen. Genet.* **216**, 60–69
46. Mayes, S. R., Cook, K. M., & Barber, J. (1990) *FEBS Lett.* **262**, 49–54
47. Pritchard, A. E., Venuti, S. E., Ghalambor, M. A., Sable, C.L., & Cummings, D. J. (1989) *Gene* **78**, 121–134
48. Böhm, R., Sauter, M., & Böck, A. (1990) *Mol. Microbiol.* **4**, 231–243
49. Lutz, S., Jacobi, A., Schlinsog, V., Böhm, R., Sawers, G., & Böck, A. (1991) *Mol. Microbiol.* **5**, 123–135
50. Menon, N. K., Robbins, J., Peck, Jr., H. D., Chatelus, C. Y., Choi, E. -S., & Przybyla, A. E. (1990) *J. Bacteriol.* **172**, 1969–1977
51. Menon, A. L., Stults, L. W., Robson, R. L., and Mortenson, L. E.(1990) *Gene* **96**, 67–74
52. Leclere, M., Colbear, A., Cauvin, B., and Vignais, P. M. (1988) *Mol. Gen. Genet.* **214**, 97–107
53. Sayavedra-Soto, L. A., Powell, G. K., Evans, H. J., and Morris, R. D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8395–8399
54. Hidalgo, E., Leyva, A., & Ruiz-Argüeso, T. (1990) *Plant Mol. Biol.* **15**, 367–370
55. Reeve, J. N., Beckler, G. S., Cram, D. S., Hamilton, P. T., Brown, J. W., Krzycki, J. A., Kolodziej, A. F., Alex, L., Orme-Johnson, W. H., & Walsh, C. T. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3031–3035
56. Voordouw, G., Menon, N. K., Legall, J., Choi, E. -C., Peck, Jr., H. D., & Przybyla, A. E. (1989) *J. Bacteriol.* **171**, 2894–2899
57. Alex, L. A., Reeve, J. N., Orme-Johnson, W. H., & Walsh, C. T. (1990) *Biochemistry* **29**, 7237–7244
58. Uffen, R. L., Colbeau, A., Richaud, P., & Vignais, P. M. (1990) *Mol. Gen. Genet.* **221**, 49–58

Chapter II

Isolation and EPR Studies of Iron-Sulfur Proteins from Complex I

SUMMARY

NADH:ubiquinone oxidoreductase (complex I) contains several iron-sulfur clusters and at least four clusters, N-1b, N-2, N-3, and N-4, are detectable in the intact enzyme by EPR spectroscopy. Complex I from bovine heart mitochondria was dissociated in the presence of detergents, and three subfractions, here called IP-1, IP-2, and FP, were obtained by ammonium acetate fractionation. IP-1 consisted predominantly of the 75kDa subunit. IP-2 was composed of about 13 polypeptides including the 49-, 30-, 23-, and 20-kDa subunits. FP consisted predominantly of the 51kDa and 24kDa subunits. These fractions contained EPR-detectable iron-sulfur clusters, some of which were similar to EPR-detectable species in the intact complex I. By combining information on subunit structure, it was proposed that the N-1b, N-2, N-3, and N-4 clusters are associated with the 24-, 23-, 51-, and 75-kDa subunits, respectively. EPR studies of IP-1 and IP-2 also revealed the presence of additional clusters and suggested that complex I contains "EPR-silent" clusters, which can not be detected in the intact enzyme. EPR spectra of IP-2 revealed the presence of a radical signal with a narrow line width at $g = 2.00$, which resembled a signal from ubisemiquinone species. These results suggested that all the redox components are located in relatively peripheral domain of the enzyme.

INTRODUCTION

Complex I contains one FMN and several iron-sulfur clusters detected by EPR spectroscopy. There is still considerable uncertainty regarding the number and type of iron-sulfur clusters and their spatial distribution in complex I (for reviews see *1*, *2*). Based on the EPR and thermodynamic analysis of iron-sulfur clusters in bovine complex I, two binuclear clusters, namely N-1a and N-1b, and four tetranuclear clusters, N-2 through N-5 (designated by Ohnishi), have been reported (*3*, *4*). However, from the spin stoichiometry relative to the flavin in these systems as well as other organisms, only four clusters, N-1b, N-2, N-3, and N-4, have been generally accepted as intrinsic redox components of complex I (*5–7*).

Analysis of the structure and localization of iron-sulfur clusters in complex I has been greatly facilitated by the use of resolved subfractions, which retain non-heme iron and acid-labile sulfide and which also elicit some distinct iron-sulfur EPR signals. The disruption of the bovine enzyme with chaotropes has proved to be particularly valuable (*8–11*). Two water-soluble fractions, the FP (flavoprotein) fraction and IP (iron-sulfur protein) fraction, have been characterized after treatment with perchlorate. We have been detrermined the subunit compositions and primary structures of some subunits, as partly described in the preceding chapter. The FP fraction appears to contain FMN and two iron-sulfur clusters and transfers electrons from NADH to ferricyanide and several other electron acceptors (*12*). The IP fraction contains at least two iron-sulfur clusters. The remainder of the subunits, known as the HP (hydrophobic protein) fraction, also contains some of iron-sulfur clusters. By further resolution of the FP and IP fractions with chaotropic agents, some iron-sulfur proteins have been isolated (*9*, *11*). EPR spectroscopic studies of these fractions have suggested that seven or eight iron-sulfur clusters are present in complex I (*11*). However, most of original line shapes of the clusters have lost in these resolved fractions, and it is obscure at present which subunits ligate these clusters.

As described below, complex I from bovine heart mitochondria have been resolved by treatment with detergents, and three distinct fractions containing iron-sulfur cluters have been isolated and characterized. EPR spectroscopic studies have revealed that the obtained fractions contained all of the iron-sulfur clusters detectable in the intact complex I. Based on these results, possible location of the iron-sulfur clusters have been discussed.

MATERIALS AND METHODS

Materials — NADH:cytochrome *c* reductase was prepared from beef heart muscle by the method of Rieske (*13*) with slight modifications. The following steps during preparation were changed from those in the original method: a starting material was a Keilin-Hartree preparation (*14*) instead of a mitochondria preparation; pretein concentration was increased from 25 to 35 mg/ml on extraction of membrane proteins. As the results of these modifications, NADH dehydrogenase activity was shown in the fraction between 0 and 35% saturation of ammonium sulfate in the presence of cholate. The NADH dehydrogenase was further purified because this fraction still exhibited a slight NADH:cytochrome *c* activity. The following fractionation procedure with ammonium sulfate was performed accoding to the method of Hatefi *et al.* (*15*). The final preparation had only NADH dehydrogenase activity. The subunit composition was almost the same as reported by Hatefi *et al.* (*16*). This preparation also contained large amounts of two polypeptides with apparent molecular weights in the region around 52,000. These polypeptides seemes to be α and β subunits of F_1-ATPase. Any procedure could hardly remove these polypeptides from the preparation without loss of NADH dehydrogenase activityl. Thus this preparation was used as a strating material for resolution experiment.

Chemicals — Deoxycholic acid and cholic acid (recrystallized) were purchased from Nakalai tesque, Inc..Sucrosemonolaurate was purchased from Mitsubishi-Kasei Food Co. Ltd.. Other chemicals were all required commercially.

Analytical Methods — Protein concentrations were measured by Biuret method (*17*) using bovine serum albumin as a standard. Polyacrylamide gels containing 15% acrylamide and 12% glycerol were prepared and run in the buffer system of Laemmli (*18*).

Resolution of Complex I — The experimental procedure described below was based partly on the method of Suzuki and Ozawa (*19*, *20*). All operations were carried out at 0–4℃. The enzyme was dissolved in 15 mM Tris-HCl buffer (pH 8.0), 0.25 M sucrose, 0.1 M KCl, and 5 mM DTT at a protein concentration of approximately 5 mg/ml and dialyzed against 10 volumes of the same buffer for 1 h at 4℃. A 10% (w/v) solution of potassium deoxycholate and a 20% (w/v) solution of potassium cholate were added to a final concentration of 1.5% and 1.0 %, respectively, and incubated at 4℃ for 3 h with moderate stirring under a nitrogen atmosphere. After the incubation, chilled ethanol was added to a final concentration of 10%

(v/v) and the resulting mixture was centrifuged for 20 min at 38000 g after 30 min of stirring. A small pellet was discarded and to the supernatant was added a solid urea to give a final concentration of approximately 2 M, and the resulting solution was subjected to the ammonium acetate fractionation.

After each addition of 50% saturation ammonium acetate, the suspension was allowed to stir for 15 min, and precipitated material was collected by centrifugation at 38000g for 20 min. The precipitate between 6 and 12% saturation was dispersed in 0.25 M sucrose, 50 mM Tris-HCl buffer, pH 8.0, 5 mM DTT. The material precipitated between 12 and 14% saturation was discarded to avoid cross-contamination. The fraction of 14–30% saturation was suspended in sucrose–Tris–DTT buffer. The supernatant was dialyzed against the same buffer and concentrated after the addtion of 0.1% sucrosemonolaurate by an ultrafiltration apparatus using an ADVANTEC UP-20 membrane (Toyo Roshi Co. Ltd.). The 6–12% and 14–30% fractions were homogenized by hand in a small volume of sucrose–Tris–DTT buffer containing 0.1% sucrose monolaurate, and solubilized by the addition of 1 N NaOH. Alternatively, the 14–30% fraction was dissolved in a large volume of the same buffer and concentrated by ultrafiltration on a ADVANTEC UK-50 membrane.

EPR Spectroscopy — Concentrated samples of protein were kept on ice in the presence of 2 mM NADH for 1 min or 5 mM dithionite (freshly prepared) for 3 min. The samples were transferred into EPR tubes and immediately frozen in liquid nitrogen. Most of EPR measurements were performed on an X-band EPR spectrometer (model EPR-200, Bruker, F.R.G.) equipped with a liquid-helium cryostat (model EPR-900, Oxford Instruments, England) at the Analytical Instrument Center of the National Institute for Basic Biology (*21*). The EPR spectra of some fractions were recorded at X-band (9.23 GHz) microwave by using a Varian X-band cavity with home-built EPR spectrometer with 100-kHz modulation at the laboratory of Dr. Hori at the Faculty of Engineering Science of Osaka University (*22*).

In a following description, N-1 type cluster detected in the intact complex I is referred to cluster N-1b, taking account of the proposal that the N-1 cluster is attributable to two species, N-1a and N-1b (*23*). The iron-sulfur clusters of complex I are defined as follows: cluster N-1b, $g_{x,y,z}$ =1.92, 1.94, 2.02; cluster N-2, $g_{x,y,z}$ = 1.92, 1.92, 2.05; cluster N-3, $g_{x,y,z}$ = 1.86, 1.93, 2.04; cluster N-4, $g_{x,y,z}$ = 1.88, 1.94, 2.10.

RESULTS

Resolution of Complex I — Complex I has been resolved by chaotropic agents (*10*, *11*) or by treatment with phospholipase at high pH(*24*), and resulting smaller fragments have been characterized. Most of these resolution methods require the vigorous, drastic treatment of the enzyme such as incubation at high temperature (e.g. 37℃) and repeating freezing and thawing. However, such conditions cause loss of intactness of the iron-sulfur clusters, leading to an irreversible alteration of their EPR signals. The resolution of the enzyme ought to be carried out in such a way that iron-sulfur clusters retained their intactness in resulting fractions. It is clear that treatment at alow temperture, e.g. 4℃, is favorable. Thus, several factors influencing the resolution were examined.at 4℃ based on the fractionation by precipitation with ammonium sulfate.

It has been reported that complex I can be disrupted with detergents such as Triton X-100, deoxycholate, and octylglucoside (*9*). Various detergents including some other ones were examined for ability to dissociate the enzyme at 4℃. Some gave the same kind of fractionation pattern, but deoxycholate gave the cleanest resolution. Deoxycholate has also been reported to be most effective to separate the IP polypeptides. Contrarily, sucrosemonolaurate was rather effective to keep the stability of the enzyme, and so, in the following experiment this detergent was added to the fractions obtained for solubilization and stability. After all, the practical procedure employed was based partly on the method of Suzuki and Ozawa, which has originally required incubation of the enzyme at 30℃ for 1 h (*19*, *20*).

After incubation of complex I at 4℃ for 3 h in the presence of 1.5% deoxycholate and 1.0% cholate, chilled ethanol was added and the precipitate, containing polypeptides of other membrane enzymes, was removed. Without addition of ethanol, separation of the subunits in the following fractionation was insufficient. The precipitate between 0 and 6% ammonium acetate saturation was white and insoluble, contained predominantly the hydrophobic polypeptides.

The precipitate of 6–12% saturation was dark brown and insoluble at neutral pH. The pellet was suspended in sucrose–Tris–DTT buffer containing 0.1% sucrosemonolaurate and solubilized by increasing the pH to 11. Alternatively, 50 mM CAPS buffer (pH 10.5) was also used for the buffer to solubilize it. The solution did not decolorized even under these

conditions. This fraction was enriched in the 75-kDa polypeptide (Fig. II-1).

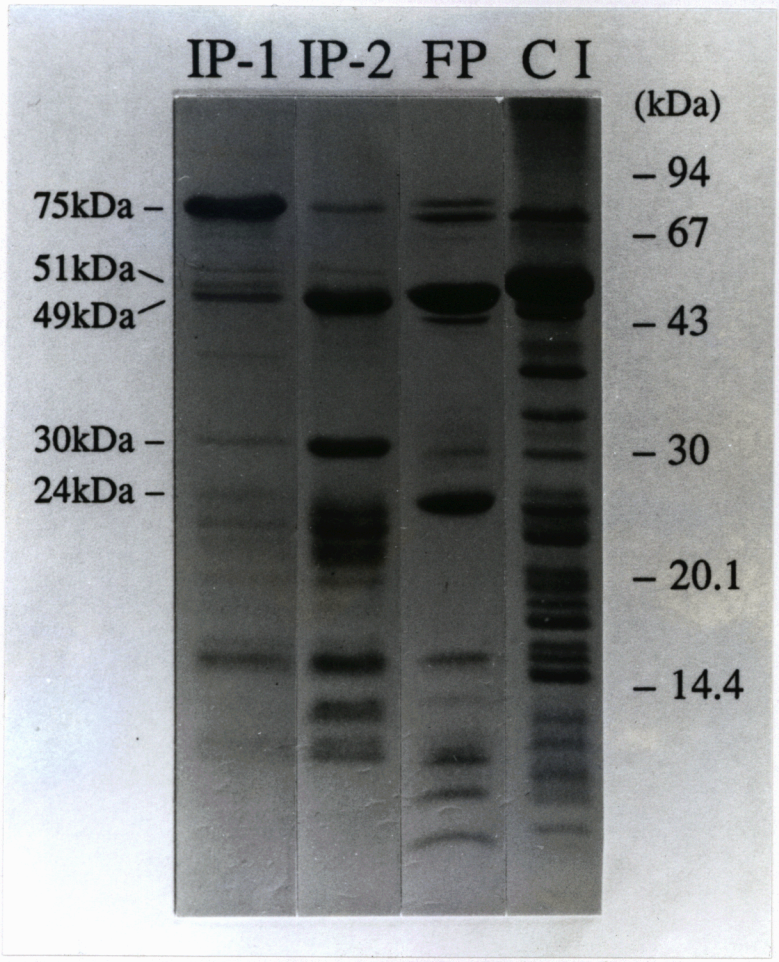

Fig. II-1. Subunit composition of fractions obtained from deoxycholate and cholate/ammonium acetate treatment of complex I. The samples applied were as follows: IP-1, material precipitated between 6% and 12% saturation with ammonium acetate; IP-2, material precipitated between 14% and 30% saturation; FP, supernatant fraction after precipitation at 30% saturation; CI, complex I.

The precipitate of 14–30% saturation was yellowish brown and soluble in a large amount of neutral (sucrose–Tris–DTT) buffer. The resulting pale yellow solution could be concentrated by ultrafiltration. As concentration proceeded, the solution became dark brown but highly viscous and so, the concentration was limited to some extent. In order to obtain a high concentration solution for EPR spectroscopy, the pellet was suspended in a small amount of the same buffer and solubilized by increasing the pH to 9.5. This fraction contained about 15 polypeptides, including the 49-, 30-, 23-, 20-, 19-, 18-, 15-, and 13-kDa polypeptides (Fig. II-1). In one-dimensional electrophoresis system, the smaller proteins were not clearly resolved. It has been shown that several of the smaller bands are composed of more than one type of polypeptide (*25*).

The supernatant at 30% saturation, which was pale yellow, was dialyzed against sucrose–Tris–DTT buffer to remove salts, and the dialyzed solution was easily concentrated by ultrafiltration. This fraction was enriched in the 51- and 24-kDa polypeptides (Fig. II-1).

Subunit compositions of these three fractions were similar to those of IP and FP obtained by treatment of chaotropes (*8*, *12*), except for one difference, that is, the 75-kDa polypeptide was separated from the other IP polypeptides, such as the 49- and 30-kDa polypeptides. The bovine 75-kDa subunit has been reported to polymerize into filamentous structures by subsequent extraction and purification (*26*). Thus the low solubility of 6–12% fraction might be due to its own structural property. In a subsequent description, the fractions of 6–12%, 14–30%, and supernatant at 30% saturation are referred to IP-1, IP-2, and FP, respectively.

