MOLECULAR PROPERTIES OF TWO REDOX PROTEINS IN LIVER MICROSOMAL MEMBRANES

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CONTENTS

PART I

DENATURATION OF CYTOCHROME b_5 BY GUANIDINE HYDROCHLORIDE: EVIDENCE FOR INDEPENDENT FOLDING OF THE HYDROPHILIC AND HYDROPHOBIC MOIETIES OF THE CYTOCHROME MOLECULE

SUMMARY

Hepatic microsomal cytochrome b_5 is an amphipathic protein consisting of a hydrophilic (heme-containing) moiety and a hydrophobic (membrane-binding) segment and exists in aqueous media as a micelle. Circular dichroism studies indicated that denaturation of cytochrome b_5 by guanidine hydrochloride is a two-stage process, the first transition occurring at the denaturant concentration of about 2.6 M and the second one at 5.0-5.5 M. A hydrophilic fragment of the cytochrome lacking the hydrophobic segment, on the other hand, underwent one-stage denaturation at a guanidine hydrochloride concentration of about 2.9 M. Detachment of the heme from the cytochrome and the fragment, measured by optical absorption, was effected at the denaturant concentrations of 2.6 and 2.9 M, respectively. Gel chromatography experiments showed that dissociation of the cytochrome micelle into the monomers took place concomitant with the second stage of denaturation. It is suggested that the two moieties of the cytochrome molecule exist as relatively independent domains undergoing unfolding separately and the hydrophobic domain is much more resistant to denaturation. It was further found that removal of the heme from the cytochrome by acid-acetone treatment rendered the hydrophilic domain unstable but did not affect the structure of the hydrophobic domain.

INTRODUCTION

Cytochrome b_5 is a typical membrane protein that is tightly bound to microsomes of various tissues. It has been solubilized

from liver microsomes with detergents and purified to homogeneity (1-3). The cytochrome thus purified, called d-$\underline{b}_5$ (detergent-solubilized cytochrome $\underline{b}_5$) to distinguish it from its heme-containing fragment, has a molecular weight of about 16,000 and is composed of a single peptide chain lacking half cystine residue (2,3). A notable feature of the d-$\underline{b}_5$ molecule is its amphipathic nature; it consists of a hydrophilic moiety to which protoheme is noncovalently attached (catalytic segment) and a hydrophobic moiety by which it binds to microsomal membranes (membrane-binding segment) (1-5). Because of this amphipathic nature, purified d-$\underline{b}_5$ exists in aqueous media in the form of a micelle or an oligomeric aggregate (1-3).

Digestion of purified d-$\underline{b}_5$ micelles or liver microsomes with trypsin results in the cleavage of cytochrome molecule at the junction between the hydrophilic and hydrophobic moieties, leading to the liberation of a hydrophilic (heme-containing) fragment. This fragment, referred to as t-$\underline{b}_5$ (trypsin-solubilized cytochrome $\underline{b}_5$), has a molecular weight of about 11,000 and exhibits the same absorption spectra as d-$\underline{b}_5$ in Soret and visible regions (1-3). However, the t-$\underline{b}_5$ molecule is incapable of binding to microsomal membranes because of the lack of the membrane-binding segment (4,5). Both primary (6,7) and three-dimensional (8,9) structures of t-$\underline{b}_5$ have been determined, and the complete sequences of porcine and equine d-$\underline{b}_5$, including the hydrophobic segment, have recently been elucidated (10,11). The study on three dimensional structure of d-$\underline{b}_5$, which is the native form of cytochrome $\underline{b}_5$, is, however, still insufficient.

To gain information about the structure of d-$\underline{b}_5$, we have carried out an investigation of the denaturation of both d-$\underline{b}_5$ and t-$\underline{b}_5$ in guanidine hydrochloride (GuHCl) by measuring both circular dichroism (CD) and optical absorption. The results obtained provide evidence that the two segments of the d-$\underline{b}_5$ molecule exist as discrete domains that have relatively independent three-dimensional structures and thus confirm the two-domain structure proposed for d-$\underline{b}_5$ by Visser et al. (12) based on different approaches.

MATERIALS AND METHODS

Enzymes and Chemicals. Cytochrome $\underline{b}_5$ (d-$\underline{b}_5$) and its hydrophilic fragment (t-$\underline{b}_5$) were purified from rabbit liver microsomes by the methods of Spatz and Strittmatter (3) and of Omura and Takesue (13), respectively. When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (using 7.5% gel) according to the method of Hinman and Philips (14), the preparation gave only a single protein band, whereas a small band due to an impurity protein was detected in the d-$\underline{b}_5$ preparation (Fig. 1). However, the content of this contaminant was estimated to be less than 5% by densitometry. Therefore, no attempts were made to further purify the preparation. Protoheme was removed from the cytochrome $\underline{b}_5$ preparations by acid-acetone treatment as described by Strittmatter (15). The apoproteins thus prepared could restore the normal shape and full strength of cytochrome $\underline{b}_5$ spectra when protohemin chloride was added according to the procedure of Strittmatter (15).

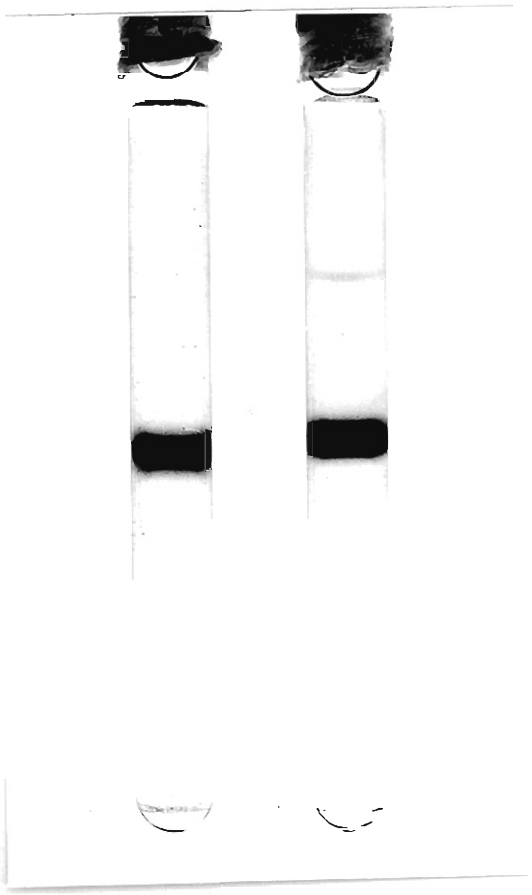

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the t-$\underline{b}_5$ and d-$\underline{b}_5$ preparations used in the present study. Left, t-$\underline{b}_5$; right, d-$\underline{b}_5$. The sample (14 μg of protein) was dissolved by heating in 10 mM Tris-acetate buffer, pH 9.0, containing 1% dodecyl sulfate and 2 M urea, and applied to a 7.5% cross-linked gel disk. Electrophoresis was performed in 0.1 M Tris-acetate, pH 9.0, containing 1% dodecyl sulfate, and 0.01% EDTA for 3 h at a current of 3 mA per tube. The gel was stained with Coomassie blue.

GuHCl of guaranteed reagent grade was purchased from Nakarai Chemical Company, Kyoto, and recrystallized as described by Nozaki and Tanford (16). Sephadex gels and blue dextran 2000 were obtained from Pharmacia and FMN from Nakarai. Bovine serum albumin (Cohn Fraction V) and horse heart cytochrome c (Type II-A) were purchased from Sigma; cytochrome c was purified by CM-Sephadex chromatography before use. The other chemicals used were of the highest quality available.

Analytical Methods. Cytochrome b_5 (both d-b_5 and t-b_5) was determined from the intensity of α-absorption band (556 nm) of the reduced form, assuming a molar extinction coefficient of 2.56×10^4 (17). The molar concentration of apocytochrome b_5 was estimated by protein determination by microbiuret method (18), using d-b_5 and t-b_5 as standard for apo-d-b_5 and apo-t-b_5, respectively. Optical absorption measurements were carried out at 25° in a Cary 14 spectrophotometer, using cells of 10 mm optical path; the concentration of cytochrome b_5 being 4 to 5 μM. CD measurements were performed also at 25° in a JASCO J-20 recording spectropolarimeter with a CD attachment, using quartz cells of 1 mm path; the concentrations of d-b_5 and t-b_5 were usually about 15 to 30 μM. The instrument was calibrated with d-10-camphorsulfonic acid (19). For both optical absorption and CD measurements, the sample was dissolved in 10 mM potassium phosphate buffer, pH 7.5, containing a desired concentration of GuHCl; the pH of the mixture was further adjusted to 7.5 with KOH. KCl was added to 0.1 M to the solutions containing no GuHCl. Prior to CD or optical absorption measurements, each solution was allowed to stand at 25° for about 24 h to ensure the establish-

ment of denaturation equilibrium. The results of CD measurements were expressed in terms of molar ellipticity [θ].