EPR Spectroscopy — In order to analyze the distribution of iron-sulfur clusters, EPR characteristics were examined in individual subfractions. Fig. II-2 presents EPR spectra of the IP-1 solution reduced with 5 mM dithionite and recorded at two different temperatures (8 and 30 K) at a fixed microwave power level of 10 milliwatt. EPR signals arising from two species of iron-sulfur clusters were discernible and exhibited distinct spin relaxation behavior and EPR line shape. Signals from a rapid relaxing species were clearly seen at 8 K with a peak at g = 2.10 and principal absorption centered at around g = 1.94. The line shape of this species was dissimilar to that of any iron-sulfur cluster detectable in intact complex I, but the g value of 2.10 agreed well with the g_z value of the N-4 cluster in intact enzyme.

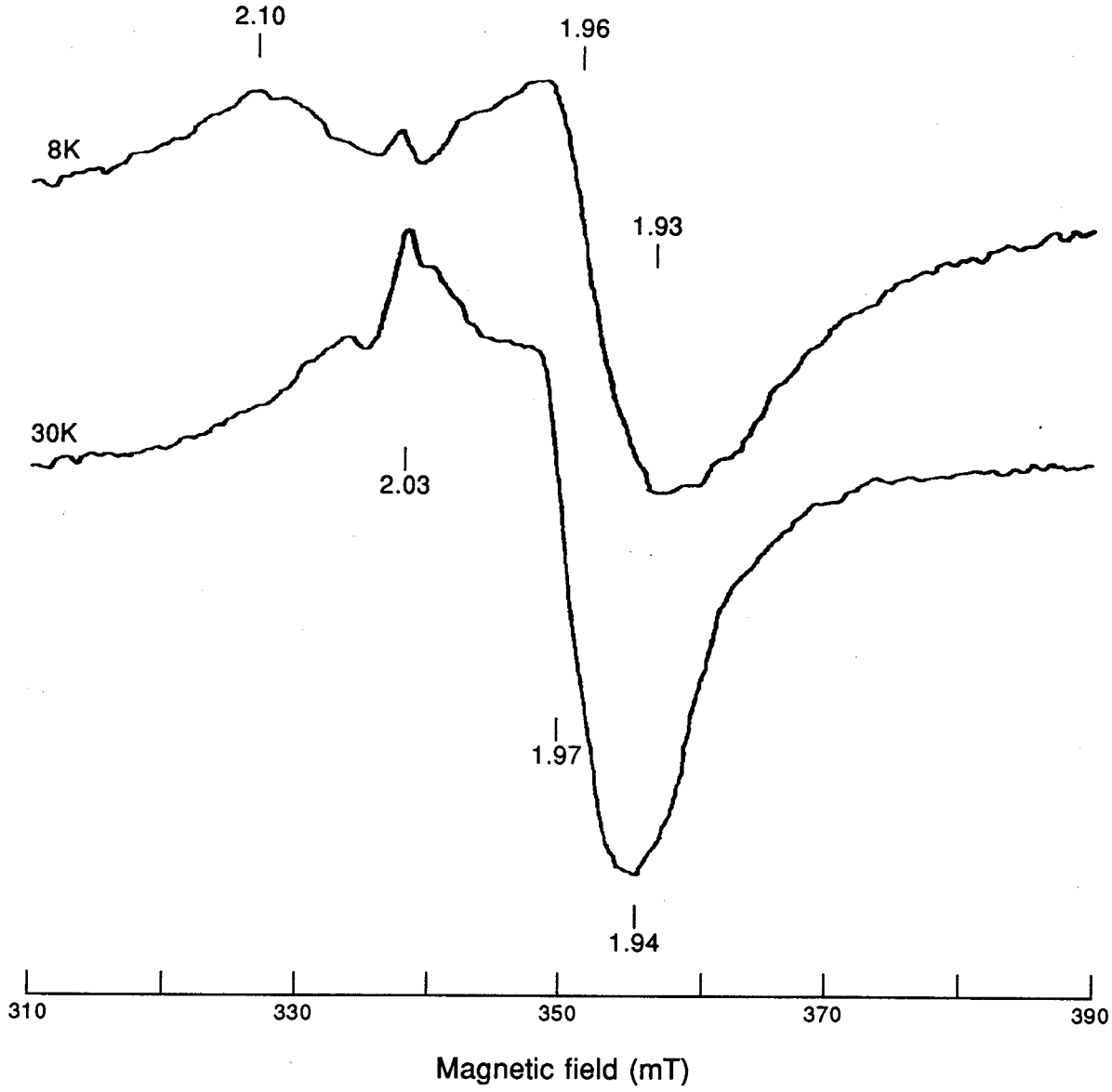

Fig. II-2. EPR spectra of IP-1. The IP-1 fraction (22 mg of protein/ml) was reduced with 5 mM dithionite, and spectra were recorded at the indicated temperatures. EPR conditions were as follows: microwave frequency 9.64 GHz; modulation amplitude 1 mT; microwave power 10 mW; time constant 0.16 s; scanning rate 240 mT/min.

Upon raising the temperature of the sample, the intensification of the EPR spectrum of an axial line shape with a peak at g = 2.03 and principal absorption centered at around g = 1.96 was recognized. Based on the relatively isotropic line shape, which was similar to that of a typical binuclear clusters of adrenodoxin, this iron-sulfur species could be assigned to be a binuclear cluster. Another possibility was that the g = 2.03 signals may arise from artificial conversion of a tetranuclear cluster to the binuclear-like form during the resolution.

IP-2 was first solubilized in a small volume of an alkaline buffer to obtain a high concentration of protein for EPR study. Fig. II-3 shows EPR spectra of dithionite-reduced IP-2 under different conditions. So many signals were observed in this sample that the spectrum of each species was difficult to be separated from each other accurately. However, it was recognized that this sample contained at least two distinct species of iron-sulfur clusters which differed in their spectral line shape and spin relaxation behavior.

The spectrum of a slowly relaxing species was selectively seen at 15K at a micropower level of 5 milliwatt. This species had a spectrum with a peak at a g = 2.04 and principal absorption centered around g = 1.93. The line shape of this species was similar to that of the N-2 cluster. Upon raising the temperature of the sample, the intensity of this spectrum decreased, whereas that of an axial line shape with a peak at g = 2.00 and principal absorption centered at around g = 1.94. In addition to these two species, a weak signal with a peak at g = 2.07 was recognized, but its EPR characteristics could not be determined. It has been known that complex I contains the fifth cluster, so-called N-5, which gives the signals with g values of 2.07 and 1.90, and that the relative concentration of this species is very low. It was not certain whether the signal observed in IP-2 corresponded to the N-5 cluster or not.

The IP-2 solution was also prepared by dissolution of the precipitated residue in a large amount of a neutral buffer followed by concentration. The spectra of this sample are shown in Fig. II-4. The intensity of the signals were relatively low, if compared to those seen in Fig. II-3. From the parallel temperature dependence of EPR signals, it was clear that the g = 2.05 signal was associated with the g = 1.92 absorbance. The signals of this species probably correspond to those of the species with g values of 2.04 and 1.93 in Fig. II-3. The axial line shape of this species was very similar to that of the N-2 cluster in intact complex I.

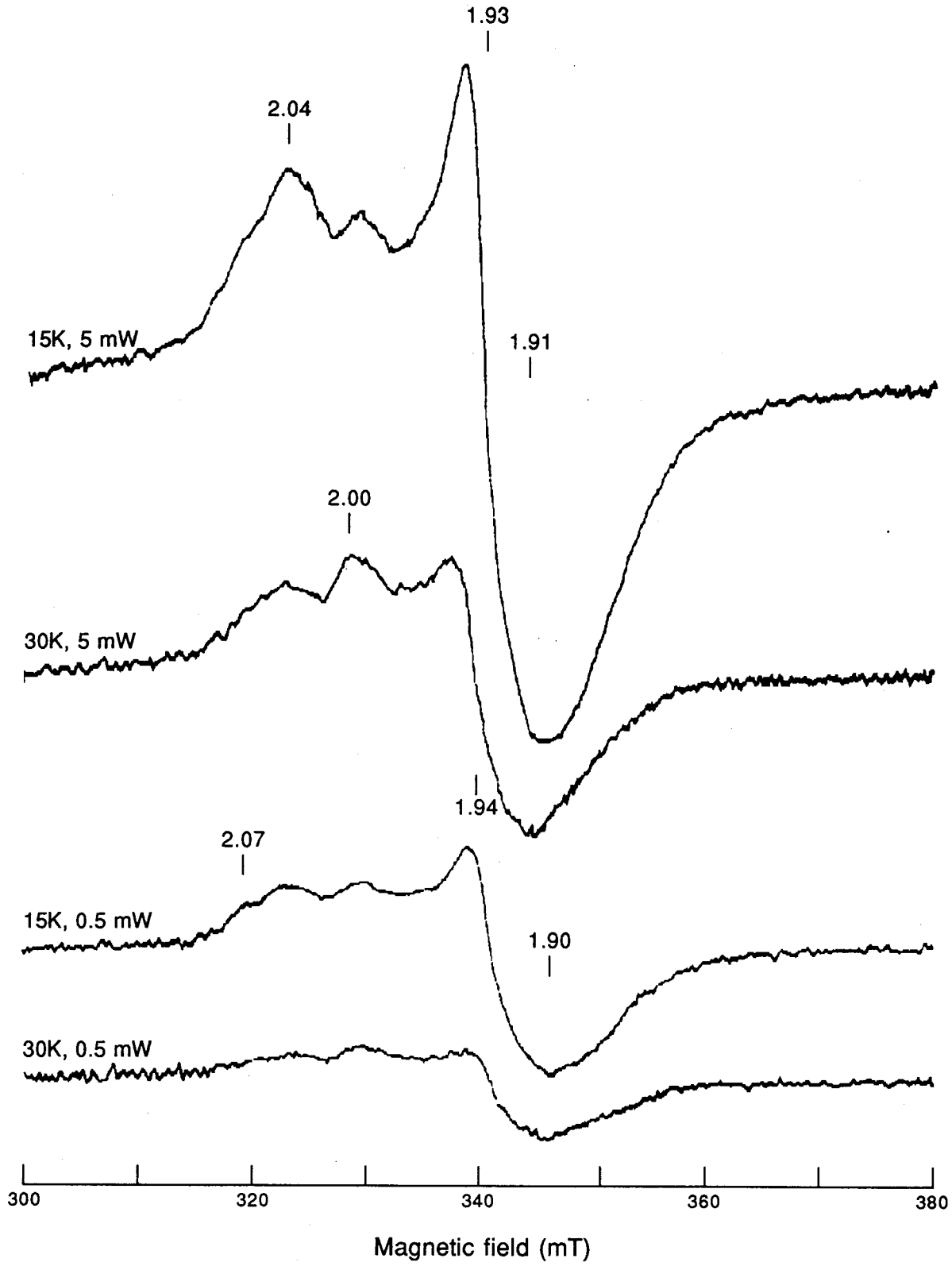

Fig. II-3. EPR spectra of IP-2 solubilized in a alkaline buffer. The IP-2 fraction (38 mg of protein/ml) was reduced with 5 mM dithionite, and spectra were recorded at the indicated conditions. EPR conditons were as follows: microwave frequency 9.227 GHz; time constant 0.04 s; scanning rate 200 mT/min.

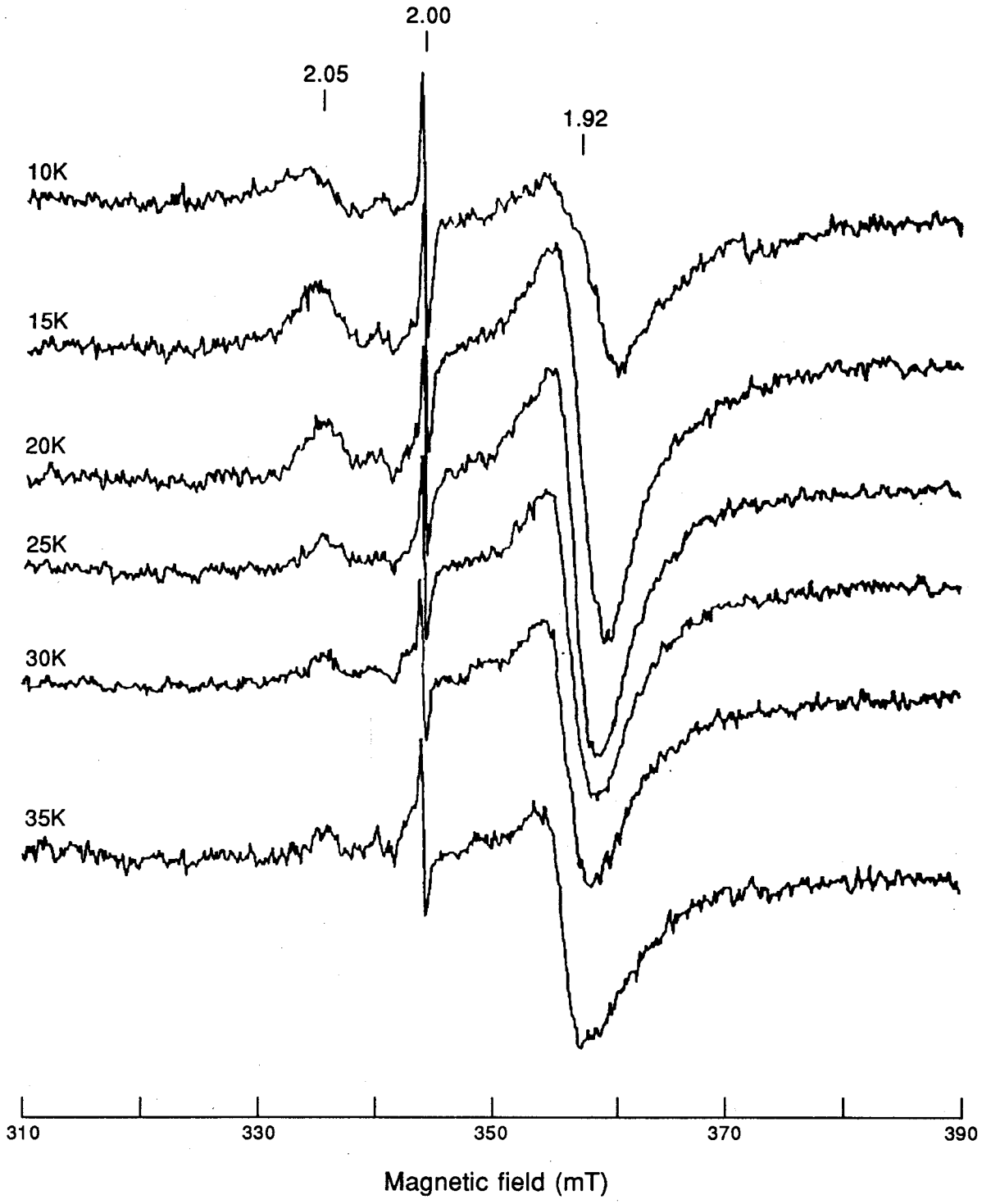

Fig. II-4. EPR spectra of IP-2 prepared by concentration of a dilute solution. The IP-2 fraction (20 mg of protein/ml) was reduced with 5 mM dithionite and spectra were recorded at the indicated temperatures. EPR conditions: microwave frequency 9.63 GHz; modulation amplitude 1 mT; time coonstant 0.16 s; scanning rate 60 mT/min; microwave power 10 mW.

Also, an intense free radical signal (g = 2.00) was observed over a wide range of temperature as shown in Fig. II-4. The sample contained no mediator dye, which gives a symmetric EPR signal centered at g = 2.00. The line width of this signal (<1 mT) was much narrower than that of such a radical and was similar to that of semiquinone associated with complex I reduced with NADH (*27*, *28*) and complex II (*29*) and complex III (*30*).

Another signal probably from an iron-sulfur cluster existed at around g = 2.00, although it was difficult to be assigned owing to overlapping of two signals. This signal seemed to exhibit similar spin relaxation behavior to that with a peak of g = 2.00.

EPR measurement of NADH-reduced FP revealed the presence of at least three distinct species of iron-sulfur clusters (Fig. II-4). A slowly relaxing species selectively seen at 50 K had a spectrum of rhombic symmetry with g values of 2.00 (g_z), 1.95 (g_y), and 1.91 (g_x). Below 15 K the signal from this species was mostly saturated, and a spectrum arising from a rapidly relaxing species was seen with g values of 2.035 (g_z), 1.95 (g_y), and 1.86 (g_x). The g values of this iron-sulfur cluster agreed well with those of the N-3 cluster in intact complex I.

In addition to these two species, this fraction contained weak signals of another species over a wide range of temperature. This species had a spectrum with g values of 2.02 (g_z), 1.94 (g_y), and 1.91 (g_x). The spectrum of this component could be clearly distinguished from the others at 50K. The line shape of this species was similar to that of the N-1b cluster observed in intact complex I.

Table II-1 summarizes the results obtained from EPR spectroscopic studies of each fraction. The accurate g values could not be determined for several spectra because they overlapped one another. Particularly, the broadening of the signals at high magnetic field region was observed in the spectra of IP-1 and IP-2, which showed axial or nearly axial line shapes. On the other hand, the spectra of FP were relatively sharp and clearly rhombic. These results implied that some of the clusters of IP-1 and IP-2 might be slightly damaged.

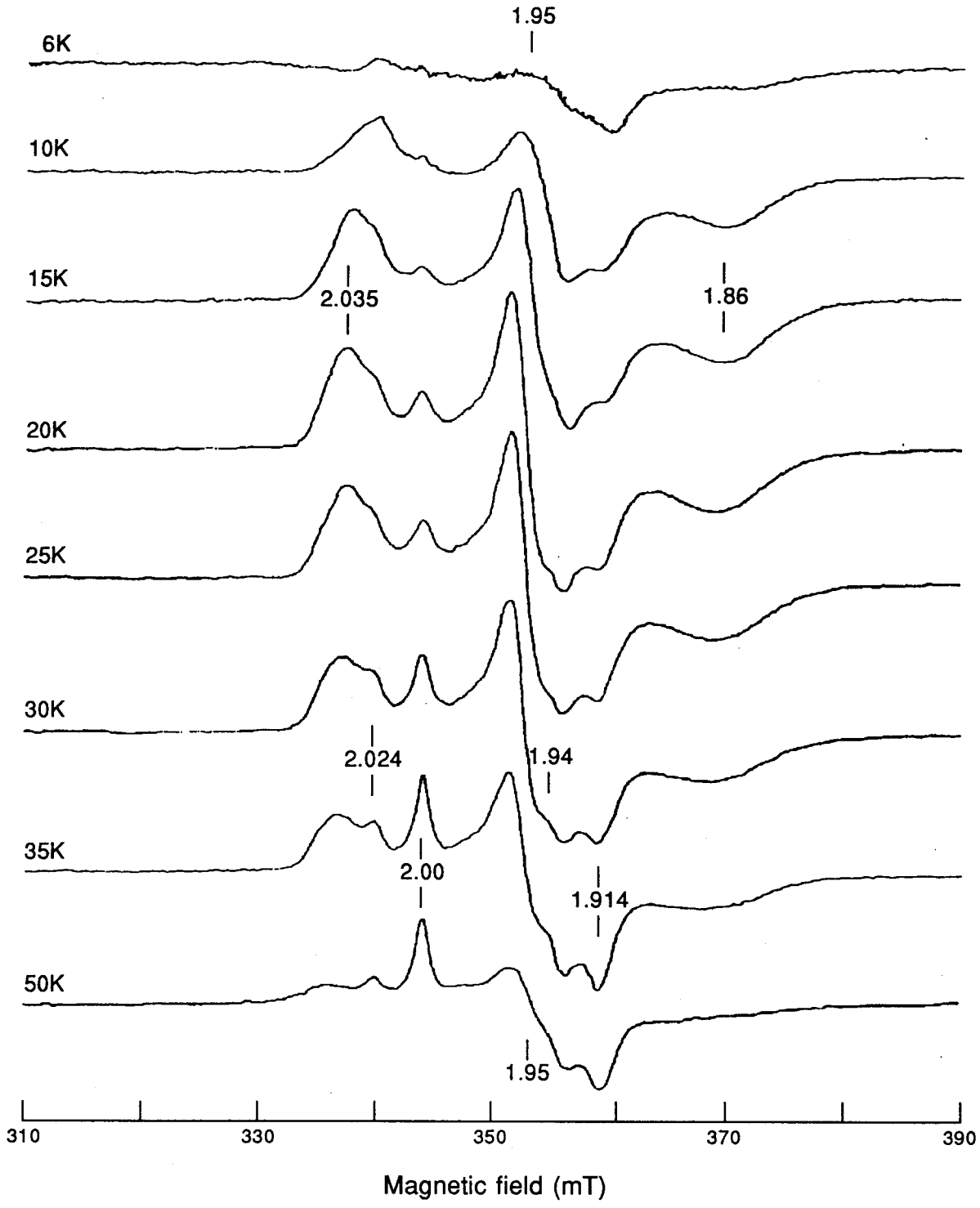

Fig. II-5. EPR spectra of FP. The FP fraction (12 mg of protein/ml) was reduced with 2 mM NADH and spectra were recorded at the indicated temperatures. EPR conditions were the same as in Fig. II-4.

Table II-1. EPR signals detected in fractions obtained from complex I.

Fraction		g_x	g_y	g_z	Probable cluster
		$g_{\parallel}$		$g_{\perp}$	
IP-1		1.93	1.96	2.10	N-4
		1.96–1.97		2.03	(N-1a)
IP-2	(sample 1)	1.92–1.93		2.04	(N-2)
		1.94–1.95		2.00	
		(1.93–1.94)		2.07	
	(sample 2)	1.95		2.05	N-2
		(1.92–1.93)		2.00	
FP		1.86	1.92	2.04	N-3
		1.91	1.94	2.02	N-1b
		1.91	1.94	2.00	

Sample 1 of IP-2 was solubilized in a small volume of a alkaline buffer. Sample 2 of IP-2 was prepared by dissolution of the precipitate in a large amount of a neutral buffer and by ultraconcentration.

DISCUSSIONS

It has been very difficult to determine which subunits chelate a specific iron-sulfur cluster in the multiple iron-sulfur clusters in complex I. One approach to study this has been the disruption of complex I, for example, with chaotropic agents, and the characterization of the smaller fragments (*10*, *11*). In particular, EPR spectroscopy has provided invaluable information on the iron-sulfur clusters of the enzyme. The advantages of this method are that the existence of a cluster can be unambiguously demonstrated, and the separation may allow previously EPR-silent centers to become visible. Several attempt has been undertaken to subfractionate complex I, but isolation and identification of the iron-sulfur proteins has been very difficult (*10*, *11*). The present investigation provided significant information regarding the iron-sulfur clusters in bovine complex I.

IP-1 — In this study the 75-kDa subunit was purified as IP-1 from the enzyme for the first time. It has been reported that the 75-kDa subunit has been dissociated from the IP fraction in the presence of deoxycholate (*9*), but its EPR spectrum has lost a characteristic line shape of iron-sulfur clusters (*11*). EPR spectroscopy of IP-1 indicated that the 75-kDa subunit contained two iron-sulfur clusters. One of them had a spectrum with a peak at $g = 2.10$, which was similar to g_x value of the N-4 cluster. This result suggests that the N-4 cluster is located in the IP-1 fraction, that is, 75-kDa subunit. However, the whole line shape of the spectra differ from each other: the IP-1 spectrum is nearly axial, whereas the N-4 spectrum is clearly rhombic. This discrepancy may resulte from slight alteration of the native structure. The N-4 cluster has never been identified so far in any fraction or protein obtained from resolved enzyme, and so this is the first report of identification of the N-4 cluster.

The other species of IP-1 did not resemble any iron-sulfur cluster detected in complex I but might be similar to the N-1 cluster. Ohnishi *et al.* observed a relatively isotropic line shape in the 75-kDa subunit from the IP fraction and assigned it to be a binuclear cluster, N-1a (*23*, *31*). The spectra of the N-1a cluster appears to be similar to that of IP-1 rather than that of their sample. Cluster N-1a was observed only at very low redox potentials and which was not reduced with NADH. Other groups have been unable to detect this cluster (*3*, *32*, *33*). Experimental evidence for the presence of two distinct species of cluster N-1 in intact enzyme have not been obtained from this work (see Chapter III). However, it has also been suggested

by other groups that cluster N-1 is certainly attributable to two clusters with axial spectra (*34*). Therefore, the N-1-like species detected in IP-1 may be one of such clusters.

IP-2 — Among its 10 or so subunits, IP-2 contained 6 subunits of the water-soluble IP fraction obtained by chaotropic treatment. In addition, IP-2 contained the 23- and 20-kDa subunits, which have been detected in some preparation of the IP fraction and considered to be water-soluble proteins (see Chapter I). Additionally, IP-2 contained some subunits, which have been contained in the HP fraction, although identification of the subunits was incomplete.

This experiment showed that one of EPR-detectable iron-sulfur clusters in intact enzyme, N-2, was present in this fraction. Previous experiments have shown that the signals of the N-2 cluster resides in the membrane-embedded hydrophobic fraction (HP) (*11*). In this resolution process, most of hydrophobic proteins were precipitated at the first step with ammonium acetate fractionation. Therefore, this discrepancy suggests that the N-2 cluster is associated with the subunit which was first recovered in hydrophilic fraction, IP-2, by this experiment. The candidate for iron-sulfur proteins will be discussed later.

Among iron-sulfur clusters of complex I, cluster N-2 has the most positive, phospholipid-dependent redox potential (*35*). It has been further reported that the rotenone-sensitive ubisemiquinone signal interacts with one of the tetranuclear clusters, most probably N-2 (*27*, *28*, *36*). These data have suggested that the N-2 cluster serves as the electron donator for the substrate ubiquinone. In this respect, it is of interest that a radical signal observed in the IP-2 spectra may be a signal attributable to ubisemiquinone.

Many investigators have assumed that complex I contains an intrinsic ubiquinone. There is a possibility that incubation of IP-2 with dithionite for a short time partially reduce an ubiquinone and produce a radical of semiquinone form. The content of ubiquinone in the IP-2 fraction was not determined in this work. It should be noted that the resolution procedure employed here was an adaptation of the method developed for preparing an ubiquinone-binding protein from complex I (*19*, *20*). Evidence from various lines indicates that ubisemiquinone exists as the ubiquinone-protein to form a stable radical in the enzyme (*27*, *28*, *36*). Suzuki and King have proposed a ubiquinone-binding protein to be a 14-kDa polypeptide isolated from the IP fraction of bovine complex I (*20*). On the other hand, Heinrich *et al.* has recently identified the 9.5-kDa subunit as a ubiquinone-binding protein in *Neurospora crassa* by photoaffinity labeling (*37*, *38*). At present it is not clear whether IP-2 contains these proteins or not.

Characterization of component polypeptides and ubiquinone content in this fraction provide information on these problems in the most direct way.

The EPR spectra of IP-2 revealed the presence of two additional species. Both species have no similarity to that of intact complex I. It is possible that the handling of the fraction affects the structure of the N-2 cluster and causes appearance of a species with different line shape. Another possible explanation is that an "EPR-silent" cluster undetectable in intact enzyme become visible after resolution of the enzyme. It has been reported previously that the IP fraction or its subfractions contain the two species dissimilar to that of any detectable cluster in complex I (*11*).

FP — EPR measurements indicated that the N-3 and N-1b clusters reside in FP. The presence of the N-1b and N-3 clusters in the so-called the soluble NADH dehydrogenase, corresponding to FP in this study, have been previously reported by Ragan *et al.* (*10, 23*). The spectra of FP are very similar to those reported for those preparations except for the existence of an additional species besides a N-1 and N-3 type species. The signals of such a species have also been observed in some of the dehydrogenase preparations, in which the signals similar to the N-1b cluster disappeared concomitantly (*10*). Ragan *et al.* have suggested that the spectrum of the slowly relaxing species rapidly changes from an N-1 type to another during preparation of the dehydrogenase and that only two clusters are present in this fraction. The present data might be regarded to support their interpretation, although there is a possibility that an extra cluster is present.

After all EPR-detectable iron-sulfur clusters in intact enzyme were identified in hydrophilic subfractions obtained from resolution of complex I with detergents.

Possible Location of Iron-Sulfur Clusters — Attempts to separate and purify individual iron-sulfur proteins of the enzyme were unsuccessful in this study except for the 75-kDa subunit. The above results alone are insufficient to make firm conclusions as to the location and structure of the iron-sulfur clusters of the enzyme. By combining our data with other information on the enzyme, however, it can be found to make positive identification in many instances.

Walker *et al.* have been characterized the primary structure of bovine complex I, and recently completed sequencing of 34 different nuclear-encoded subunits (*39–51*), including those sequenced as given in the preceding chapter. Also, the primary structure of a fair number

of subunits have become available from other organisms such as *Neurospora crassa* and *Paracoccus denitrificans* (*1*, *52–54*). Furthermore, the detection of sequence homologies with other redox proteins and the existence of conserved characteristic motifs has permitted the relation of individual subunits to distinct redox centers, respectively.

As summarized in Table II-2, at least four subunits are thought to involve in formation of iron-sulfur clusters. These subunits have cysteine motifs characteristic of some iron-sulfur proteins. The sequence of the 75-kDa subunit, IP-1, suggests that it contains at least one tetranuclear cluster (*46*). The sequence of the 23-kDa subunit, present in IP-2, shows similarity to those of bacterial type ferredoxins and suggests that it contains two tetranuclear clusters (*39*). The 51-kDa subunit of FP appears to contain a tetranuclear cluster, in addition to the binding sites of FMN and NADH (*44*). The sequence of the 24-kDa subunit, also present in FP, has four conserved cysteine residues. Although its cysteine arrangement is not similar to that of any known iron-sulfur protein, this subunit has been proposed to contain an iron-sulfur cluster (*43*). These data strongly suggest that the cluster N-1b, -2, -3, and -4 of complex I are associated with the 24-, 23-, 51-, and 75-kDa subunits, respectively.

In addition to typical cysteine motifs, the 75-kDa subunit contains seven cysteines that are conserved among the homologous proteins. Particularly, the spacing of the three cysteines among them agreed with that of the sequence of endonuclease III in *Escherichia coli*, which contains an iron-sulfur cluster (*55*, *56*). The three-dimensional structure of this DNA repair enzyme indicates that four cysteines, including three ones mentioned above, ligate a tetranuclear iron-sulfur cluster (*57*). Thus, it is possible that another tetranuclear cluster is present in the 75-kDa subunit and gives the signals of the second species observed in the IP-1 spectra.

Two unidentified species have been observed also in IP-2 by the EPR spectra. IP-2 contains the other candidates of iron-sulfur proteins in addtion to the 23-kDa subunit. The complete cDNA sequence of the 20-kDa subunit has recently been determinied by using sequence information presented in the preceding chapter (*50*). It contains three cysteines that are conserved across a wide range of species. Another protein detected in IP-2, the 19-kDa subunit, contains eight conserved cysteines (*40*). These subunits could possibly be additional iron-sulfur proteins, which might be EPR-silent in intact enzyme and become visible in a subfraction, IP-2, although there is no direct evidence to support this suggestion at present. It should be confirmed whether an "EPR-silent" cluster exists in complex I or not. The study of

an "EPR-silent" cluster ought to be pursued further not only in order to confirm its presence, but also to elucidate the electron transfer pathway within complex I.

Table II-2. Possible location of iron-sulfur clusters in complex I.

Fraction	Cluster		Possible location	
	(*g*)	Type	Subunit	Cysteine clusters
FP	2.04	N3	51-kDa	CGQCTPCREX$_{31}$TICA
FP	2.02	N1b	24-kDa	CTTTPCMLX$_{30}$VECLGACV
IP-1	2.10	N4	75-kDa	CIQCIRCIRX$_{39}$DICPV
IP-2	2.04	N2	23-kDa	CIACKLCEAVCPX$_{27}$CIYCGFCQEACP
IP-1	2.03	N1a	75-kDa	CX$_{10}$CRMCX$_{15}$CX$_{35}$CPICX$_{5}$CD
IP-2	2.00	—	(20-kDa	3 conserved cysteines)
IP-2	2.07	—	(19-kDa	8 conserved cysteines)

Dashes (—) show the clusters which have not been detected in intact complex I. The 20-kDa and 19-kDa are listed as a candidate for an iron-sulfur protein. Two signals with *g*-values of 2.00 and 2.07 in IP-2 cannot be assigned to an specific subunit.

The dissociation of complex I also provides important information about both the structure of the enzyme and the electron pathway from NADH to ubiquinone. The results from this work imply that the hydrophilic fractions obtained by dissociation of complex I with detergents contain all of the subunits with the prosthetic groups detected in intact enzyme. Many subunits in the fractions obtained can also be considered from their sequences to be water-soluble. It is possible that these proteins lies mostly in the peripheral region of the inner membrane, presumably on the matrix side.

In this respect, it is also worth noting that a smaller form of NADH:ubiquinone oxidoreductase is produced in *Neurospora crassa* mitochondria by inhibition of the organeller protein synthesis (*58*). This small form consists of approximately 13 subunits and contains FMN and the iron-sulfur clusters, N-1b, N-3, and N-4, but is devoid of cluster N-2 (*59*). It is uncertain at present whether this small form contains the subunits corresponding to those of the fractions in this work, because its subunit composition have not been characterized so extensively. Recently, three-dimensional structure of complex I from *Neurospora crassa* was determined by electron microscopic study and it was revealed that the enzyme has an L-shaped structure with two distinguishable arms, one of which protrudes from the membrane to the matrix (*60*). Furthermore, three-dimensional reconstruction of the small form has shown only peripheral domain of the enzyme (*60*). It seems reasonable that such a bulky structure is composed of hydrophilic proteins like those of the fractions obtained in this work. In that case it can be proposed that the electron route from NADH to ubiquinone exists within the hydrophilic domain, and the hydrophobic domain mainly participate in binding of the enzyme to the membrane. The function of the hydrophobic domain is of interest, since the membrane anchoring portion may be important to drive proton gradient formation. Among the subunits in the fractions obtained in this work, the 75-kDa subunit has been reported to be transmembranous and the 23-kDa and some smaller subunits has been contained in the hydrophobic protein fraction by resolution with chaotropic agents (*2*, *61*). There is another possibility that the putative bulky structure composed of the subunits of IP-1 and IP-2 also are embedded in the membrane partly. All these ideas, however, remain speculative and await further experimentation.