Sephadex G-200 Gel Chromatography. A Sephadex G-200 column (1.9 x 100 cm) was used for determination of apparent molecular weight (micelle size) for d-b_5. The column was equilibrated with 10 mM potassium phosphate buffer, pH 7.5, containing a desired concentration of GuHCl. For experiments in the absence of GuHCl, KCl was added to the buffer to 0.1 M. Elution was conducted with the equilibration buffer at 25°; the elution rate was controlled to 6-8 r/h and the operation pressure was less than 10 cm. Fractions of 2 g were collected. For each GuHCl concentration to be studied, the column was calibrated by applying 0.9 ml of the elution buffer containing 5 mg of blue dextran 2000 (for determination of void volume), 1 mg of FMN (for determination of internal volume), 10 mg of bovine serum albumin and 0.1 μmole of cytochrome c (as molecular weight markers). Elution of these substances was monitored by measuring the absorbance at 280 nm; blue dextran, FMN, and cytochrome c also were detected by measuring the absorbance at 630, 460, and 409 nm, respectively. A solution containing about 80 nmoles of d-b_5, 1 mg of blue dextran 2000, and 1 mg of FMN in 0.95 ml of the elution buffer was applied to the calibrated column, and the elution of d-b_5 was monitored by measuring the absorbance at 280 nm. At GuHCl concentrations causing no detachment of the heme, it was also measured at 413 nm. The K_d value was calculated according to the equation of Gelette (20).

RESULTS

CD Studies of Denaturation of Cytochrome b_5. Fig. 2 shows the far-ultraviolet CD spectra of d-b_5 and t-b_5 that had been incubated for 24 h in the absence of GuHCl is similar to those reported by Schnellbacher and Lumper (21), Huntley and Strittmatter (22), and Visser et al.(12), though minor quantitative differences are noted in the four spectra. It can be seen that the negative CD peak around 222 nm, clearly seen for both d-b_5 and t-b_5 in the absence of GuHCl, is greatly decreased in the presence of the denaturant, indicating that both proteins had undergone unfolding during incubation with GuHCl. The CD spectra in 7 M GuHCl suggest that the proteins were almost completely unfolded at this concentration of the denaturant. Since exactly the same CD spectra were obtained after incubation for additional 24 h, it seemed that the denaturation equilibrium had been reached within the initial 24 h.

In Fig. 3, we plotted the [θ] value at 222 nm attained after 24 h incubation against the GuHCl concentration in the incubation medium. It will be seen that the destruction of the secondary structure of t-b_5, as monitored by the decrease of the negative CD peak, proceeded in one stage as a function of the denaturant concentration; the transition midpoint was seen at a GuHCl concentration of about 2.9 M. This denaturation behavior of t-b_5 is in good agreement with that reported by Schnellbacher and Lumper (21). The denaturation of d-b_5, on the other hand, was clearly a two-stage event having two transition midpoints at GuHCl concentrations of about 2.6 and 5.0-5.5 M. This anomaly was not due to artifacts in CD measurements caused by scattering

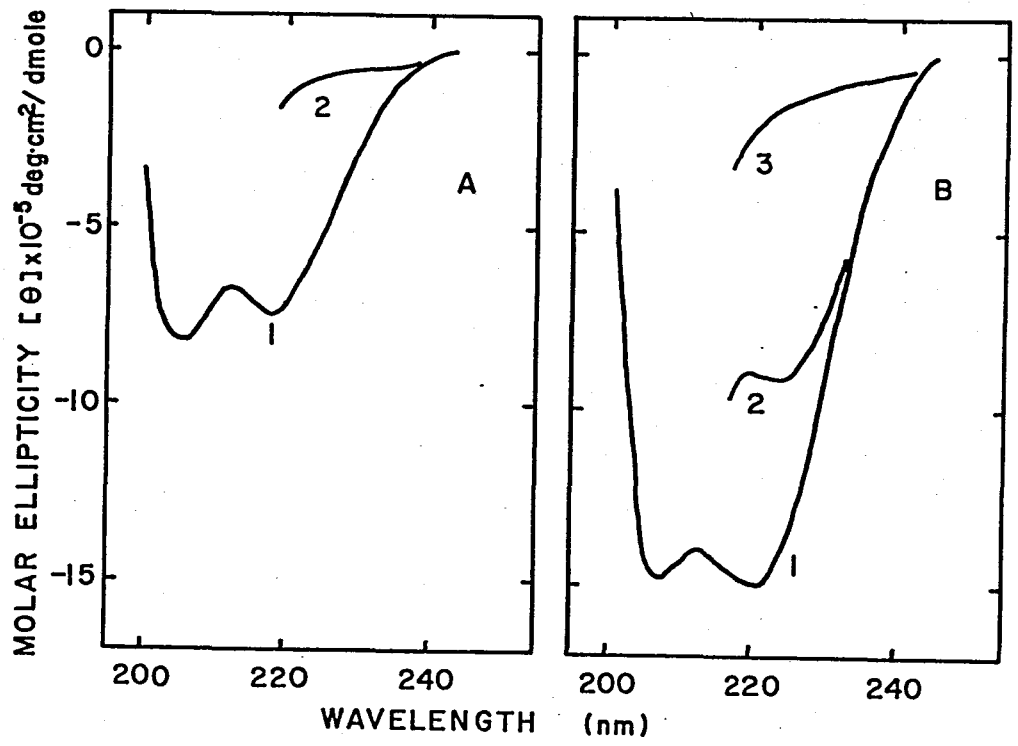

Fig. 2. Far ultraviolet CD spectra of t-b_5 and d-b_5 in the absence and presence of GuHCl. (A) Spectra of t-b_5 in 0 M (curve 1) and 6.8 M GuHCl (curve 2); (B) spectra of d-b_5 in 0 M (curve 1), 4.0 M (curve 2), and 6.9 M GuHCl (curve 3). Measurements were made after incubation of the sample at 25° in 10 mM potassium phosphate buffer, pH 7.5, containing indicated concentration of GuHCl (KCl was added to a final concentration of 0.1 M to the solution containing no GuHCl). Cytochrome b_5 concentrations were about 20.0 and 15.5 μM for t-b_5 and d-b_5, respectively. CD spectra of both t-b_5 and d-b_5 in the absence of GuHCl were also measured at cytochrome b_5 concentrations of about 10.0 and 7.7 μM, respectively, to obtain the spectra in the shorter wavelength region.

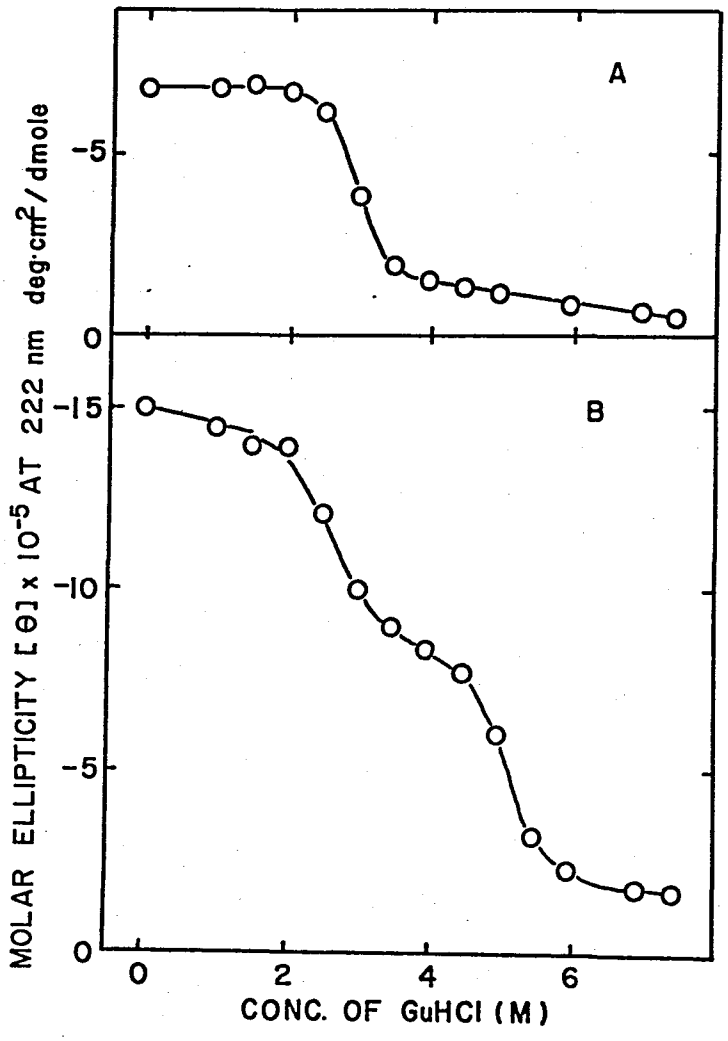

Fig. 3. Effects of GuHCl concentration on molar ellipticities at 222 nm of t-$\underline{b}_5$ and d-$\underline{b}_5$. (A) t-$\underline{b}_5$; (B) d-$\underline{b}_5$. The measurements were carried out as described in Fig. 2, except that the GuHCl concentration was varied as indicated.

effects or absorption statistics, since solutions of d-$\underline{b}_5$ were completely transparent at any GuHCl concentrations. Instead, it was suggested that, at the GuHCl concentration range around 4 M, the molecule of d-$\underline{b}_5$, but not t-$\underline{b}_5$, assumed a conformation that was intermediate between those of native and completely denatured state. To see if this intermediate state represented a stable structure, d-$\underline{b}_5$ was converted to the completely denatured state in 6.9 M GuHCl and then diluted with buffer to the denaturant concentrations of 5.8, 5.0, 4.4, 3.9, and 3.5 M. The molar ellipticities of the diluted solutions had now return to the values observable when the native protein was incubated in the presence of respective concentrations of GuHCl. Therefore, it was certain that at the GuHCl concentrations around 4 M, d-$\underline{b}_5$ could assume a stable, intermediate structure capable of undergoing the second stage unfolding at higher concentrations of the denaturant. Since the transition midpoint observed for the denaturation of t-$\underline{b}_5$ (about 2.9 M) was similar to that for the first-stage denaturation of d-$\underline{b}_5$ (about 2.6 M), it seemed likely that the first stage represented the unfolding of the hydrophilic moiety of d-$\underline{b}_5$.