Acknowledgments

I would like to thank Dr. H. Hori (Osaka University) and Dr. S. Itoh and Ms. M. Iwaki (National Institute for Basic Biology) for their help with recording the EPR spectra. I also thank Mr. T. Kubota, M. Kawamoto, and T. Matsunaga in this laboratory for their help with preparing the complex I.

REFERENCES

1. Weiss, H., Friedrich, T., Hofhaus, G., and Preis, D. (1991) *Eur. J. Biochem.* **197**, 563–576
2. Ragan, C. I. (1987) *Curr. Top. Bioenreg.* **15**, 1–36
3. Beinert, H. and Albracht, S. P. J. (1982) *Biochim. Biophys. Acta* **683**, 245–277
4. Ohnishi, T. and Salerno, J. C. (1982) in *Iron Sulfur Proteins* (Spiro, T. J., ed) Vol. 4, pp. 285–327, J. Wiely & Sons, Inc., New York
5. Thayer, W. S., Ohnishi, T., and Rubin, E. (1980) *Biochim. Biophys. Acta* **591**, 22–36
6. Albracht, S. P. J., van Verseveld, H. W., Hagen, W. R., and Kalkman, M. L. (1980) *Biochim. Biophys. Acta* **593**, 173–186
7. Hearschen, D. O., Dunham, W. R., Albracht, S. P. J., Ohnishi, T., and Beinert, H. (1981) *FEBS Lett.* **133**, 187–190
8. Hatefi, Y. and Stempel, K. E. (1969) *J. Biol. Chem.* **244**, 2350–2357
9. Ragan, C. I., Galante, Y. M., and Hatefi, Y. (1982) *Biochemistry* **21**, 2518–2524
10. Ragan, C. I., Galante, Y. M., Hatefi, Y., and Ohnishi, T. (1982) *Biochemistry* **21**, 590–594
11. Ohnishi, T., Ragan, C. I., and Hatefi, Y. (1985) *J. Biol. Chem.* **260**, 2782–2788
12. Galante, Y. M. and Hatefi, Y. (1979) *Arch. Biochem.* **192**, 559–568
13. Rieske, J. S. (1967) *Methods Enzymol.* **10**, 381–384
14. Yoshikawa, S., Choc, M. G., O'Toole, M. C., and Caughey, W. S. (1977) *J. Biol. Chem.* **252**, 5498–5508
15. Hatefi, Y. (1978) *Methods Enzymol.* **53**, 11–14
16. Cleeter, M. W. J. and Ragan, C. I. (1985) *Biochem. J.* **230**, 739–746
17. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
18. Laemmli, U. K. (1970) *Nature* **227**, 680–685
19. Suzuki, H. and Ozawa, T. (1984) *Biochem. Int.* **9**, 563–568
20. Suzuki, H. and Ozawa, T. (1986) *Biochem. Biophys. Res. Commun.* **138**, 1237–1242
21. Itoh, S., Tang, X. S., and Satoh, K. (1986) *FEBS Lett.* **205**, 275–281
22. Hori, H., Masuya, F., Tsubaki, M., Yoshikawa, S., and Ichikawa, Y. (1992) *J. Biol. Chem.* **267**, 18377–18381
23. Ohnishi, T., Blum, H., Galante, Y. M., and Hatefi, Y. (1981) *J. Biol. Chem.* **256**, 9216–9220

24. Paech, C., Friend, A., and Singer, T. P. (1982) *Biochem. J.* **203**, 477–481
25. Heron, C., Smith, S., and Ragan, C. I. (1979) *Biochem. J.* **181**, 435–443
26. Price, M. G. and Gomer, R. H. (1989) *Cell Motil. Cytockek.* **13**, 274–287
27. Suzuki, H. and King, T. E. (1983) *J. Biol. Chem.* **258**, 352–358
28. Burbaev, D. Sh., Moroz, I. A., Kotlyar, A. B., Sled, V. D., and Vinogradov, A. D. (1989) *FEBS Lett.* **254**, 47–51
29. Ruzicka, F. J., Beinert, H., Schepler, K. L., Dunham, W. K., and Sands, R. H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2886–2890
30. Meinhardt, S. W., Yang, X., Trumpower, B. L., and Ohnishi, T. (1987) *J. Biol. Chem.* **262**, 8702–8706
31. Ingledew, W. J. and Ohnishi, T. (1980) *Biochem. J.* **186**, 111–117
32. Albracht, S. P. J. and Subramanian, J. (1977) *Biochim. Biophys. Acta* **462**, 36–48
33. Kowal, A. T., Morningstar, J. E., Johnson, M. K., Ramsey, R. R., and Singer, T. P. (1986) *J. Biol. Chem.* **261**, 9239–9245
34. Albracht, S. P. J., Dooijewaard, G., Leeuwerik, F. J., van Swol, B. (1977) *Biochim. Biophys. Acta* **459**, 300–317
35. Ohnishi, T., Leigh, J. S., Ragan, C. I., and Racker, E. (1974) *Biochem. Biophys. Res. Commun.* **56**, 775–782
36. Kotlyar, A. B., Sled, V. D., Burbaev, D. Sh., Moroz, I. A., and Vinogradov, A. D. (1990) *FEBS Lett.* **264**, 17–20
37. Heinrich, H. and Werner, S. (1992) *Biochemistry* **31**, 11413–11419
38. Heinrich, H., Azevedo, J. E., and Werner, S. (1992) *Biochemistry* **31**, 11420–11424
39. Dupuis, A., Skehel, J. M., and Walker, J. E. (1991) *Biochemistry* **30**, 2954–2960
40. Dupuis, A., Skehel, J. M., and Walker, J. E. (1991) *Biochem. J.* **277**, 11–15
41. Fearnley, I. M., Runswick, M. J., and Walker, J. E. (1989) *EMBO J.* **8**, 665–672
42. Fearnley, I. M., Finel, M., Skehel, J. M, and Walker, J. E. (1991) *Biochem. J.* **278**, 821–829
43. Pilkington, S. J. and Walker, J. E. (1989) *Biochemistry* **28**, 3257–3264
44. Pilkington, S. J., Skehel, J. M., Gennis, R. B., and Walker, J. E. (1991) *Biochemistry* **30**, 2166–2175
45. Pilkington, S. J., Skehel, J. M., and Walker, J. E. (1991) *Biochemistry* **30**, 1901–1908
46. Runswick, M. J., Gennis, R. B., Fearnley, I. M., and Walker, J. E. (1989) *Biochemistry* **28**, 9452–9459
47. Runswick, M. J., Fearnley, I. M., Skehel, J. M., and Walker, J. E. (1991) Biochemistry

FEBS Lett. **286**, 121–124

48. Skehel, J. M., Pilkington, S. J., Runswick, M. J., Fearnley, I. M., and Walker, J. E. (1991) *FEBS Lett.* **282**, 135–138
49. Walker, J. E., Arizmendi, J. M., Dupuis, A., Fearnley, I. M., Finel, M., Medd, S. M., Pilkington, S. J., Runswick, M. J., and Skehel, J. M. (1992) *J. Mol. Biol.* **226**, 1051–1072
50. Arizmendi, J. M., Runswick, M. J., Skehel, J. M., and Walker, J. E. (1992) *FEBS Lett.* **301**, 237–242
51. Arizmendi, J. M., Skehel, J. M., Runswick, M. J., Fearnley, I. M., and Walker, J. E. (1992) *FEBS Lett.* **313**, 80–84
52. Yagi, T. and Dinh, T. M. (1990) *Biochemistry* **29**, 5515–5520
53. Xu, X., Matsuno-Yagi, A., and Yagi, T. (1992) *Arch. Biochem. Biophys.* **296**, 40–48
54. Xu, X., Matsuno-Yagi, A., and Yagi, T. (1992) *Biochemistry* **31**, 6925–6932
55. Asahara, H., Wistort, P. M., Bank, J. F., Bakerian, R. H., and Cunningham, R. P. (1989) *Biochemistry* **28**, 4444–4449
56. Cunningham, R. P. , Asahara, H., Bank, J. F., Scholes, C. P., Salerno, J. C., Surerus, K., Münck, E., McCraken, J., Peisach, J., and Emptage, M. H. (1989) *Biochemistry* **28**, 4450–4455
57. Kuo, C. H., McRee, D. E., Fisher, C. L., O'Handley, S. F., Cunningham, R. P., and Tainer, J. A. (1992) *Science* **258**, 434–440
58. Friedrich, T., Hofhaus, G., Ise, W., Nehls, U., Schmitz, B., and Weiss, H. (1989) *Eur. J. Biochem.* **180**, 173–180
59. Wang, D.-C., Meihardt, S. W., Sackmann, U., Weiss, H., and Ohnishi, T. (1991) *Eur. J. Biochem.* **197**, 257–264
60. Hofhaus, G., Weiss, H., and Leonard, K. (1992) *J. Mol. Biol.* **221**, 1027–1043
61. Patel, S. D., Cleeter, M. W. J., and Ragan, C. I. (1988) *Biochem. J.* **256**, 529–535

Chapter III

EPR Studies of Complex I Modified with Inhibitory Reagents

SUMMARY

Reduction levels of iron-sulfur clusters of NADH:ubiquinone oxidoreductase (complex I) were measured after treatment of two inhibitory reagents, *N*-bromosuccinimide and *p*-chloromercuriphenylsulfonic acid. Both reagents destroyed the signal from the N-4 cluster at relatively low concentrations, while other signals were little affected. The signal from the N-3 cluster increased with decreasing that from the N-4 cluster, implying the interaction between the N-3 and N-4 cluster. These results suggested that the N-4 cluster is on a surface side and probably on a gate region of the electron flow in the complex. Based on these results, electron transfer pathway within complex I are proposed.

INTRODUCTION

NADH:ubiquinone oxidoreductase (complex I) contains FMN and at least four iron-sulfur clusters, but pathway of the electron transfer through these redox components and the site of energy coupling are still uncertain. Most of our information have been based on EPR studies of the constituent iron-sulfur clusters. On the basis of results of EPR measurements, there is still a controversial discussion favoring different functional models (*1–3*).

Potentiometric titrations combined with EPR spectroscopy have revealed a major potential gap of about 300 mV between the cluster N-2 ($E_{m,7}$ = –30 mV) and the other clusters, N-1b, N-3, and N-4 ($E_{m,7}$ = –360 to –260 mV) (*4*). Analysis of the electron transfer pathway in complex I has been hampered by the lack of suitable inhibitors that block the electron flow at distinct sites (*5*, *6*) and by the failure of rapid mixing and freezing techniques in combination with EPR spectroscopy in resolving the fast intramolecular electron transfer steps (*7*, *8*). Complex I catalyzes electron transfer from NADH to a variety of electron acceptors such as ubiquinone analogues and ferricyanide (*9*, *10*). The reduction of ubiquinone and its analogue is rotenone-sensitive, whereas that of ferricyanide is rotenone-insensitive, which shows different sites for electron donation from the complex. Studies with sulfhydryl and other reagents have shown several inhibition sites characterized by selective inhibition with different acceptors (*11*, *12*). It has been thought that there are two specific sites accessible for inhibitors, the NADH-binding site and the site for the substrate ubiquinone. However, they are the sites for the electron input and output, and such investigations give less information for electron pathway within the enzyme.

On the other hand, information for the structure of complex I has been accumulated. The topographical organization of the subunits have been studied by labeling and chemical cross-linking them with hydrophobic and hydrophilic reagents (*13–15*). Recently, three-dimensional structure of complex I from *Neurospora crassa* have been reported to show an L-shaped structure with two arms (*16*). The enzyme has been resolved with chaotropic agents into smaller fragments (*17*, *18*). Also, the preceding chapter presented that complex I could be dissociated with mild detergents and the possible location of iron-sulfur clusters could be assigned to respective subunits. Taking these information into account, we should reconsider many proposals for electron transfer pathway.

Krishnamoorthy and Hinkle have measured electron transfer activities and steady state reduction levels of iron-sulfur clusters of complex I in mitochondria and submitochondrial particles after treatment with various reagents (*1*), which is rather informative to design a new approach to study the electron flow in the complex. In this chapter, effects of two inhibitory reagents, *N*-bromosuccinimide (NBS) and *p*-chloromercuriphenylsulfonic acid (PCMPS) are investigated to elucidate the reduction levels of the clusters in purified complex I. Based on the results and other information, electron transfer pathway within the enzyme is discussed.

MATERIALS AND METHODS

Complex I was prepared as described in the preceding chapter (*19*, *20*). The reagents used for chemical modification were obtained from Wako Pure Chemical Industries or Nakalai Tesque, Inc..

Chemical modifications were performed by the addition of the reagent to a suspension of complex I (final protein concentration of 1 mg/ml), and incubation at 25℃ for 30 min under nitrogen. In the case of NBS, the enzyme was suspended in 0.25 M sucrose and 10 mM K-MOPS, pH 7.5, whereas in the case of PCMPS in 0.25 M sucrose and 10 mM K-MES, pH 6.5. The excess reagent was removed by pelleting the suspension at 30,000 g, and the resulting pellet was resuspended in 0.25 M sucrose, 50 mM Tris-HCl buffer, pH 8.0, to a final concentration of about 20 mg of protein/ml.

The suspensions were incubated on ice with 2 mM NADH for 1 min and/or 5 mM dithionite for 3 min. It has been demonstrated that NADH alone gives maximal reduction of the iron-sulfur clusters in complex I (*21*). The samples were transferred into EPR tubes and immediately frozen in liquid nitrogen. EPR measurements were performed as described in the preceding chapter (*22*). In this chapter, N-1 type cluster detected in the intact enzyme is referred to cluster N-1 or N-1b. N-1 was estimated from the amplitude of the g = 2.02 line at 30K. N-2 was estimated from the area under the g = 2.05 line at 10K. Reduction of N-3 and N-4 were estimated by the areas covered by the troughs at g = 1.86 and g = 1.88, respectively. N-4 was also determined from the area under the g_z (2.10) line. The g_z (2.04) line of N-3 could not be used for the estimation owing to its overlap with those from other clusters.

RESULTS

Fig. III-1 shows the spectra of the iron-sulfur clusters in the complex I prepared in this work. Four iron-sulfur clusters of complex I can be recognized after full reduction with NADH and dithionite. The signal of respective cluster exhibits a distinct relaxation behavior and so distinguishable by changing the temperature of the sample. In order to observe the reduction level of each cluster, EPR measurements of the modified complex I were carried out at 10K and 30K. At 10K the intensity of the g_x lines of cluster N-3 and N-4 are easily estimated, whereas they are saturated at 30K. The signals of cluster N-1 and N-2 can be estimated at 30K without overlap with N-3 and N-4. The EPR spectrum recorded at the sample temperature of 50K showed only the signals arising from the N-1 cluster. It could be clearly seen that two troughs are present at around g = 1.94. It was difficult to be answered without the help of computer simulation of the spectrum whether the particular line shape of this spectrum is caused by a superposition of two axial lines or by a rhombic line. In the following description, the term N-1 refers to N-1b.

Figs. III-2 and III-3 show EPR spectra of complex I treated with NBS, which oxidizes tryptophan residues. When reduced with NADH, the signal of N-4 was lost more than 90% by the treatment with 40 μM NBS. Higher concentrations of NBS resulted in progressive loss of the signal from the N-4 cluster. The progressive loss of the N-4 signal seemed to saturate at 160 μM. The N-4 signal was not restored even when the sample was reduced with dithionite, as shown in Fig. III-3. These results suggested that NBS affected the N-4 cluster itself, rather than electron transfer of N-4. Contrarily, the signal intensity of the N-3 cluster increased on treatment of NBS. The signals of other clusters were little affected by treatment with lower concentration of the reagent, but as increasing the concentration of the reagent they were gradually decreased. Among all of the clusters, N-3 was least affected with NBS, suggesting that the N-3 cluster is hard to access from the environment. In addition, it should be noted that the N-1 cluster, which is known not to be reduced with dithionite, was reduced with dithionite in modified enzyme, although the level of reduction with dithionite was slightly lower than that with NADH. This implies that experimental procedures may harm intact reactivity of the enzyme or redox components.

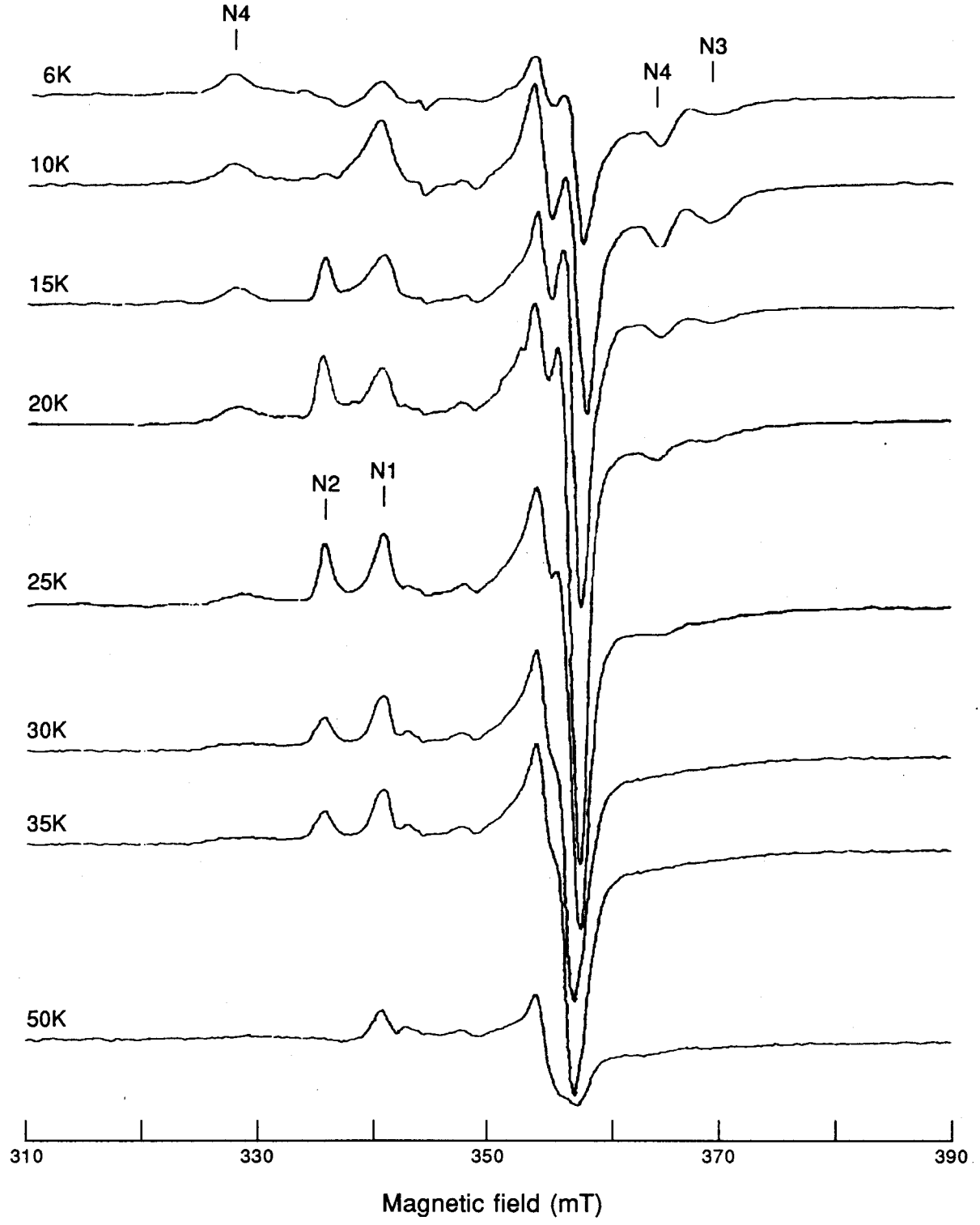

Fig. III-1. EPR spectra of intact complex I. Complex I (20 mg of protein/ml) was reduced with 5 mM dithionite and 2 mM NADH, and recorded at the indicated temperatures. EPR conditions: microwave frequency 9.63 GHz; modulation amplitude 1 mT; microwave power 10 mW; time constant 0.16 s; scanning rate 60 mT/min.

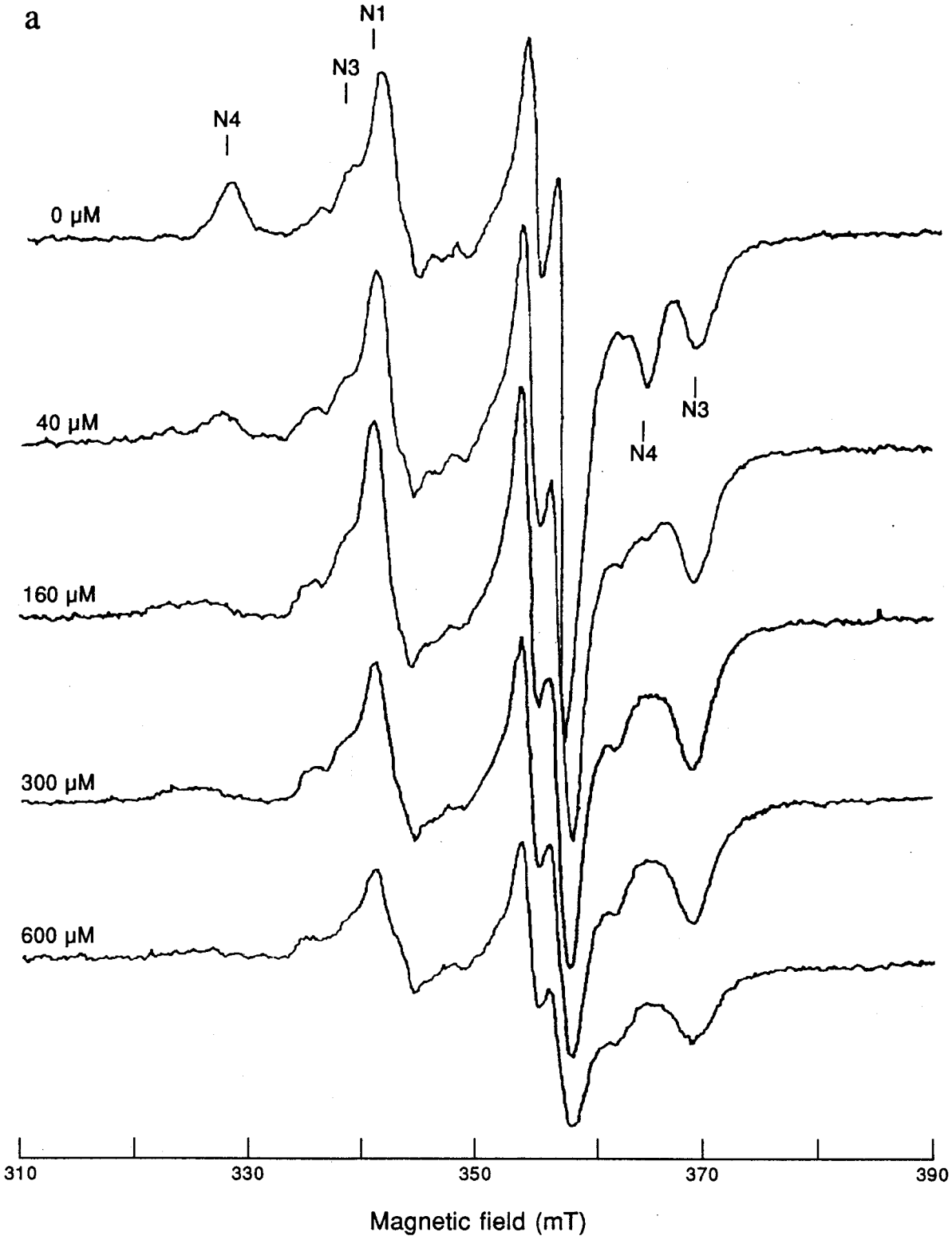

Fig. III-2. EPR spectra of NADH-reduced complex I treated with NBS. Complex I was treated with NBS at the indicated concentrations. The samples were reduced with 2 mM NADH, and spectra were recorded at 10K (a) and 30K (b). Other EPR conditions were the same as in Fig. III-1.

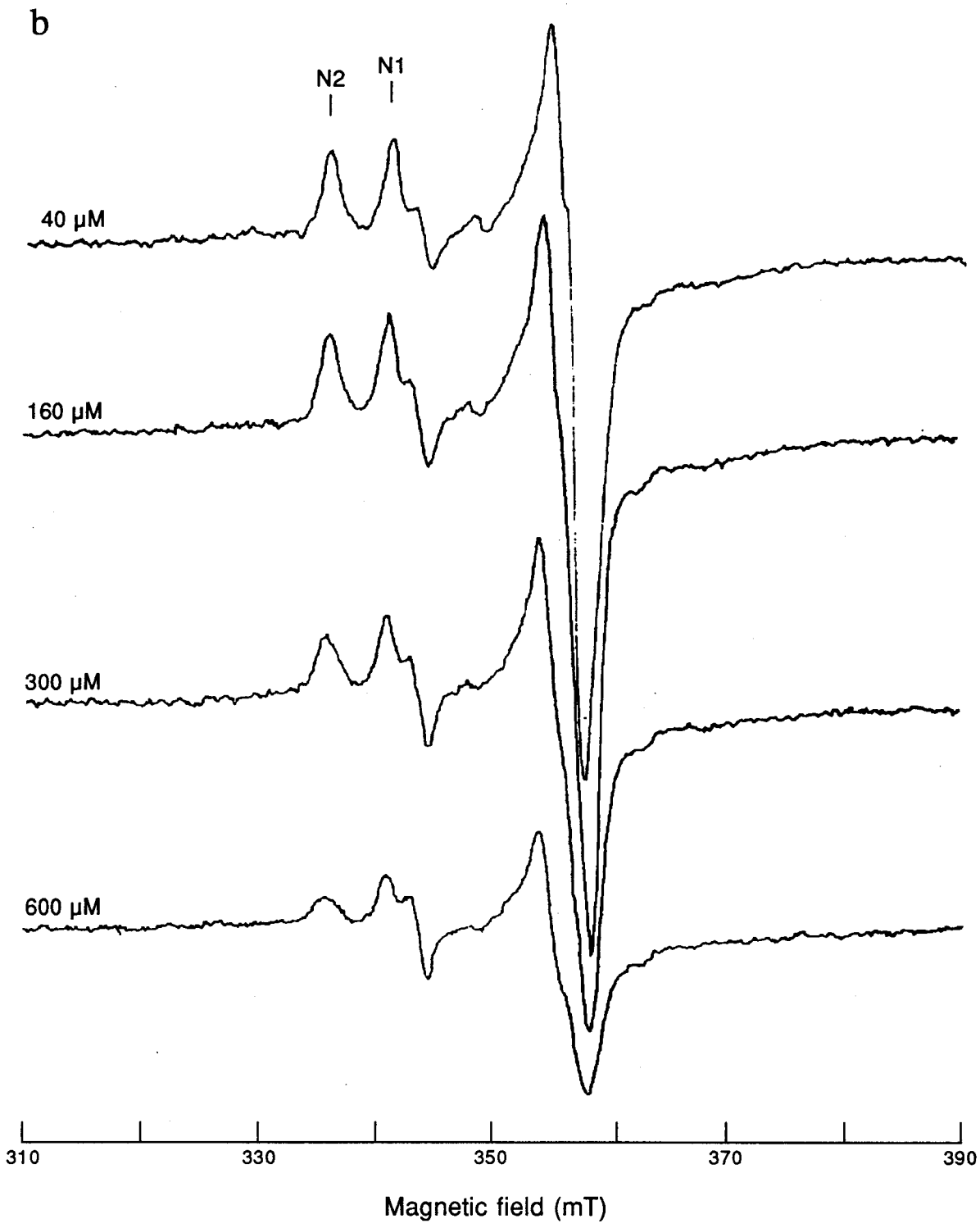

Fig. III-2. (continued) (b) spectra recorded at 30K.

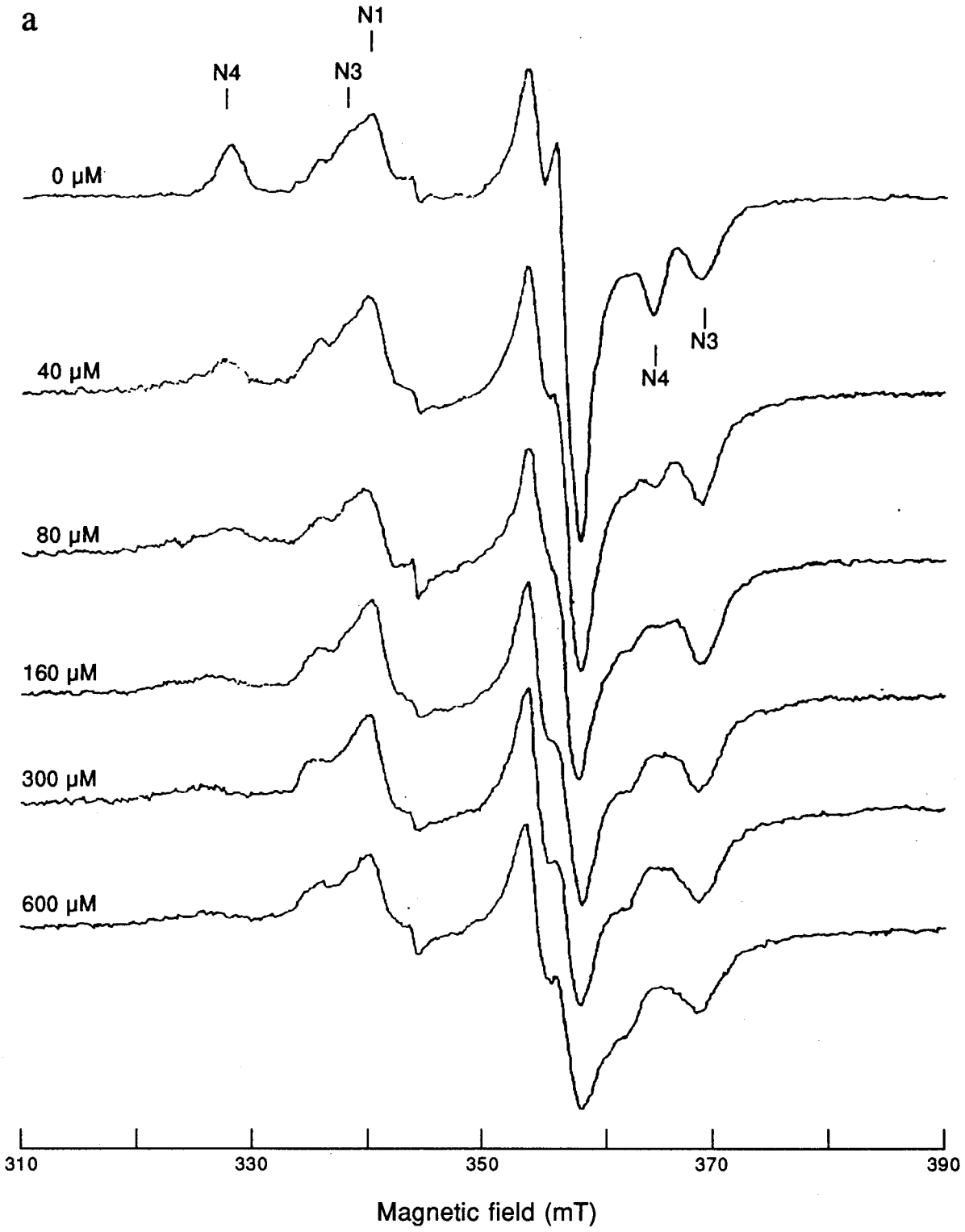

Fig. III-3. EPR spectra of dithionite-reduced complex I treated with NBS. Complex I were treated with NBS at the indicated concentrations. The samples were reduced with 5 mM dithionite, and spectra were recorded at 10K (a) and 30K (b). Other EPR conditions were the same as in Fig. III-1.