Detachment of Heme. It is well known that the oxidized form of cytochrome $\underline{b}_5$ shows an intense Soret absorption peak at 413 nm due to uniquely coordinated protoheme. If the structure to which the heme is noncovalently bound is destroyed as a result of unfolding, the heme should be detached from the protein, causing a marked decrease in absorbance at 413 nm. We took advantage of this fact to examine if the first-stage denaturation of d-$\underline{b}_5$ corresponded to the unfolding of the heme-containing

moiety. Fig. 4 shows that the detachment of the heme from d-$\underline{b}_5$ and t-$\underline{b}_5$ occurred at about 2.6 and 2.9 M GuHCl, respectively. Therefore, it was likely that the heme detachment from d-$\underline{b}_5$ took place concomitant with the first-stage unfolding and that from t-$\underline{b}_5$ was accompanied by the destruction of the conformation of the whole molecule. Also note that the heme detachment from d-$\underline{b}_5$ was one-stage process. At higher GuHCl concentrations than those causing the heme liberation, both d-$\underline{b}_5$ and t-$\underline{b}_5$ showed an absorption spectrum characteristic of free protohemin chloride. These results, together with the CD data described above, supported the view that the first-stage denaturation of d-$\underline{b}_5$ represented the unfolding of the heme-containing, hydrophilic moiety of the molecule and that the hydrophobic segment could undergo unfolding only at a higher GuHCl concentration of 5.0-5.5 M. If this is really the case, then the one is led to the conclusion that the hydrophilic and hydrophobic moietys of the d-$\underline{b}_5$ molecule, though linked to each other covalently, constitute separate domains that can undergo unfolding independently.

Denaturation of Apo-Cytochrome $\underline{b}_5$. The hydrophilic moiety of d-$\underline{b}_5$ as well as t-$\underline{b}_5$ have been shown to be very resistant to proteolytic attack; they become susceptible to protease digestion only after removal of the heme (2). It is therefore likely that the presence of the heme stabilizes the structure of t-$\underline{b}_5$ and that of the hydrophilic moiety of d-$\underline{b}_5$ against proteolysis. If this is so and the d-$\underline{b}_5$ molecule consists of the independent domains, as discussed above, then it is expected that removal of the heme from d-$\underline{b}_5$ by acid-acetone treatment will affect the denaturation be-

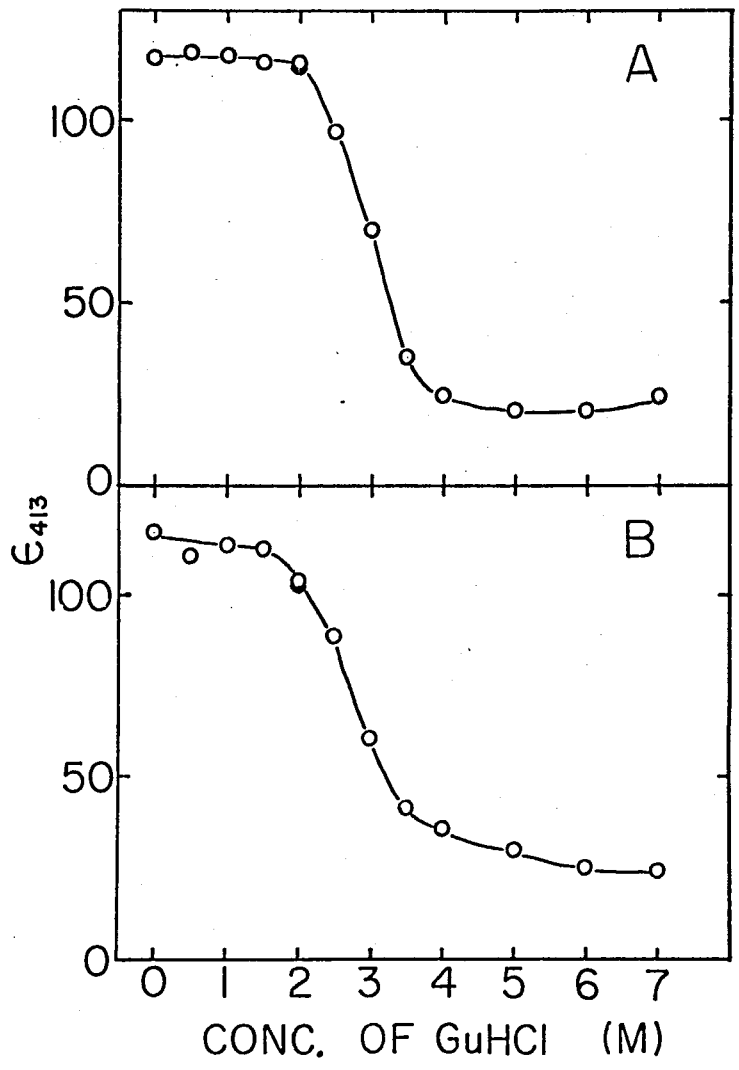

Fig. 4. Detachment of heme from t-$\underline{b}_5$ and d-$\underline{b}_5$ as a function of GuHCl concentration. (A) t-$\underline{b}_5$; (B) d-$\underline{b}_5$. The sample dissolved in 10 mM potassium phosphate buffer, pH 7.5, containing indicated concentration of GuHCl (KCl was added to a final concentration of 0.1 M to the solution containing no GuHCl) was incubated at 25° for 24 h and its absorbance at 413 nm (Soret peak of oxidized cytochrome $\underline{b}_5$) was determined. The concentrations of both t-$\underline{b}_5$ and d-$\underline{b}_5$ were 4-5 μM. The absorbance of each solution is expressed in terms of E_{413}, $\underline{i}.\underline{e}.$, milimolar extinction coefficient at 413 nm.

havior of the hydrophilic domain but will not affect the conformation of the hydrophobic domain. To test this possibility, we studied the denaturation behavior of apo-d-$\underline{b}_5$ and apo-t-$\underline{b}_5$ in GuHCl again by following the intensity of the negative CD peak at 222 nm.

As can be seen in Fig. 5, the denaturation of apo-t-$\underline{b}_5$ began to occur at very low concentrations of GuHCl and the degree of unfolding increased continually as the denaturant concentration was increased until almost complete unfolding was attained at about 4 M. This pattern was in sharp contrast to that for holo-t-$\underline{b}_5$, where no denaturation was observable up to about 2 M GuHCl and a rather sudden unfolding took place thereafter (cf.Fig. 3A). It was concluded that removal of the heme caused a considerable conformational change so that the protein was rendered more labile to the denaturant. The denaturation behavior of apo-d-$\underline{b}_5$ was quite similar to that of apo-t-$\underline{b}_5$ up to about 4 M GuHCl, except that a substantial portion of ordered structure was still remaining at this concentration of the denaturant. However, this remaining structure underwent almost complete unfolding upon raising the GuHCl concentration to 5.0-5.5 M. Actually, the GuHCl concentration required for this transition and the magnitude of decrease in $[\theta]$ at 222 nm associated with this change were very similar to those observed for the second-stage denaturation of holo-d-$\underline{b}_5$ (cf. Fig. 3B). These results led us to conclude that the ordered structure of the hydrophobic domain of d-$\underline{b}_5$ remained unaffected even after the heme derivation which caused the destabillization of the hydrophilic domain.

Aggregation State of Cytochrome $\underline{b}_5$ in the Presence of GuHCl. As mentioned earlier, d-$\underline{b}_5$ exists in aqueous solution as a

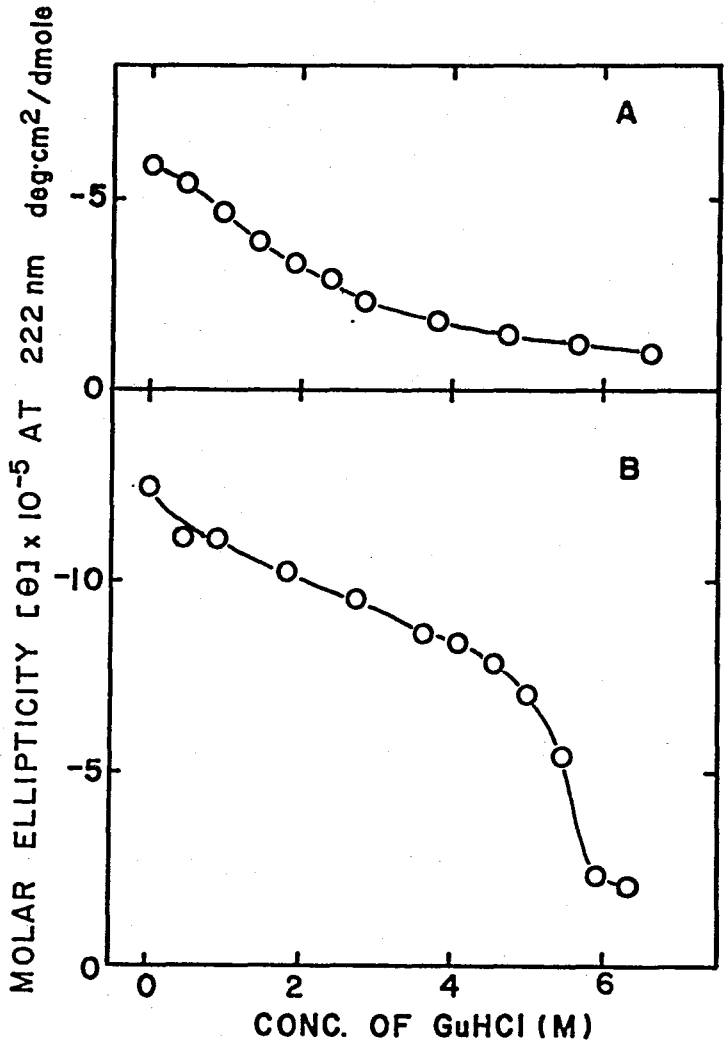

Fig. 5. Denaturation of apo-t-$\underline{b}_5$ and apo-d-$\underline{b}_5$ as a function of GuHCl concentration. (A) apo-t-$\underline{b}_5$; (B) apo-d-$\underline{b}_5$. The experiments were conducted as described in Fig. 3, except that apo-cytochrome $\underline{b}_5$ preparations were used.

micelle or an oligomeric aggregate because of its amphipathic character (1-3). To see if the aggregation state of d-$\underline{b}_5$ has anything to do with its denaturation behavior, we estimated the micelle size of d-$\underline{b}_5$ at several GuHCl concentrations by gel chromatography on a Sephadex G-200 column, using bovine serum albumin and cytochrome $\underline{c}$ as reference standard. The results obtained in these determinations are summarized in Table I. From the $\underline{K}_d$ value obtained in the absence of GuHCl, it was estimated that the apparent molecular weight of d-$\underline{b}_5$ falls in the region of 300,000, a value considerably larger than that of 120,000 reported previously (1-3). Although the reason for this discrepancy is not clear, it is to be noted that the buffer used in the present study contained 0.1 M KCl, whereas buffers of low ionic strength were employed in the previous studies. The data shown in Table I also indicate that the $\underline{K}_d$ values for both bovine serum albumin and cytochrome $\underline{c}$ were considerably smaller in 4 and 6 M GuHCl than at lower concentrations of the denaturant, because of considerable increase in Stokes radius of these proteins upon denaturation (23). A similar reduction of $\underline{K}_d$ value was also seen for d-$\underline{b}_5$ at 4 M GuHCl, indicating that the effective size of d-$\underline{b}_5$ micelle had increased upon exposure to this concentration of the denaturant probably because of the unfolding of the hydrophilic moiety. At any rate, it can be concluded from Table I that d-$\underline{b}_5$ exists in the form of a micelle (aggregate) up to 4 M GuHCl, indicating that the first-stage denaturation of d-$\underline{b}_5$ between 2 and 3 M GuHCl did not affect the micellar state of the molecule. Judging from the $\underline{K}_d$ values, it seems that d-$\underline{b}_5$ was completely dissociated into the monomers in the presence of

Table I

K_d values of d-b_5, bovine serum albumin, and cytochrome *c* in gel chromatography on a Sephadex G-200 column at several GuHCl concentrations.

GuHCl concentration (M)	K_d		
	d-b_5	Bovine serum albumin	Cytochrome *c*
0[a]	0.050	0.343	0.683
1.5	0.056	0.379	0.706
4	0.017	0.153	0.559
6	0.454	0.128	0.555

[a] KCl was added to a final concentration of 0.1 M.

6 M GuHCl. It is likely that dissociation took place concomitantly with the second-stage denaturation at 5.0-5.5 M GuHCl.

DISCUSSION

Previous studies have shown that the molecule of d-$\underline{b}_5$, which consists of a single peptide chain, is amphipathic in nature and is composed of a hydrophilic moiety carrying protoheme and a hydrophobic moiety responsible for the binding of the cytochrome to microsomal membranes (1-5). It also has been found that trypsin and other proteases remove the hydrophobic segment from the cytochrome, releasing the hydrophilic portion as a heme-containing fragment (t-$\underline{b}_5$) (1-3). The amphipathic nature of the cytochrome has been supported by Robinson and Tanford (24), who reported that deoxycholate, Triton X-100, and phosphatidylcholine can bind to d-$\underline{b}_5$, but not to t-$\underline{b}_5$. Delinger *et al.*(25) also reported, based on spin label studies, that d-$\underline{b}_5$, but not t-$\underline{b}_5$, can immobilize a certain amount of phospholipid molecules in lipid bilayer membranes.

The results described above provide evidence that the hydrophilic and hydrophobic regions of the d-$\underline{b}_5$ molecule exist as relatively independent structural domains differing from each other in susceptibility to the denaturing action of GuHCl. Thus, the hydrophilic domain undergoes unfolding with concomitant release of the heme at about 2.6 M GuHCl, whereas the destruction of the ordered structure of the hydrophobic domain requires a GuHCl concentration as high as 5.0-5.5 M. Because of the lack of the hydrophobic segment, t-$\underline{b}_5$ suffers denaturation and at the same

time loses the heme at the denaturant concentration similar to that causing the unfolding of the hydrophilic domain of d-$\underline{b}_5$. These situations may be illustrated schematically as shown in Fig. 6. This conclusion is in agreement with the two-domain structure proposed for d-$\underline{b}_5$ by Visser et al.(12) based on the determination of Stokes radii and CD spectra of the intact cytochrome and its hydrophilic and hydrophobic fragments in deoxycholate solution. These workers have also presented evidence that the two domains of d-$\underline{b}_5$ are linked to each other by an interdomain peptide segment of about 15 Amino acid residues.

Although the denaturation behavior of t-$\underline{b}_5$ is similar to that of the hydrophilic domain of d-$\underline{b}_5$, they are significantly different in the following two respects. First, while the unfolding midpoint for t-$\underline{b}_5$ is seen at about 2.9 M GuHCl, that for the hydrophilic moiety of d-$\underline{b}_5$ is reproducibly lower by about 0.3 M. The same differnce also can be detected in the concentration of GuHCl causing the detachment of the heme from both proteins. Secondly, t-$\underline{b}_5$ is very stable in 0 to 2 M GuHCl, whereas the hydrophilic domain of d-$\underline{b}_5$ undergoes slight but clearly detectable unfolding in this range of the GuHCl concentration. A likely reason for these differences may be the micellar dispersion of d-$\underline{b}_5$ as opposed to the monomeric dispersion of t-$\underline{b}_5$. As a second possibility, it may be assumed that the conformation of t-$\underline{b}_5$ is somehow stabilized owing to the removal of the hydrophobic domain. However, since both d-$\underline{b}_5$ and t-$\underline{b}_5$ show exactly the same absorption and CD spectra in the Soret and visible regions (1-3,16), the structure around the heme in t-$\underline{b}_5$ must not be significantly different from that in d-$\underline{b}_5$. At present, the available data are

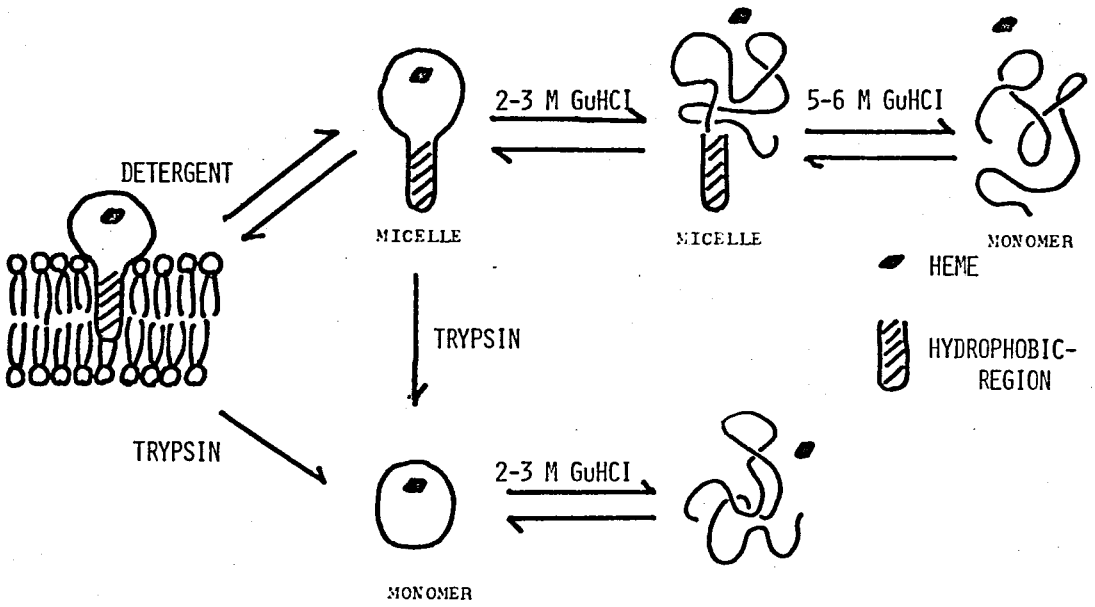

Fig. 6. Schematic illustration of structural transitions of t-b_5 and d-b_5 induced by GuHCl.

insufficient to decide which of the two possibilities is actually the case.