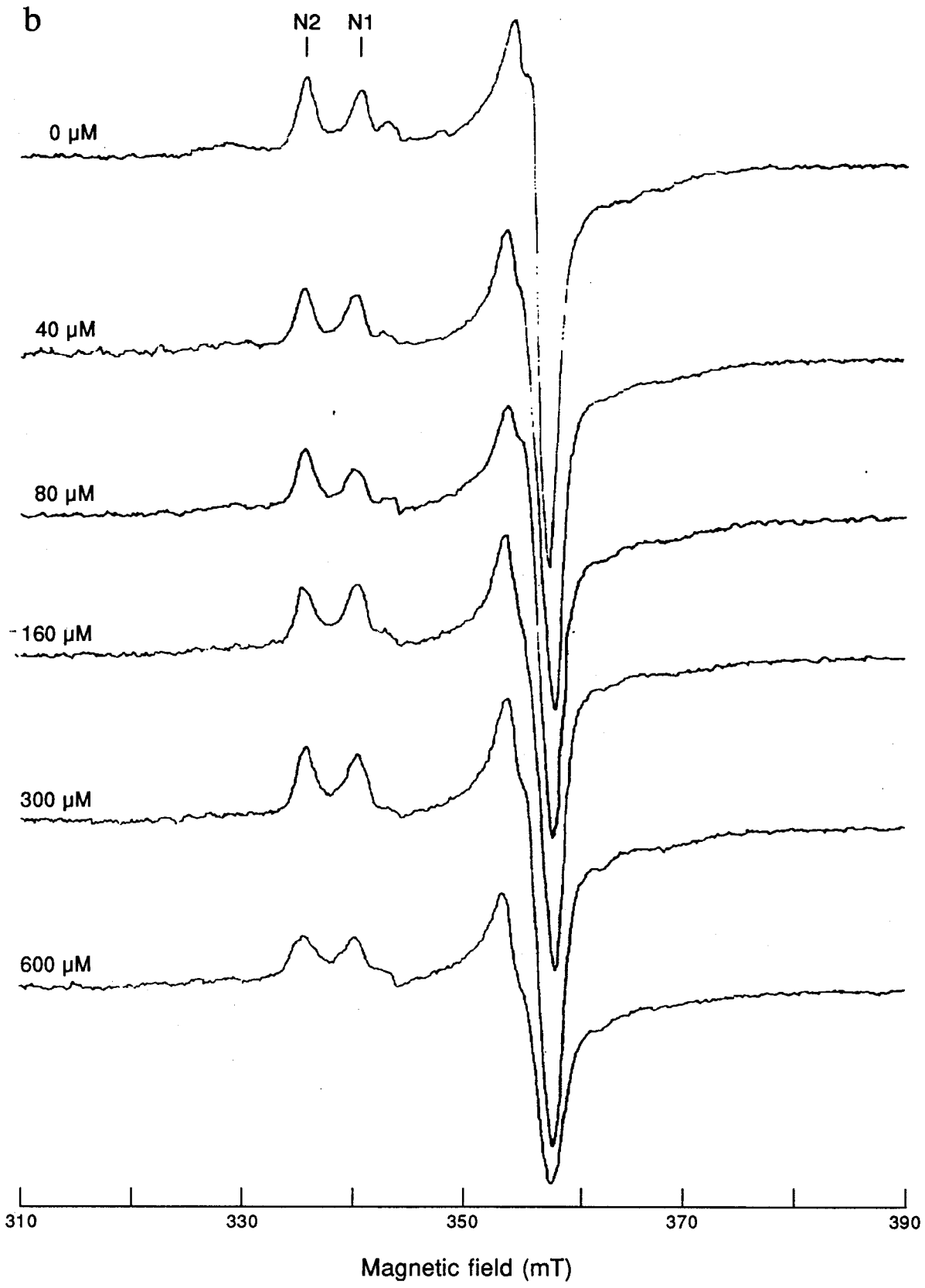

Fig. III-3. (continued) (b) spectra recorded at 30K.

p-Chloromercuriphenylsulfonic acid (PCMPS) is one of sulfhydryl reagents. Reaction of PCMPS even at a low concentration (30 μM) with complex I resulted in the loss of the N-4 signal to a considerable extent (Fig. III-4). As the concentration of PCMPS increased , the signal intensity of N-4 progressively decreased. The loss of the N-4 signal was hardly reversed by reaction with dithionite (Fig. III-5). It has been reported that treatment of PCMPS-treated complex I with DTT restored the N-4 signal (*1*), but such restoration did not observed in this study.

Also in this case, the signal of the N-3 cluster increased on the treatment with the reagent. This increased intensity of N-3 might be the result of relieving of spin-spin interaction. These results suggested that spin-spin interaction existed between N-3 and N-4, and that destruction of N-4 resulted in relieving of this interaction which causes the increase of the N-3 signal intensity.

It has been reported that reaction of PCMPS (200 μM) with NADH-preconditioned complex I resulted in very low levels of reduction of all the clusters (*1*). Figs. III-6 and III-7 present EPR spectra of NADH-preconditioned complex I treated with various concentration of PCMPS. When reduced with NADH (Fig. III-6), the reduction levels of all clusters still remained high at the low concentration (30 μM) of PCMPS. Higher concentrations (above 100 μM) of the reagent caused progressive loss of all of the signals. The signal of N-4 also tended to be affected at lower concentrations than the others were. Comparing to the spectra in Fig. III-4, the reduction levels of all signals in Fig. III-6 became lower at higher concentrations of PCMPS. Another important feature was that reduction level of N-1 was lower in Fig. III-6 than in Fig. III-4. The decreased intensity of the N-1 signal in NADH-preconditioned complex I was apparent in the case of reduction with dithionite (Fig. III-7). Particularly, EPR spectra recorded at 30K revealed loss of the N-1 signal even at low concentrations, as shown in Fig. III-7b. A previous study revealed that dithionite restored all the signals except for N-4 (*1*). If compared the spectra in Fig. III-6 to those in Fig. III-7, the statement is likely to be true to a certain extent that dithionite is effective for restore of some signals.

When the enzymes were treated with the reagents at high concentrations, the troughs low-field to g = 1.86 signal of the N-3 cluster were observed in the spectra recorded at 10K. These signals might arise from the N-5 cluster (g_x =2.07 and g_z = 1.90). These signals appeared under the conditions, which affected the reduction levels of the signals from the other clusters.

Although the relative concentration of this cluster was very low, these results suggested that the N-5 cluster also interacts with some of the other clusters.

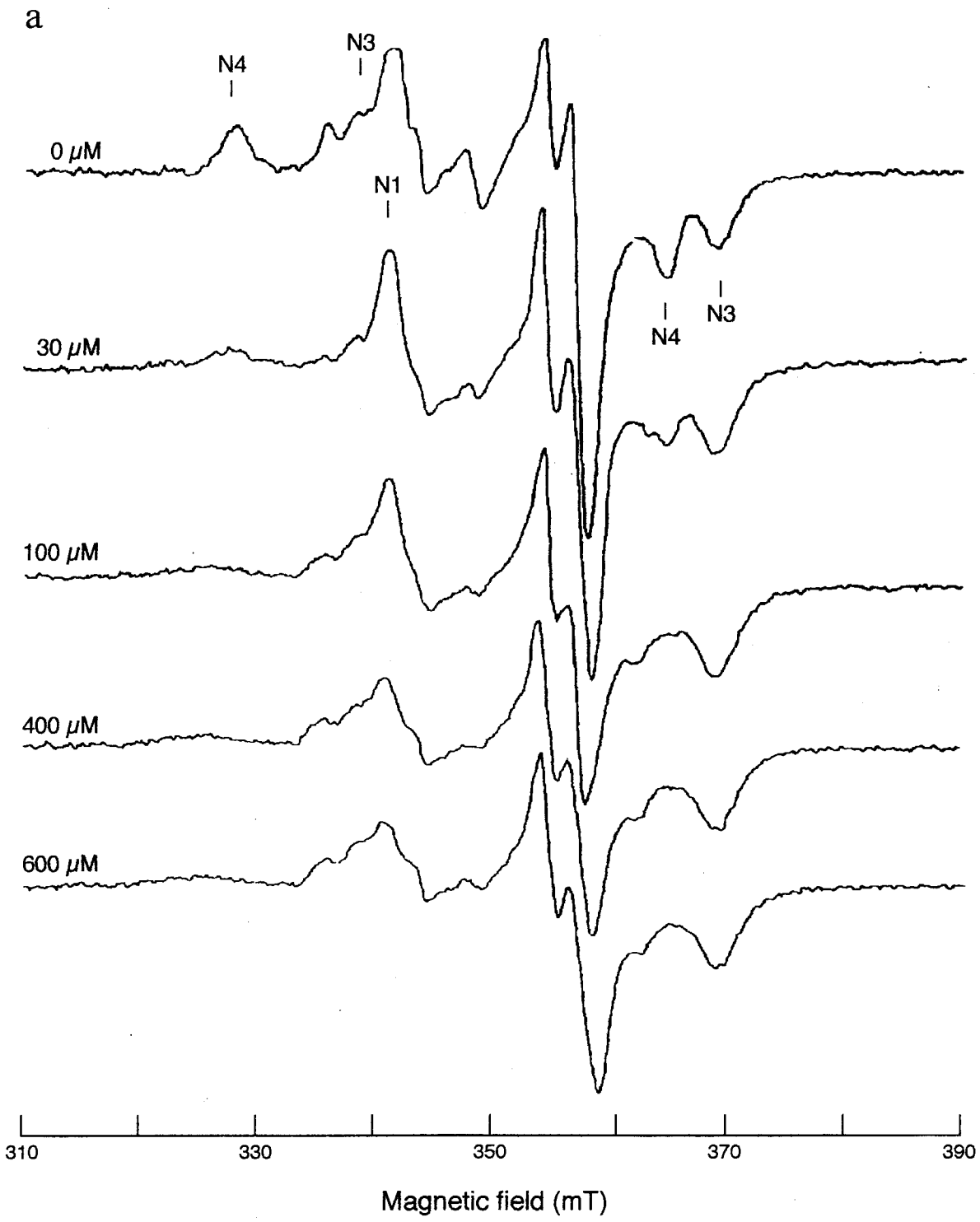

Fig. III-4. EPR spectra of NADH-reduced complex I treated with PCMPS. Complex I was treated with PSMPS at the indicated concentrations. The samples were reduced with 2 mM NADH, and spectra were recorded at 10K (a) and 30K (b). Other EPR conditions were the same in Fig. III-1.

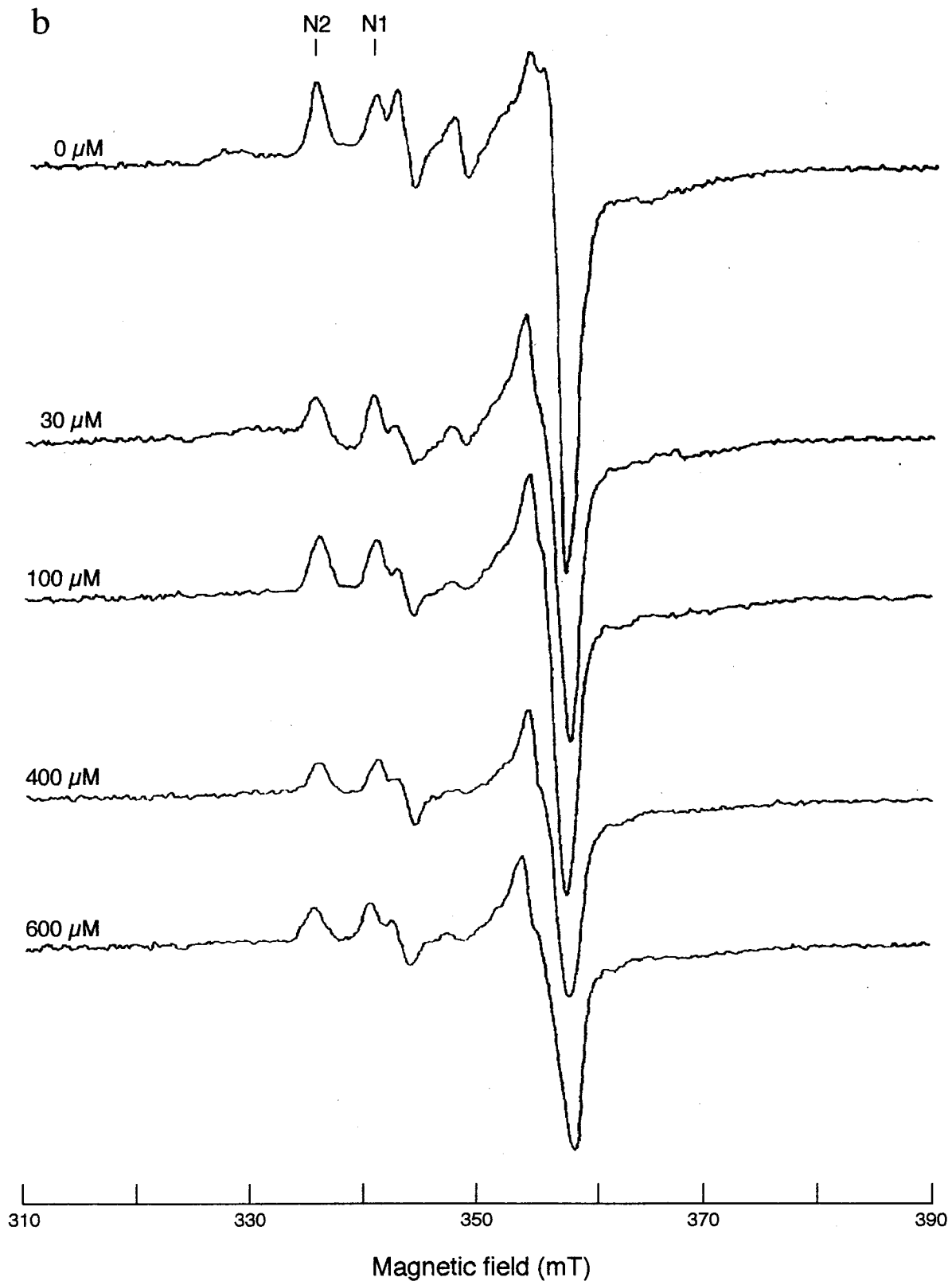

Fig. III-4. (continued) (b) spectra recorded at 30K.

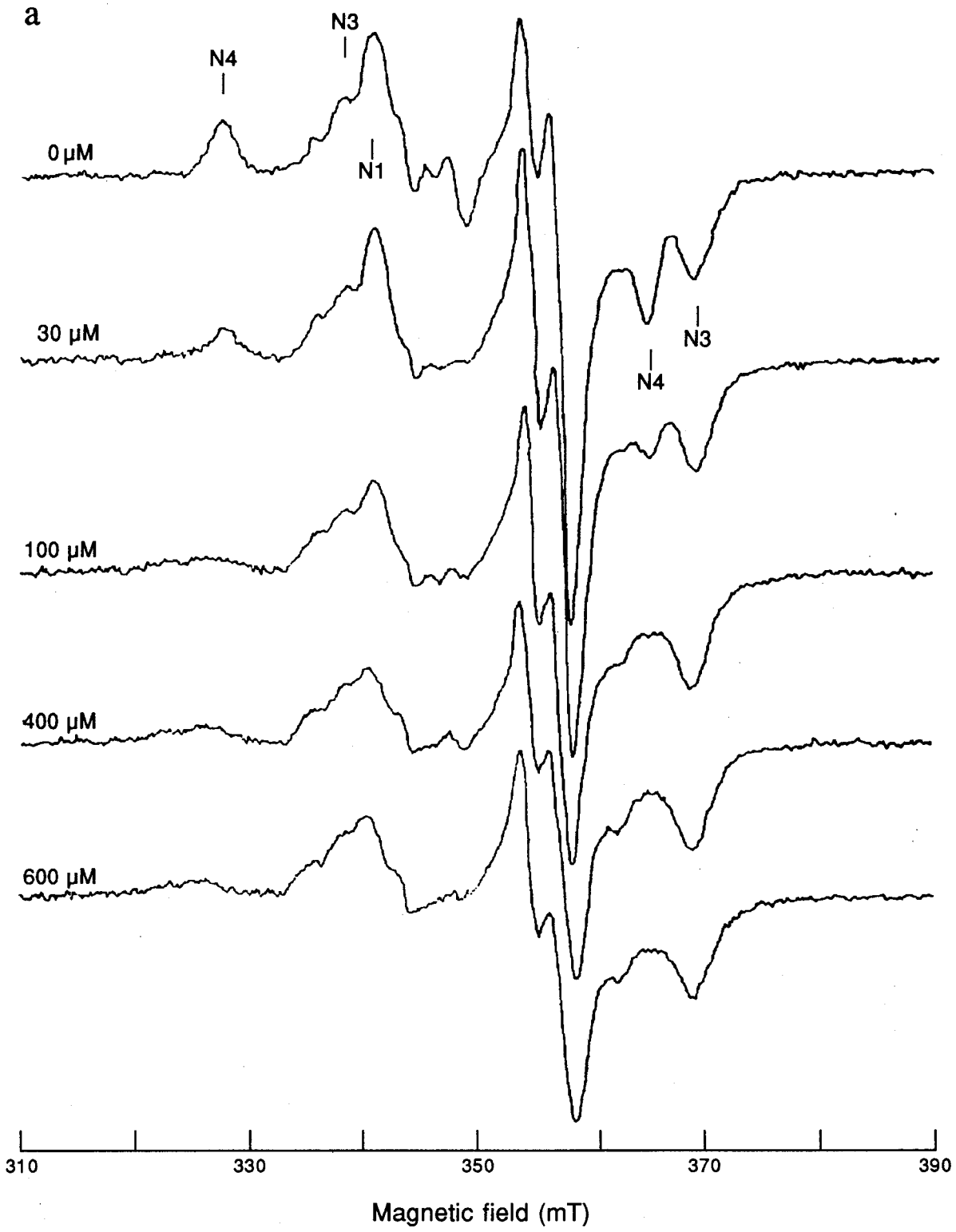

Fig. III-5. EPR spectra of dithionite-reduced complex I treated with PCMPS and then with DTT. Complex I was treated with PCMPS at the indicated concentrations and then with 2 mM DTT. The samples were reduced with 5 mM dithionite, and spectra were recorded at 10K (a) and 30K (b). Other EPR conditions were the same as in Fig. III-1.