A remarkable feature of the d-$\underline{b}_5$ molecule is the unusually high stability of its hydrophobic domain. As mentioned above, a GuHCl concentration as high as 5.0-5.5 M is required to destroy the structure of this domain. The results described above suggest that this stability is related to the micellar dispersion of the d-$\underline{b}_5$ molecules. It is shown from gel chromatography experiments that the micellar structure of d-$\underline{b}_5$ is still retained even in 4 M GuHCl, where its hydrophilic domain is in a completely unfolded state, and that the denaturation of the hydrophobic domain takes place apparently concomitantly with the dissociation of the micelle into the monomers. This suggests, though not necessarily proves, that the rigidity of the hydrophobic domain is maintained by an intermolecular hydrophobic interaction, which is responsible for the micelle formation. It is conceivable that unfolding of the hydrophobic domain occurs only when the micelle structure is destroyed and the hydrophobic domain is exposed to the aqueous phase. If this is correct, it may be assumed that the hydrophobic domain of microsomal bound cytochrome $\underline{b}_5$ is stabilized by its interaction with the apolar interior of the membrane matrix.

The conclusion drawn that the molecule of cytochrome $\underline{b}_5$ consists of two discrete domains that undergo denaturation independently is rather unique, since denaturation of most single-chain proteins is thought to be a one-stage, cooperative process (26). However, the two-domain structure is not restricted to cytochrome $\underline{b}_5$. for example, CD (27,28) as well as X-ray crystallographic (29,30) studies of Bence Jones proteins have shown that the constant and

variable halves of these proteins exist as independently folded regions. Azuma *et al.*(31,32) have further reported that the structural transition of Bence Jones proteins induced by GuHCl and urea occurs in two stages corresponding to the unfolding of the constant half and that of the variable half of the molecule.

Nothing is as yet understood of the functional implications of the two-domain structure of cytochrome b_5, however, this two-domain structure is shared by at least one other membrane protein, *i.e.*, NADH-cytochrome b_5 reductase of liver microsomes, undergoes two-stage denaturation as a function of GuHCl concentration, as will be reported in Part III.

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PART II

NATURE OF TRYPSIN ATTACK ON CYTOCHROME b_5 AND FURTHER EVIDENCE FOR TWO-DOMAIN STRUCTURE OF THE CYTOCHROME MOLECULE

SUMMARY

Rabbit cytochrome $\underline{b}_5$ was incorporated into single-walled phosphatidylcholine liposomes, and the cytochrome $\underline{b}_5$-liposome complex thus formed was digested with trypsin. Protein chemical characterization indicated that the main products formed were 1) a hydrophilic (heme-containing) fragment of the cytochrome corresponding to the sequence consisting of the masked NH_2-terminus through residue 88, 2) a hydrophobic peptide which spans residue 91 to the COOH-terminus, and 3) a dipeptide, seryl-lysine, derived from residues 89 and 90. The hydrophobic peptide was obtained in the form of its complex with liposome. It was concluded that trypsin cleaved rather specifically the peptide bonds between residues 88 and 89 (Arg-Ser) and between residues 90 and 91 (Lys-Leu), although some other bonds such as that between residues 93 and 94 (Lys-Pro) were also attacked to lesser extents. Tryptic digestion of free cytochrome $\underline{b}_5$ also resulted in the cleavage of the same peptide bonds. The circular dichroism spectrum of intact cytochrome $\underline{b}_5$ (or its complex with liposomes) in the far ultraviolet region was closely similar in both shape and magnitude to the sum of spectra of the hydrophilic fragment and the hydrophobic peptide (or its complex with liposomes). This indicates that the tryptic cleavage of the cytochrome molecule does not induce any significant changes in the conformations of the hydrophilic and hydrophobic domains of the molecule and thus provide further evidence that the three-dimensional structures of the two domains are independent from each other.

INTRODUCTION

Cytochrome $\underline{b}_5$ is a typical membrane protein and has been purified from liver microsomes of several species of animals (1-4). It is composed of a single peptide chain and is folded in such a way that the molecule has both a hydrophilic moiety to which protoheme is noncovalently attached and a hydrophobic peptide segment (1-3). Available evidence indicates that the hydrophobic segment is required for the cytochrome to bind tightly to various natural and artificial membranes (1-3, 5-10, 41-43). Digestion of microsomal membranes or purified cytochrome $\underline{b}_5$ with proteases such as trypsin results in the cleavage of the cytochrome molecule at the junction between the hydrophilic and hydrophobic regions, leading to the liberation of a hydrophilic, heme-containing fragment (1-3, 11-15), and this fragment has been shown to have been derived from the NH_2-terminal portion of the parent molecule (4). The primary structures of such fragments obtained from 7 species of animals have been determined (16-19), and the complete sequences including the COOH-terminal hydrophobic segment of porcine and equine cytochrome $\underline{b}_5$ have recently been elucidated (20, 21).

Visser *et al.* (22) have reported that the hydrophilic and hydrophobic moieties of cytochrome $\underline{b}_5$ exist as two globular domains which are connected to each other by a helix-rich link peptide consisting of 15 amino acid residues. From studies of the denaturation of rabbit cytochrome $\underline{b}_5$ by guanidine hydrochloride, we have also provided evidence that the two domains of the cytochrome molecule are folded independently from each other (23).

In preliminary experiments, however, it was not possible for us to isolate the link peptide from the tryptic digest of rabbit cytochrome $\underline{b}_5$. Therefore, we decided to perform a detailed study of the products formed from the rabbit protein by tryptic digestion to elucidate the possible importance of the link peptide in the structure and function of cytochrome $\underline{b}_5$.

In this paper, we report that rabbit cytochrome $\underline{b}_5$ which has been incorporated into phosphatidylcholine liposome is cleaved by trypsin to form three main products, i.e. a hydrophilic, heme-containing fragment (NH_2-terminus through residue 88) a hydrophobic peptide in association with liposome (residue 91 through COOH-terminus), and a dipeptide derived from residues 89 and 90. The same main products have been formed upon tryptic digestion of free, unbound cytochrome $\underline{b}_5$ and no peptide having 15 amino acid residues has been detectable. Although we have not studied porcine cytochrome $\underline{b}_5$, the sequence data available for this protein (18, 20) predicts that trypsin would cleave it at the same sites as in the case of the rabbit protein. The presence of link peptide reported by Visser et al.(22) has, therefore, to be reexamined. This paper also reports that the circular dichroism (CD) spectrum of intact cytochrome $\underline{b}_5$ in the far ultraviolet region closely resembles the sum of spectra of the hydrophilic and hydrophobic fragments of the cytochrome, a finding which provides further evidence for the independently folded two-domain structure of cytochrome $\underline{b}_5$ (23).

MATERIALS AND METHODS

Materials. Cytochrome b_5 was purified from rabbit liver microsomes by the method of Spatz and Strittmatter (3); it was homogeneous when subjected to polyacrylamide gel electrophoresis (20% cross-linked gel) in the presence of sodium dodecyl sulfate and urea by the method of Hinman and Philips (24). Egg-yolk phosphatidylcholine was prepared as described by Singleton et al. (25); its purity was confirmed by thin-layer chromatography. Single-walled liposomes of phosphatidylcholine were prepared by the method of Huang (26). Bovine pancreatic trypsin (Type I) and soybean trypsin inhibitor (Type II) were obtained from Sigma, and yeast carboxypeptidase Y from Oriental Yeast Company, Tokyo. Sodium dodecyl sulfate (sequanal grade) and sodium deoxycholate were purchased from Pierce Chemical Company and Difco, respectively. Anhydrous hydrazine was prepared from a commercial product by distillation of the azeotropic mixture with toluene over calcium oxide (27). Guanidine hydrochloride of guaranteed reagent grade was purchased from Nakarai Chemical Company, Kyoto, and recrystallized as described by Nozaki and Tanford (28). Sephadex gels and polyamide sheets were purchased from Pharmasia Fine Chemicals and Chen Chin Trading Company, respectively. The other chemicals used were of the highest quality available.

Preparation of Cytochrome b_5-Liposome Complex. A solution (1.0 ml) containing 250 μM cytochrome b_5, 2.1-2.5 mM single-walled phosphatidylcholine liposomes, 50 mM Tris-HCl buffer (pH 8.0), and 1 mM EDTA was incubated at 0° for 20-24 h. The mixture was then layered over 30 ml of a linear sucrose concentration gradient (5-40%, w/v) containing 50 mM Tris-HCl buffer (pH 8.0)

and 1 mM EDTA and centrifuged at 23,000 rpm at 2° for 24 h in an SW 25.1 rotor of a Beckman L-2 centrifuge or in an RPS 25 rotor of a Hitachi 55P centrifuge. After centrifugation, 10-drop fractions were collected from the bottom of the tube and assayed for cytochrome $\underline{b}_5$. The sucrose concentration of each fraction was determined by using an Abbe refractometer. Of the two red bands formed, the lower one located at about 25% sucrose contained the liposomes to which cytochrome $\underline{b}_5$ had bound ("cytochrome $\underline{b}_5$-liposome complex") and the upper one at about 11% sucrose unbound cytochrome $\underline{b}_5$. Occasionally a faint red band was formed between the two red bands at 19-20% sucrose, but its nature was not studied. Usually about 60% of the cytochrome added was recovered in the complex. The fractions containing the complex were combined and dialyzed against 10 mM sodium phosphate buffer (pH 7.5) at 4° for more than 16 h. For determination of phospholipid phosphorous, however, dialysis was carried out against 10 mM Tris-HCl buffer (pH 8.0).

Tryptic Digestion of Cytochrome $\underline{b}_5$-Liposome Complex. The cytochrome $\underline{b}_5$-liposome complex, which had been dialyzed against 10 mM sodium phosphate buffer (pH 7.5), was concentrated to about 2oo uM with respect to the cytochrome with the aid of an Amicon 8 MC micro-filtration system using an XM 50 membrane. Trypsin, dissolved in 2 mM HCl, was added to the concentrated solution to a trypsin to cytochrome $\underline{b}_5$ ratio of 1:100 (w/w), and the mixture was incubated at 0° for 20-24 h. The digestion was stopped by adding an amount of trypsin inhibitor equivalent to that of trypsin and the mixture was allowed to stand at 0° for 2-3 h. The mixture was then applied to a Sephadex G-75 (or G-100)

column of an appropriate size which had been equilibrated with 10 mM sodium phosphate buffer (pH 7.5) and elution was conducted with the same buffer. A colorless protein was eluted at the void volume, and a red band containing cytochrome $\underline{b}_5$ chromophore was eluted thereafter. The former was the hydrophobic tail of the cytochrome still in association with the liposome ("tail-liposome complex"), and the latter was the hydrophilic, heme-containing fragment ("hydrophilic fragment "). The fractions containing the tail-liposome complex and hydrophilic fragment were separately combined. When necessary, the combined fractions were passed through a Sephadex G-25 (coarse) column equilibrated with 0.1 M ammonium bicarbonate, and the eluted samples were lyophilized.

Tryptic Digestion of Free Cytochrome $\underline{b}_5$. To a solution containing 250-400 μM cytochrome $\underline{b}_5$, 20 mM Tris-acetate buffer (pH 8.1), and 0.2 mM EDTA was added trypsin (dissolved in 2 mM HCl) to a trypsin to cytochrome $\underline{b}_5$ ratio of 1:100 (w/w), and the mixture was allowed to stand at 0° for 20-24 h. The digestion was stopped with trypsin inhibitor as described above. The digest was then applied to a Sephadex G-75 (or G-100) column of an appropriate size which had been equilibrated with 20 mM Tris-acetate buffer (pH 8.1) containing 0.2 mM EDTA, and elution was performed with the same buffer. A colorless protein was eluted at the void volume and identified as an aggregate of the hydrophobic fragment of the cytochrome. The hydrophilic, heme-containing fragment was eluted as a red band at a later stage and used as such for characterization. The fractions containing the hydrophobic fragment were combined, lyophilized, dissolved in 20 mM Tris-acetate buffer (pH 8.1) containing 0.2 mM EDTA and 0.4%

sodium deoxycholate (w/v), and subjected to a second gel chromatography on a Sephadex G-75 (or G-100) column by using the Tris-acetate-deoxycholate buffer as equilibration and elution medium.

Isolation of Small Peptides from Tryptic Digest of Cytochrome b_5-Liposome Complex. The cytochrome b_5-liposome complex containing about 5 μmoles of the cytochrome was digested with trypsin and the digest was subjected to Sephadex G-75 (or G-100) gel chromatography as described above. After elution of the tail-liposome complex and hydrophilic fragment, elution was further continued until fractions absorbing at 240 nm were obtained. These fractions were combined and lyophilized. The lyophilized sample was dissolved in 0.1 M ammonium bicarbonate and applied to a Sephadex G-15 column (1.9 x 100 cm) equilibrated with o.1 M ammonium bicarbonate. Elution was conducted with the same solution and 5-ml fractions were collected. Materials absorbing at 230 nm were thereby eluted as shown in Fig. 5. Four portions (Fractions 1 through 4) were separately collected as indicated in Fig. 5 and concentrated by lyophilization. The lyophilized samples were then subjected to high-voltage paper electrophoresis at pH 3.4 by using pyridine-acetic acid-water (1:10:289, v/v/v) as solvent as described by Iwanaga et al.(29). The instrument used and details of the operation were described by Takahashi et al. (30). Peptide spots were located by spraying a buffered ninhydrin reagent (31) or 0.001% fluorescamine. Each spot was eluted from the paper with 0.01 N NH_4OH, lyophilized, and subjected to amino acid analysis.

Analytical Methods. Phospholipid phosphorous was determined by the method of Bartlette (32) after digestion with sulfuric

acid and H_2O_2. Cytochrome b_5 and its hydrophilic fragment were determined from the intensity of the oxidized Soret peak at 413 nm, assuming a molar extinction coefficient of 1.17×10^5 (3,11). The hydrophobic fragment of cytochrome b_5 was determined from the absorbance at 280 nm, assuming a molar extinction coefficient of 1.93×10^4. This value was obtained from the fact that the hydrophobic fragment contains 2 tyrosines and 3 tryptophans (cf. Table I) and that tyrosine and tryptophan residues have molar extinction coefficients of 1340 and 5550, respectively, at 280 nm (33). The cytochrome b_5 in the cytochrome-liposome complex had practically the same molar extinction coefficient (error, within 5%) at 413 nm as the free cytochrome, when the former was estimated by reduced minus oxidized difference spectrophotometry. The protein content in the tail-liposome complex was directly measured by the method of Lowry et al. (34), using the hydrophobic fragment of the cytochrome as a standard. All the optical absorbance measurements were carried out at room temperature in a Cary 14 (Hitachi 323) spectrophotometers using cells of 10 or 2 mm path.

Amino Acid Analyses. Samples (0.01-0.06 μmole) were hydrolyzed at 110° for 24, 48, and 72 h in an evacuated (0.1 mm Hg), sealed tube containing 3.0 ml of twice distilled 5.8 N constant-boiling HCl. After the hydrolysis the acid was removed by evaporation under reduced pressure (0.001 mm Hg) in a desiccator containing KOH pellets. All the analyses were performed in a JOEL model JLC-5AH amino acid analyzer by the method of Spackman et al. (35). Tryptophan contents in the hydrophobic fragment and tail-liposome complex were calculated from the

tryptophan/tyrosine ratios, which were determined from the absorption spectra of the hydrophobic fragment and delipidated tail-liposome complex dissolved in neutral and alkaline guanidine hydrochloride solutions as described by Edelhoch (36). Delipidation was carried out with 90% acetone. Tryptophan contents in cytochrome $\underline{b}_5$ and its hydrophilic fragment were determined by measuring the fluorescence of tryptophan residues in 6 M guanidine hydrochloride as described by Pajot (37).

$\underline{NH_2}$-Terminal Analyses. The sequences of NH_2-terminal region of the hydrophilic fragments of cytochrome $\underline{b}_5$ and of the tail-liposome complex were determined by manual Edman degradation (38). Edman degradation of tail-liposome complex was performed directly or after delipidation with 90% acetone. The phenylisothiohydantoin-derivatives obtained were determined semi-quantitatively by measuring the absorbance at 269 nm (39) and quantitatively by gas-liquid chromatography (40). The other details of Edman sequence analysis including the identifications of the phenylisothiohydantoin-derivatives were performed as described by Takahashi et al. (30).

COOH-Terminal Analyses. The COOH-terminal residues of cytochrome $\underline{b}_5$, and the tail-liposome complex were identified by hydrazinolysis at 80° for 18 h (4) as described by Schroeder (44), and the recovery of released amino acid was determined by using the amino acid analyzer. Yeast carboxypeptidase Y was also used for COOH-terminal analysis. To a solution containing 130-300 μM cytochrome $\underline{b}_5$, its hydrophilic, or the tail-liposome complex, 0.1 M pyridine-acetate buffer (pH 5.5), and 0.2% sodium dodecyl sulfate (w/v) was added carboxypeptidase Y to a molar peptidase:

sample ratio of 1:600, and the mixture was incubated at 30°. At appropriate time intervals, small portions of the mixture were withdrawn and heated in a boiling water bath for 5 min to stop the digestion. The samples were then lyophilized and the amino acids released were analyzed by using the amino acid analyzer.

CD Measurements. CD measurements were carried out at 20-25° in a JASCO J-20 recording spectropolarimeter equipped with a CD attachment, using quartz cells of 1 mm path. The concentration of the sample was 7-15 μM. The results were expressed in terms of both molar ellipticity, [θ], and mean residue molar ellipticity, $[\theta]_R$. The latter was calculated by using the values of 114.5, 114.9, and 114.4 for the average residue molecular weights of cytochrome b_5, the hydrophilic fragment, and the hydrophobic fragment (tail-liposome complex), respectively. These values were obtained from the amino acid composition data reported in Table I.

Electron Microscopy. The cytochrome b_5- or tail-liposome complexes dissolved in 10 mM sodium phosphate buffer (pH 7.5) was placed on a specimen mesh coated with a collodion film. The samples was then stained with 1% phosphotungstic acid (pH 7.0) containing 0.01% bovine serum albumin and observed in a Hitachi HU-12 electron microscope.