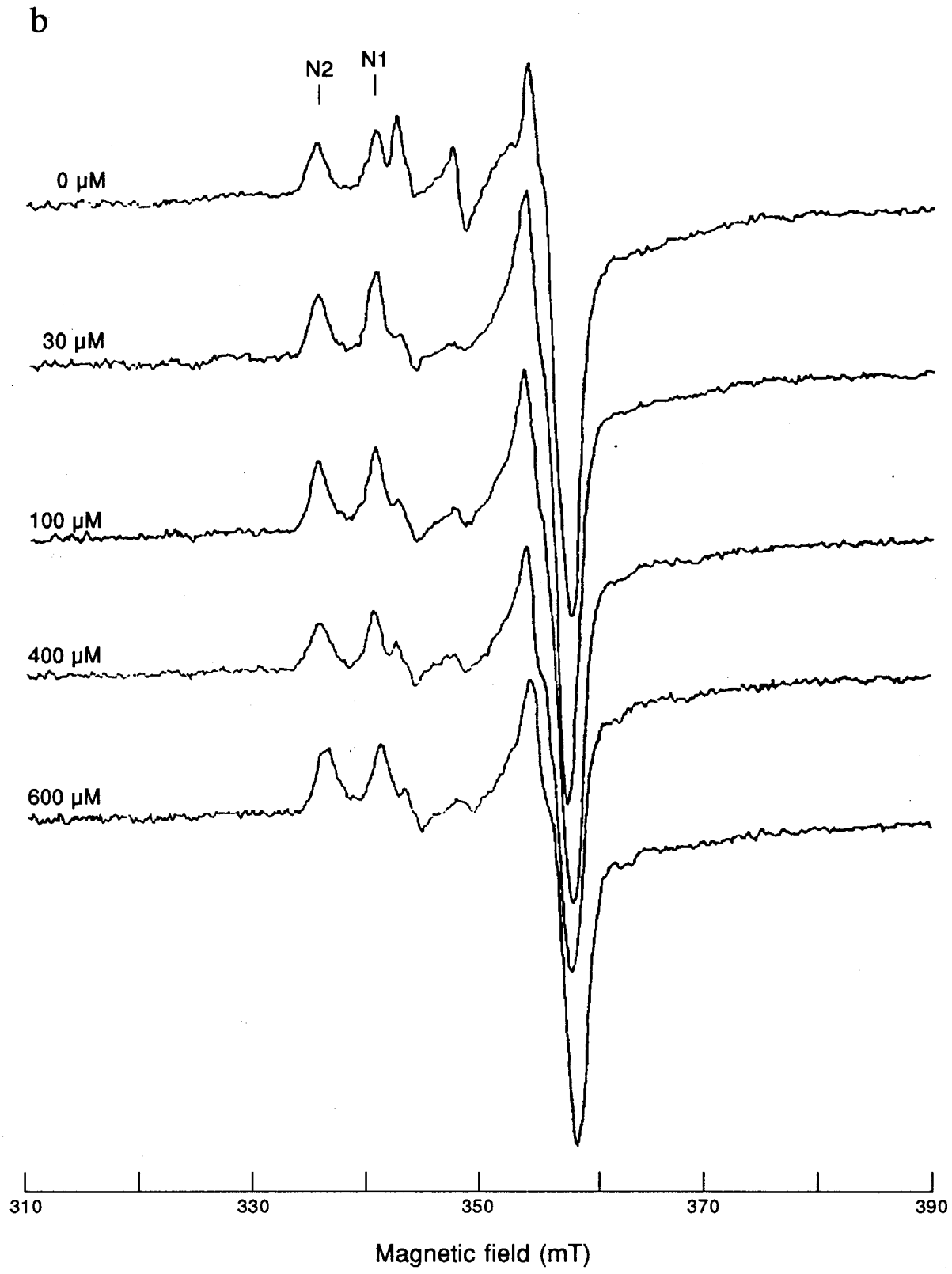

Fig. III-5. (continued) (b) spectra recorded at 30K.

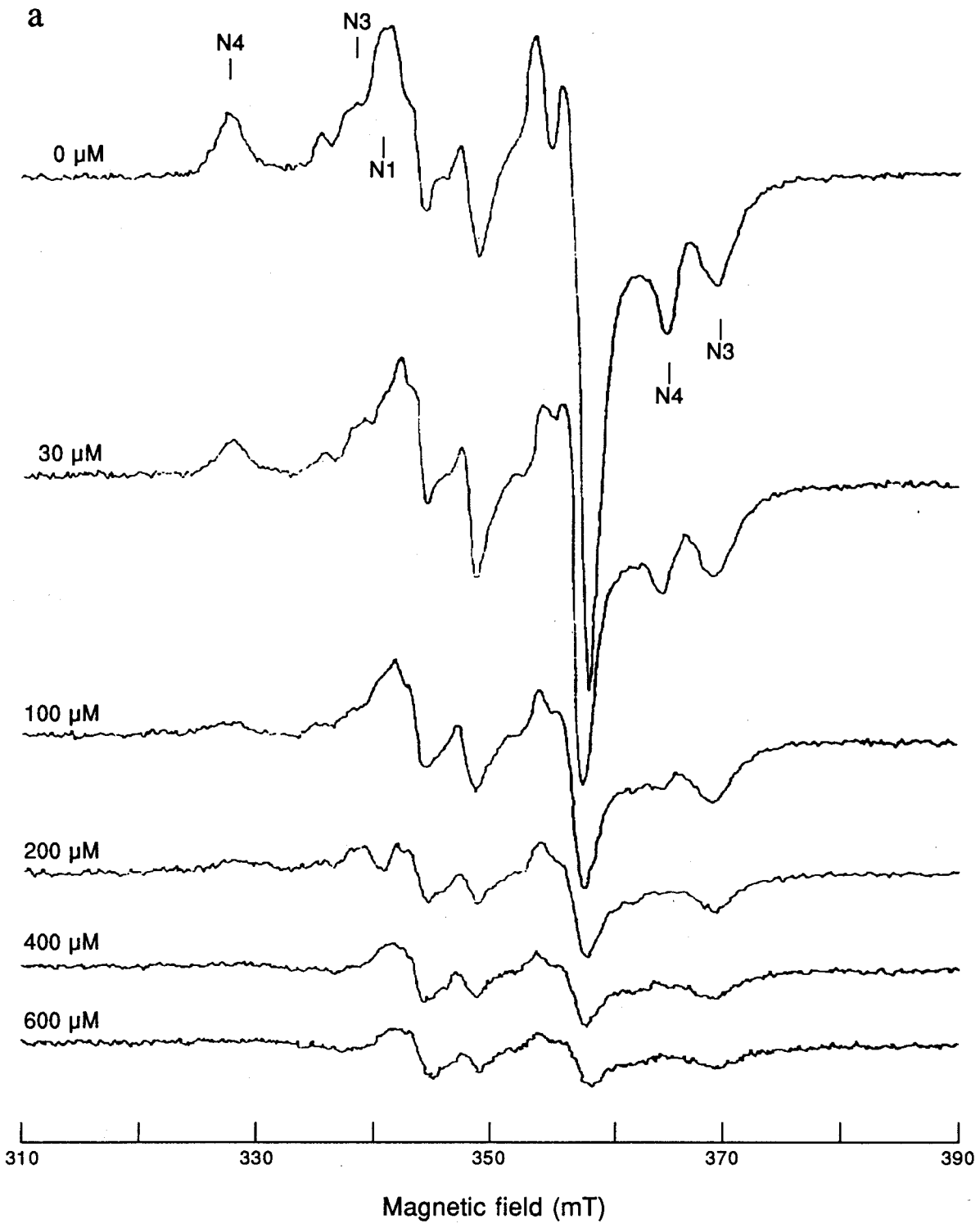

Fig. III-6. EPR spectra of NADH-reduced complex I treated with PCMPS after precondition with NADH. Complex I was preincubated with 1 mM NADH and the treated with PCMPS at the indicated concentrations. The samples were reduced with 2 mM NADH, and spectra were recorded at 10K (a) and 30K (b). Other EPR conditions were the same as in Fig. III-1.

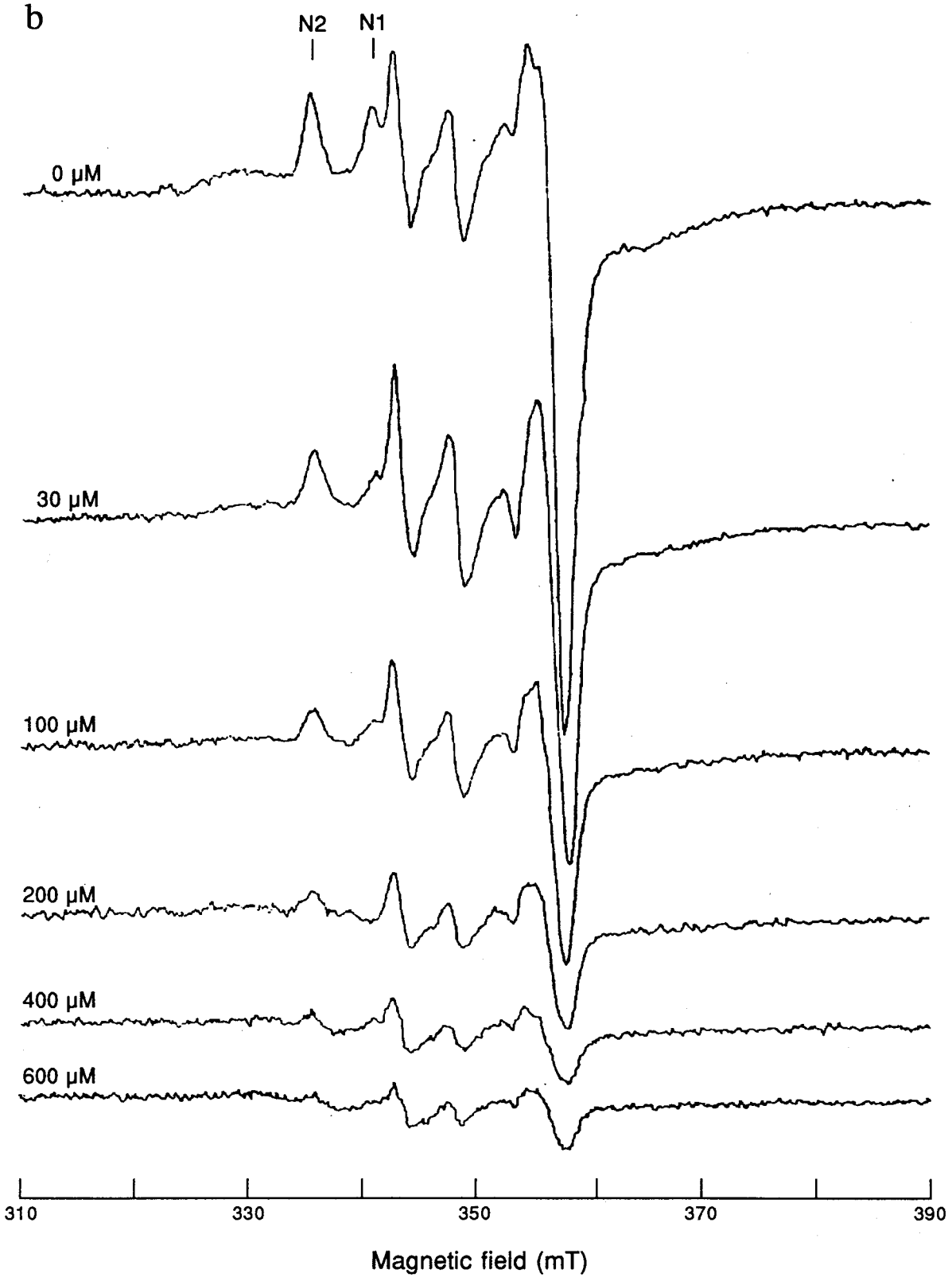

Fig. III-6. (continued) (b) spectra recorded at 30K.

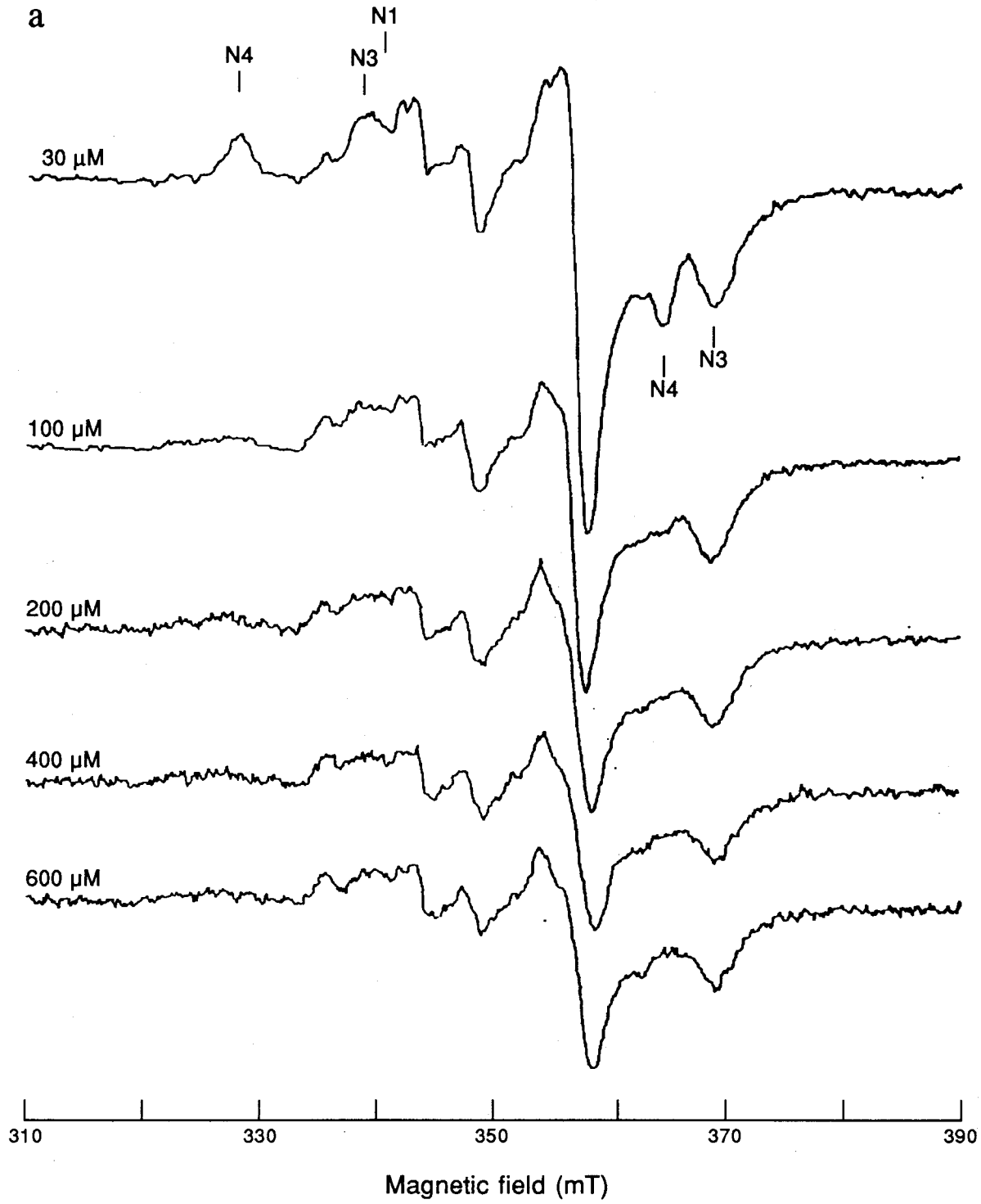

Fig. III-7. EPR spectra of dithionite-reduced complex I treated with PCMPS after precondition with NADH. Complex I was preincubated with 1 mM NADH and then treated with PCMPS at the indicated concentrations. The samples were reduced with 5 mM dithionite, and spectra were recorded at 10K (a) and 30K (b). Other EPR conditions were the same as in Fig. III-1.

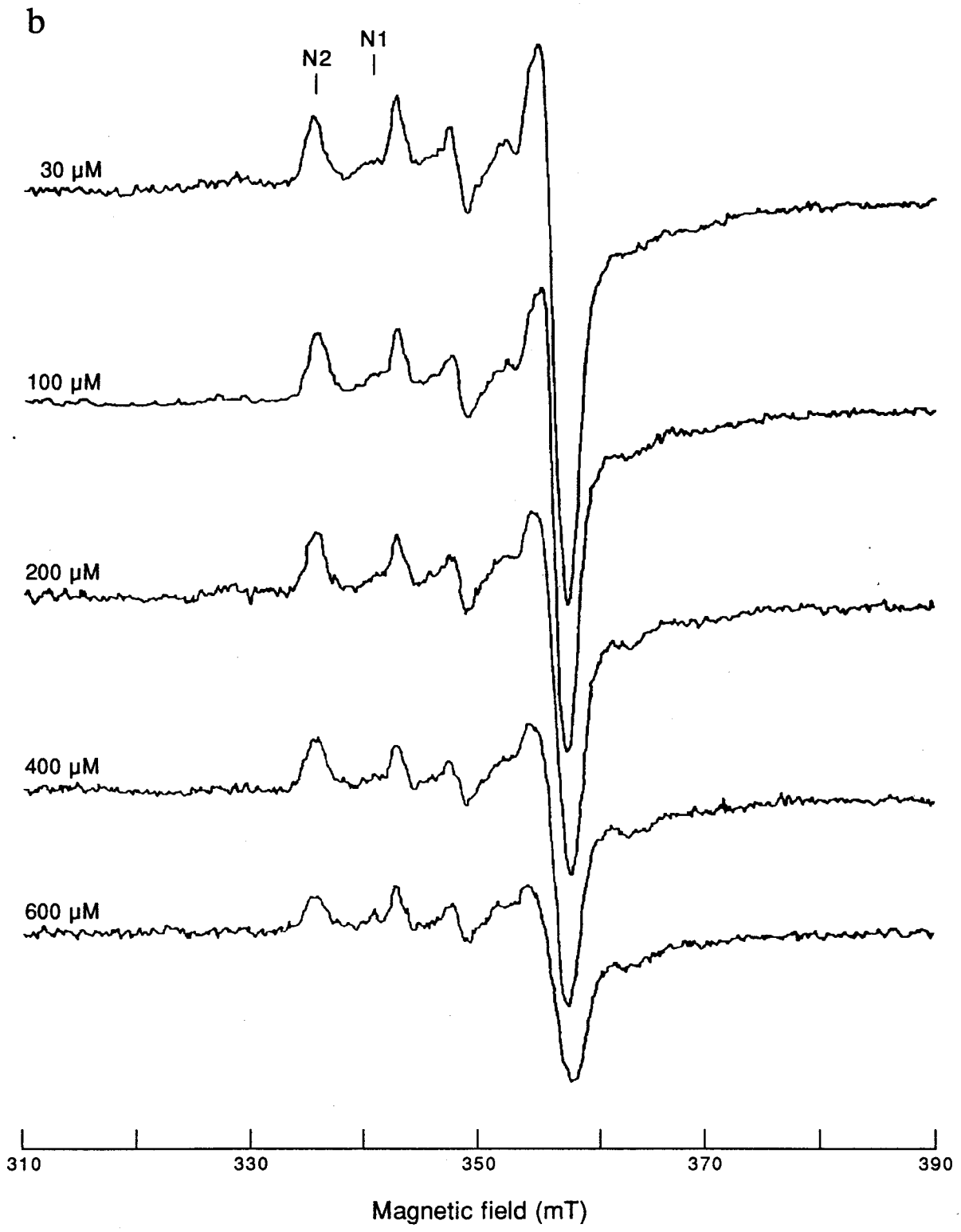

Fig. III-7. (continued) (b) spectra recorde at 30K.