RESULTS

Cytochrome b_5-Liposome Complex and Its Tryptic Digestion. When rabbit cytochrome b_5 was incubated with single-walled

phosphatidylcholine liposomes under the conditions described in "Materials and Methods", about 60% of the cytochrome added formed a complex with liposomes. The cytochrome $\underline{b}_5$-liposome complex thus formed had a buoyant density of about 1.16 in a sucrose density gradient and could be easily separated from the unbound cytochrome by the density gradient centrifugation (45). From the centrifugation pattern obtained it appeared that the complex was homogeneous with respect to buoyant density (45). The isolated complex contained 1 mole of cytochrome $\underline{b}_5$ per 10-11 moles of phosphatidylcholine. Many vesicular profiles having diameters of 300-600 Å were seen in negative staining electron micrographs of the complex preparation, and the surface of the vesicles was distinctly rough (Fig. 1). The rough appearance seemed to be due to the hydrophilic head of the bound cytochrome molecules, because it has been shown that cytochrome $\underline{b}_5$ binds to membranes by inserting its hydrophobic tail into the apolar interior of the membrane matrix (41-43). When this complex was treated with trypsin and the digest was subjected to Sephadex G-75 (or G-100) gel chromatography as described in "Materials and Methods", a fraction containing colorless protein was eluted at the void volume and a red fraction was eluted at a later stage, as shown in Fig. 2. The absorption spectrum of the latter fraction was that of cytochrome $\underline{b}_5$ and it was concluded that this fraction contained a hydrophilic fragment of the cytochrome. The former fraction was, therefore, assumed to be the hydrophobic fragment that was still associated with liposomes (tail-liposome complex). In fact, negative

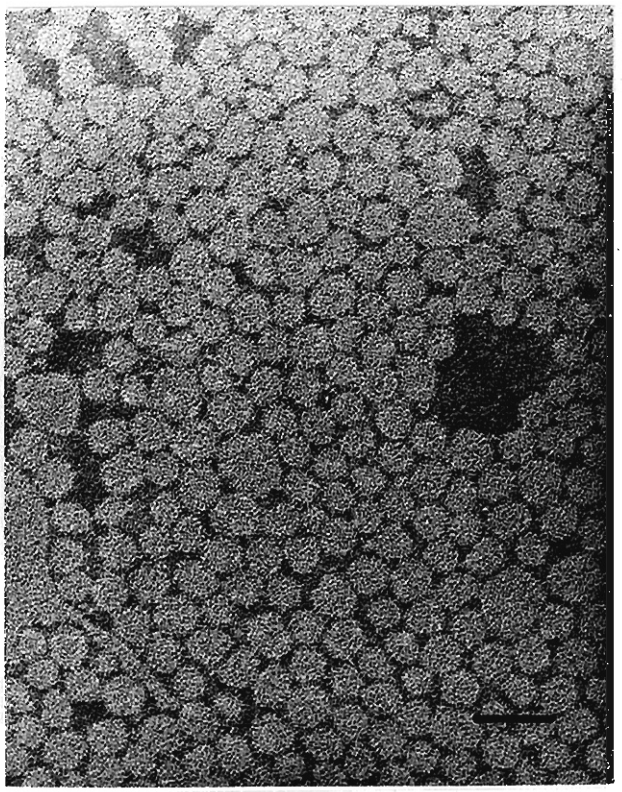

Fig. 1. Negative-staining electron micrograph of the cytochrome b_5-liposome complex. The complex prepared as described in Materials and Methods was dialyzed against 10 mM sodium phosphate buffer (pH 7.5), stained with 1% phosphotungstic acid (pH 7.0) containing 0.01% bovine serum albumin, and observed in a Hitachi HU-12 electron microscope. The final magnification is x 100,000 and the bar is equivalent to 1000 Å.

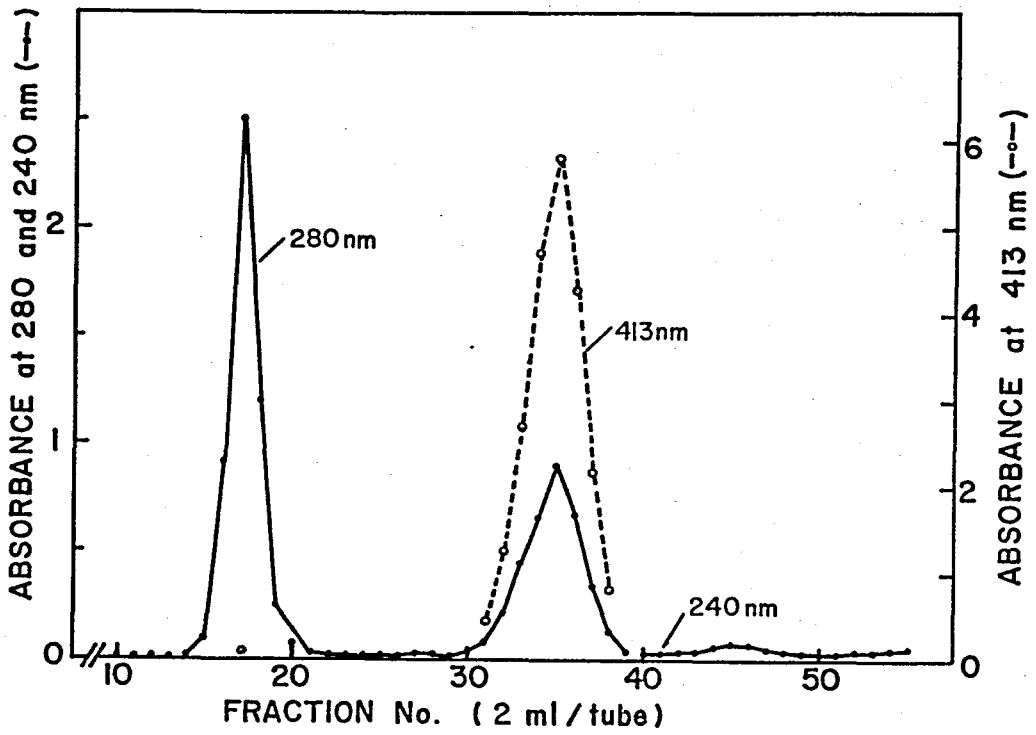

Fig. 2. Sephadex G-100 gel chromatography of trypsin-treated mixture of cytochrome $\underline{b}_5$-liposome complex. The cytochrome $\underline{b}_5$-liposome complex was digested with trypsin as described in Materials and Methods and applied to a Sephadex G-100 column (1.6 x 51 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.5). Absorbance at 280, 240 nm (-●-) and 413 nm (-○-) were measured and plotted. The fractions containing the tail-liposome complex and hydrophilic fragment were collected separately.

staining electron microscopy showed that this fraction contained numerous vesicular structures, the diameter of which was similar to that of the original cytochrome b_5-liposome complex (Fig. 3). No cytochrome b_5 chromophore could be detected in the tail-liposome complex fraction, indicating that all the cytochrome molecules in the original complex had been cleaved by trypsin. This in turn indicated that the site of tryptic cleavage in all the cytochrome molecule is located outside of the liposomal vesicles, because it is highly unlikely that the vesicular membrane is permeable to high-molecular compounds such as trypsin.

Amino Acid Compositions of the Products. To characterize the two products obtained by tryptic digestion of the cytochrome b_5-liposome complex, their amino acid compositions as well as that of intact cytochrome b_5 were determined and the results are summarized in Table I. The composition of the hydrophilic fragment determined here is in good agreement with that deduced from the primary structure reported for a heme-containing tryptic fragment of rabbit cytochrome b_5 (16-18), except that the proline content obtained in this study is somewhat higher. Since the sequence data indicates that 2 prolines are present in this fragment, this value rather than 3 (expected from the present result) was adopted to express the amino acid composition. The amino acid composition of intact rabbit cytochrome b_5 is, however, significantly different from that reported by Spatz and Strittmatter (3) for the same protein. While our data indicate that the cytochrome is composed of 133 amino acid residues, it should contain 145 residues according to their results. The reason for this dicrepancy is unclear, but it should be

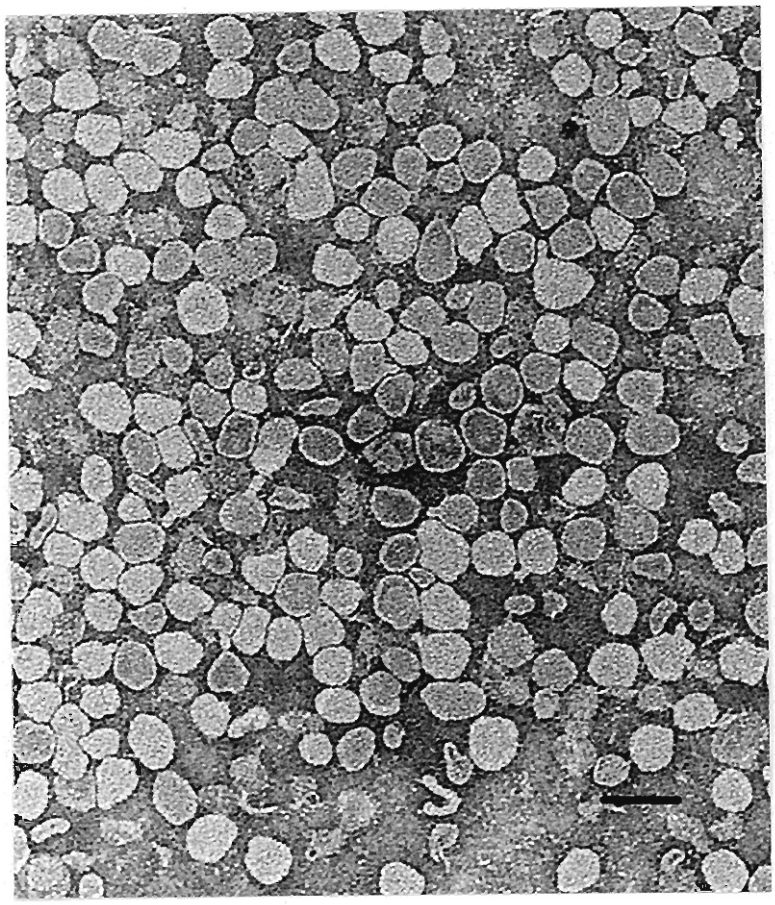

Fig. 3. Negative-staining electron micrograph of the tail-liposome complex. The cytochrome $\underline{b}_5$-liposome complex was digested with trypsin and the digest was subjected to Sephadex G-75 gel chromatography as described in Materials and Methods. The tail-liposome complex fractions were combined and observed in the electron microscope after staining with phosphotungstic acid. The final magnification is x100,000 and the bar is equivalent to 1000 Å.