DISCUSSIONS

Experiments in this chapter are reexamination of the effects of chemical modification on purified complex I. When treated with mitochondria and submitochondrial particles, NBS inhibited electron transfer activities from NADH to several acceptors (*1*). In this experiment NBS destroyed the signal from the N-4 cluster even at low concentration such as 40 μM. Under such conditions, other clusters including N-2, which is thought to be a donator of electrons to an acceptor, was highly reduced with NADH. Furthermore, rotenone-sensitive electron transfer from NADH to ubiquinone analogue is little affected at low concentrations of NBS (*1*). Destruction of the N-4 cluster was also observed in the enzyme modified by low concentrations of PCMPS. The presence of multiple reaction sites with PCMPS or other sulfhydryl reagents has been revealed previously (*1*, *11*), and the site associated with the loss of the N-4 signal is one of them. These data suggest a non-linear arrangement of the iron-sulfur clusters in the enzyme. For example, it can be proposed that the N-4 cluster is on a branched pathway of electron transfer and serves as an electron pool with buffering function. Destruction of the N-4 cluster by NBS or PCMPS and concomitant increase of the N-3 cluster suggests a spin coupling between the two clusters. Also it could be said that the N-4 cluster show a high accessibility to the reagents. This implies that the subunit with N-4 is exposed to the environment. Contrary to this, the N-3 cluster is likely to be located in relatively intrinsic area of the enzyme.

Prolonged incubation with NADH was shown to affect the N-1 cluster (*7*). Similar results were obtained in this experiment (Figs. III-6 and III-7). However, a difference between two experiments is that the N-1 signal was not restored even by dithionite in this study. Tyler has suggested that superoxide generated during preconditioning with NADH could be the cause of inactivation of the enzyme (*23*). The extent to which such inactivation occurred may explain that discrepancy. Although it is uncertain what is responsible for inactivation of the N-1 cluster, it might be probable that N-1 is located close to the NADH-binding site in the enzyme.

Many schemes have been proposed for electron transfer pathway and mechanism of energy coupling remains controversial. Bakkar and Albracht have suggested a linear pathway of electron flow through the iron-sulfur clusters (*8*). Their proposed arrangement of iron-sulfur clusters is similar to the order based on their midpoint potentials. Krishnamoorthy and Hinkle

have proposed a scheme very similar to the "b-cycle" at the coupling site 2 (*1*). In spite of the differences, it has been generally accepted that the N-2 cluster donate electrons to the substrate ubiquinone. Therefore, the arrangement of the other iron-sulfur clusters, N-1b, N-3, and N-4, is the subject to be elucidated. In the following I have attempted to combine the data so far obtained with other available information particularly for the structure of the subunits and homologues to discuss the electron pathway in complex I.

The electron input is thought to occur at the 51-kDa subunit. This subunit has been reported to be the NADH-binding subunit of complex I (*24*, *25*). EPR studies of subfractions from the enzyme has revealed the presence of the N-3 cluster in the FP fraction, probably in the 51-kDa subunit (see Chapter II). In view of the suggested interaction between FMN and N-3 (*4*) and the likelihood that FMN is the primary oxidant of NADH, FMN can be assigned to be located on the 51-kDa subunit. It is also conceivable that the 24-kDa subunit (in FP) bears the N-1b cluster. The N-4 cluster could be assigned to the 75-kDa subunit as suggested in this experiment. The 75-, 51-, and 24-kDa subunits are very likely to constitute a structural and functional unit. These three subunits are closely related to subunits of the NAD-reducing hydrogenase from the bacterium *Alcaligenes eutrophus* (*26*). This soluble [NiFe]-type hydrogenase consists of four subunits, namely α, β, γ, and δ. Sequence similarities are present between the α subunit and the 24- and 51-kDa subunits, and between the γ subunit and the N-terminal region of the 75-kDa subunit (*27*). The $\alpha\gamma$ dimer exhibits diaphorase activity and contain at least two iron-sulfur clusters (*28*), which give the EPR signals similar to N-3 and N-1b (*29*, *30*). The absence of the N-4 type signal may coincide with the suggestion that N-4 is dispensable in the electron flow of the enzyme under the conditions studied.

The reduction level of the clusters in modified enzyme has suggested that the N-1b cluster is located close to the NADH-binding site and that N-1b cluster is the primary acceptor of electrons from NADH. However, the NADH-binding site is present on the 51-kDa subunit and the N-1b cluster is probably present in the 24-kDa subunit (*24*, *26*, *31*). The location of the FMN-binding site is less clear because of insufficient data for the consensus sequence motif, but the 51-kDa subunit contains a pair of glycine residues in predicted β-α-β structure that might be the FMN-binding site (*32*). Therefore, it is reasonable to assume that the initial stage of electron transfer occurs in the 51-kDa subunit and that electrons flow from NADH primarily to FMN and then to the N-3 cluster. The 51- and 24-kDa subunits are contained in the same

subfraction (FP) as shown in the preceding chapter and so the 24-kDa subunit probably lies close to the 51-kDa subunit in the enzyme. Therefore, the N-1b cluster may be located close to the NADH-, FMN-, and N-3 binding sites. On the other hand, Krishnamoorthy and Hinkle have suggested that cluster N-1 lies before N-3 (*1*). This proposal is consistent with the data that midpoint potential of the N-1 cluster is lower than that of the N-3 cluster. However, their midpoint potentials are in a small range, and the midpoint potentials of the iron-sulfur clusters of complex I also have been shown to be dependent on the preparations of the enzyme (*1*, *3*). It seems reasonable at present that the N-1, N-3, and N-4 cluster are almost isopotential. Therefore, the N-3 cluster can be considered as the first iron-sulfur cluster to be reduced by flavin.

The N-terminal region of the 75-kDa subunit has sequence similarities not only to the subunit of the NAD-reducing hydrogenase, but also to the N-terminal region of the [Fe]-type hydrogenases. Homologous regions present in all these sequences contain the cysteine motifs (see Table II-2). These similarities suggest that the N-terminal region of the 75-kDa subunit constitute a structural domain with iron-sulfur clusters. The 75-kDa subunit have been cross-linked to the 51-kDa subunit and exposed to the matrix in mitochondria (*14*, *15*). These suggestions may support the proposals that N-4 in the 75-kDa subunit interacts with N-3 in the 51-kDa subunit and that N-4 is exposed to the environment. From the results obtained in this work, it can be assumed that N-4 builds a electron pool or a gate of the electron flow. This assumption may be supported by the small range in the midpoint potentials of N-1, N-3, and N-4.

It is almost certain that the N-2 cluster is located on an output side in electron transfer pathway. From the results presented in Chapter II, it is probable that the 23-kDa subunit, which probably contains N-2, constitutes another structural unit together with the other subunits in IP-2. Formate hydrogenlyase (*hyc*) operon of *E. coli* contains eight genes, the products of five of which, HycC, HycD, HycE, HycF, and HycG, have sequence similarity to subunits of complex I, that is, the ND4 (or ND5), ND1, 49-kDa, 23-kDa, and 20-kDa subunits, respectively (*33*, *34*). The formate hydrogenlyase complex appears to comprise the formate dehydrogenase and the hydrogenase 3. The hydrogenase 3 is one of three [NiFe]-type hydrogenases in *E. coli*. The *hyc* operon is thought to encode the components of the hydrogenase, although formate hydrogenlyase has not yet been isolated. The hycE is a putative

gene for the large subunit of hydrogenase 3, and surprisingly, its gene product shows a significant similarity to the 49-kDa subunit of complex I. The HycG and HycF proteins are very similar to the 20-kDa and 23-kDa subunits of complex I, respectively.

These similarities invite speculation about the phylogenetic relationship between bacterial hydrogenase and complex I. From the standpoint of electron transfer, the NAD-reducing hydrogenase and formate hydrogenlyase (hydrogenase 3) can be considered as a model of an input and output segment in electron transfer in complex I, respectively. Both hydrogenases are [NiFe]-type hydrogenases and contains catalytic core subunits, that is, they are so-called large and small subunits. The NAD-linked hydrogenase is water-soluble, while the hydrogenase 3 is bound to the membranes. During the oxidation of hydrogen catalyzed by the membrane-bound hydrogenases, the electrons from hydrogen are injected into the respiratory chain apparently at the level of ubiquinone and transferred to oxygen. The 49- and 20-kDa subunits have been proposed to be evolutionarily related to large and small subunits of nickel-containing hydrogenases, respectively (as mentioned in Chapter I discussion). If that is the case, the 49- and 20-kDa subunits must be responsible for linking different reactions, oxidation of NADH and reduction of ubiquinone.

One important difference among these redox systems is that complex I catalyses proton translocation across the membranes, whereas both hydrogenases lack energy-transducing functions. This may suggest that the subunits lacking similarity to both hydrogenases may function in the energy coupling in complex I. Weiss *et al.* hypothesize that the ND1 protein is involved in proton translocation (*35*). It has been indicated that ND1 protein bears DCCD-binding site and rotenone-binding site (*36*, *37*). Therefore, it may be conceivable that ND1 subunit is involved in proton translocation. It should be pointed out that the HycD, a putative component of the formate hydrogenlyase, shows a significant similarity to the ND1 protein. Even if the ND1 builds a channel for protons, some other components must be involved in energy coupling. The linkage of two different reactions may be essential for bears the energy-transducing functions. A major potential gap exists between the cluster N-2 and the others. N-2 is located on a electron output site, while the others are on a electron input site. The electron connector between the low-potential clusters (N-1, -3, and -4) and the high-potential cluster (N-2) could be some form of an internal ubiquinone (*38*) or "EPR-silent" clusters. This idea is purely speculative and awaits a further characterization of the electron transfer in complex I.

Acknowledgments

I would like to thank Dr. S. Itoh and Ms. M. Iwaki (National Institute for Basic Biology) for their help with recording the EPR spectra. I also thank Mr. T. Kubota, M. Kawamoto, and T. Matsunaga for their help with preparing complex I.

REFERENCES

1. Krishnamoorthy, G. and Hinkle, P. C. (1988) *J. Biol. Chem.* **263**, 17566–17575
2. van Belzen, R. and Albracht, S. P. J. (1989) *Biochim. Biophys. Acta* **974**, 311–320
3. Kotlyar, A. B., Sled, V. D., Burbaev, D. Sh., Moroz, I. A., and Vinogradov, A. D. (1990) *FEBS Lett.* **264**, 17–20
4. Inglew, W. J. and Ohnishi, T. (1980) *Biochem. J.* **186**, 111–117
5. Hatefi, Y. (1985)*Annu. Rev. Biochem.* **54**, 1015–1069
6. Ragan, C. I. (1987) *Curr. Top. Bioenerg.* **15**, 1–36
7. Orme-Johnson, N. R., Hansen, R. E., and Beinert, H. (1974) *J. Biol. Chem.* **249**, 1922–1927
8. Bakker, P. T. A. and Albracht, S. P. J. (1986) *Biochim. Biophys. Acta* 850, 413–428
9. Hatefi, Y. and Stiggall, D. L. (1976) in *The Enzymes* (3rd ed., ed. Boyer, P. D.), pp. 175–297, New York: Academic.
10. Hatefi, Y. and Stempel, K. E. (1969)*J. Biol. Chem.* **244**, 2350–2357
11. Singer, T. P. and Gutman, M. (1971) *Adv. Enzymol.* **34**, 79–153
12. Harmon, H. J. and Crane, F. L. (1976) *Biochim. Biophys. Acta* **440**, 45–58
13. Smith, S. and Ragan, C. I. (1980) *Biochem. J.* **185**, 315–326
14. Patel, S. D. and Ragan, C. I. (1988) *Biochem. J.* **256**, 521–528
15. Patel, S. D., Cleeter, M. W. J., and Ragan, C. I. (1988) *Biochem. J.* **256**, 529–535
16. Hofhaus, G., Weiss, H., and Leonard, K.(1992) *J. Mol. Biol.* **221**, 1027–1043
17. Ragan, C. I., Galanti, Y. M., Hatefi, Y., and Ohnishi, T. (1982) *Biochemistry* **21**, 590–594
18. Ohnishi, T., Ragan, C. I., and Hatefi, Y. (1985)*J. Biol. Chem.* 260, 2782–2788
19. Rieske, J. S. (1967) *Methods Enzymol.* **10**, 381–384
20. Hatefi, Y. (1978)*Methods Enzymol.* **53**, 11–14
21. Albracht, S. P. J., Dooijewaard, G., Leeuwerik, F. J., and van Swol, B. (1977) *Biochim. Biophys. Acta* **459**, 300–317
22. Itoh, S., Tang, X. S., and Satoh, K. (1986) *FEBS Lett.* **205**, 275–281
23. Tyner, D. D. (1974) *Biochim. Biophys. Acta* **396**, 335–346
24. Chen, S. and Guillory, R. J. (1981) *J. Biol. Chem.* **256**, 8318–8323
25. Deng, P. S. K., Hatefi, Y., and Chen, S. (1990) *Biochemistry* **29**, 1094–1098
26. Tran-Betcke, A., Warnecke, U., Böcker, C., Zaborosch, C., and Friedrich, B. (1990) *J. Bacteriol.* **172**, 2920–2929

27. Pilkington, S. J., Skehel, J. M., Gennis, R. B., and Walker, J. E. (1991) *Biochemistry* **30**, 2166–2175
28. Schneider, K., Cammack, R., and Schlegel, H. G. (1984) *Eur. J. Biochem.* **142**, 75–84
29. Schneider, K., Cannack, R., Schlegel, H. G., and Hall, D. O. (1979) *Biochim. Biophys. Acta* **578**, 445–461
30. Schneider, K., Cannack, R., and Schlegel, H. G. (1984) *Eur. J. Biochem.* **142**, 75–84
31. Pilkington, S. J. and Walker, J. E. (1989) *Biochemistry* **28**, 3257–3264
32. Preis, D., Weidner, U., Conzen, C., Azwvedo, J. E., Nehls, U., Röhlen, D., van der Pas, J., Sackman, U., Schneider, R., D.Werner, S., and Weiss, H.(1991) *Biochim. Biophys. Acta* **1090**, 133–138
33. Böhm, R., Sauter, M., and Böck, A. (1990) *Mol. Microbiol.* **4**, 231–243
34. Lutz, S., Jacobi, A., Schlinsog, V., Böhm, R., Sawers, G., and Böck, A. (1991) *Mol. Microbiol.* **5**, 123–135
35. Friedrich, T., Strohdeicher, M., Hofhaus, G., Preis, D., Salm, H., and Weiss, H. (1990) *FEBS Lett.* **265**, 37–40
36. Yagi, T. and Hatefi, Y. (1988)*J. Biol. Chem.* **263**, 16150–16155
37. Earley, F. G. P., Patel, S. D., Ragan, C. I., and Attardi, G. (1987) *FEBS Lett.* **219**, 108–113
38. Weiss, H., Friedrich, T., Hofhaus, G., and Preis, D. (1991) *Eur. J. Biochem.* **197**, 563–576

PUBLICATION LIST

1. The amino acid sequences of two 13kDa polypeptides and partial amino acid sequence of 30kDa polypeptides of complex I from bovine heart mitochondrial: Possible location of iron-sulfur clusters

   Ryoji Masui, Sadao Wakabayashi, Hiroshi Matsubara, Youssef Hatefi

   Journal of Biochemistry, **109**, 534–543 (1991)

2. The amino acid sequences of the 9kDa polypeptide and partial amino acid sequence of the 20kDa polypeptide of mitochondrial NADH:ubiquinone oxidoreductase

   Ryoji Masui, Sadao Wakabayashi, Hiroshi Matsubara, Youssef Hatefi

   Journal of Biochemistry, **110**, 575–582 (1991)