Table I. Amino acid compositions of the liposome complex of rabbit cytochrome $\underline{b}_5$, its hydrophilic fragment, and the tail-liposome complex. Values of residues per mole were calculated by extrapolation to zero time hydrolysis or from average values obtained after hydrolysis for 24, 48, and 72 h for the cytochrome- and tail-liposome complexes; the values for the hydrophilic fragment was estimated based on the results obtained after 24 h hydrolysis only. The values represent residues per mole. Figures in parentheses indicate the nearest integers

Amino acid	A Hydrophilic fragment	B Tail-liposome complex	C A + B	D cytochrome $\underline{b}_5$-liposome complex
Lysine	8.1 (8)	1.0 (1)	9	10.1 (10)
Histidine	7.0 (7)	0.0 (0)	7	7.0 (7)
Arginine	3.0 (3)	1.0 (1)	4	4.1 (4)
Aspartic acid	10.1 (10)	4.6 (5)	15	14.3 (15)
Threonine	5.8 (6)	4.2 (4)	10	10.1 (10)
Serine	4.8 (5)	4.6 (5)	10	10.5 (11)
Glutamic acid	12.9 (13)	1.1 (1)	14	14.4 (14)
Proline	2.6*(2)*	2.1 (2)	4	4.4*(4)*
Glycine	5.9 (6)	0.2 (0)	6	6.3 (6)
Alanine	5.0 (5)	4.0 (4)	9	9.0 (9)
Half cystine	0.0 (0)	0.0 (0)	0	0.0 (0)
Valine	4.0 (4)	2.9 (3)	7	6.9 (7)
Methionine	0.0 (0)	2.6 (3)	3	2.5 (3)
Isoleucine	3.6 (4)	4.0 (4)	8	7.6 (8)
Leucine	8.1 (8)	5.1 (5)	13	13.0 (13)
Tyrosine	3.1 (3)	1.9 (2)	5	5.0 (5)
Phenylalanine	3.1 (3)	0.0 (0)	3	3.1 (3)
Tryptophan	1**	3**	4	4**
Total residues	88	43	131	133
Formula weight	10,107	4,919		15,223

*Since proline content obtained for the hydrophilic fragment was higher than that established by sequence data (18), the values established were adopted not only for the hydrophilic fragment but also for the cytochrome $\underline{b}_5$-liposome complex.

**Tryptophan contents were determined as described in Materials and Methods.

mentioned that recent sequence studies have established that both porcine and equine cytochrome $\underline{b}_5$'s are composed of 133 residues (20,21). The data for the tail-liposome complex show the presence of 0.2 mole of glycine per mole of the tail protein, but this was not considered to be significant. The sum of amino acid compositions of the hydrophilic fragment and the tail-liposome complex can account for that of intact cytochrome $\underline{b}_5$, except that one serine and one lysine are missing in the two fragments formed. This suggested that a dipeptide consisting of these amino acids had also been produced by tryptic digestion.

$\underline{NH_2}$- and COOH-Terminal Structures of the Products. Edman degradation of as much as about 0.3 umole of the hydrophilic fragment yielded no NH_2-terminal amino acid reactive with phenylisothiocyanate, indicating that the terminus was blocked. Since the masked NH_2-terminus has also been reported for intact cytochrome $\underline{b}_5$ (4), it was concluded that the NH_2-terminus of the intact molecule was preserved in the hydrophilic fragment. Manual Edman degradation of the tail-liposome complex, on the other hand, revealed an NH_2-terminal sequence of NH_2-Leu-(Ser)-Lys-Pro-Met-. This is identical with the sequence from residues 91 to 95 reported for a rabbit cytochrome $\underline{b}_5$ fragment extracted with crude pancreatic lipase (18), although in our hands the second residue (serine) could not be clearly identified. Edman degradation of the tail-liposome complex also gave another NH_2-terminal sequence, NH_2-Pro-Met-, in a much lower yield (less than 15% of that of the main sequence). It is likely that the component showing this NH_2-terminal sequence was produced from the main component by removal of the NH_2-terminal tripeptide,

Leu-Ser-Lys.

Hydrazinolysis of both intact cytochrome b_5 and tail-liposome complex yielded aspartic acid as the COOH-terminal residue in an yield of 0.3-0.5 mole per mole. The COOH-terminal structure of both samples was also studied by using carboxypeptidase Y. In Fig. 4 are compared the time courses of amino acid release from the intact cytochrome and tail-liposome complex by carboxypeptidase Y digestion; only those amino acids that emerged within the first 5 min of digestion are plotted for the sake of simplicity. As can be seen, the profiles of amino acid release from the two preparations were closely similar to each other, indicating that they possess the same COOH-terminal structure. Fig. 3 also shows that both preparations have a COOH-terminal amino acid of -Asp-COOH. Carboxypeptidase Y digestion of the hydrophilic fragment, on the other hand, released only insignificant amounts of serine and lysine even after incubation for 30 min in the presence of 0.2% sodium dodecyl sulfate. It seemed that the COOH-terminal residue of the hydrophilic fragment is resistant to the action of carboxypeptidase Y.

Small Peptides Produced by Tryptic Digestion. It was found that a fraction absorbing at 240 nm was eluted of the tail-liposome complex and hydrophilic fragment when the tryptic digest was subjected to Sephadex G-75 (or G-100) gel chromatography. This fraction obtained from the complex containing about 0.5 μmole of cytochrome b_5 was further subjected to gel chromatography on a Sephadex G-15 column. The elution profile in this chromatography is shown in Fig. 5. Four different portions of the 230

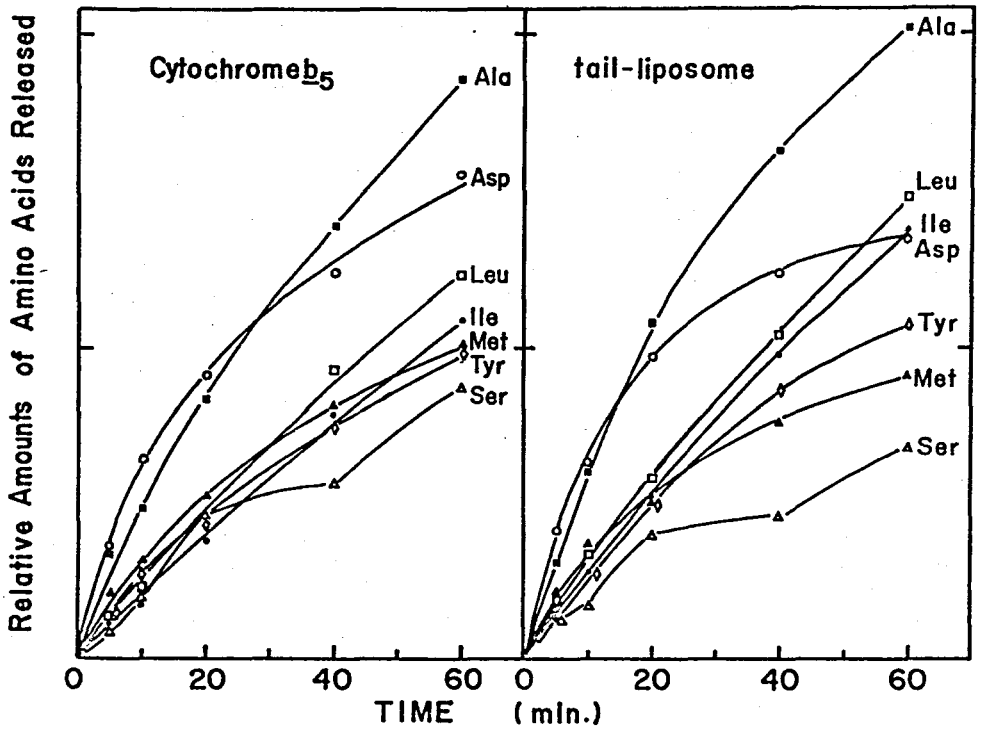

Fig. 4. Time courses of amino acid release from the cytochrome b_5- and tail-liposome complexes by the action of carboxypeptidase Y. The carboxypeptidase Y digestion was carried out as described in Materials and Methods. Only the release of those amino acids that emerged within the first 5 min of incubation are plotted.

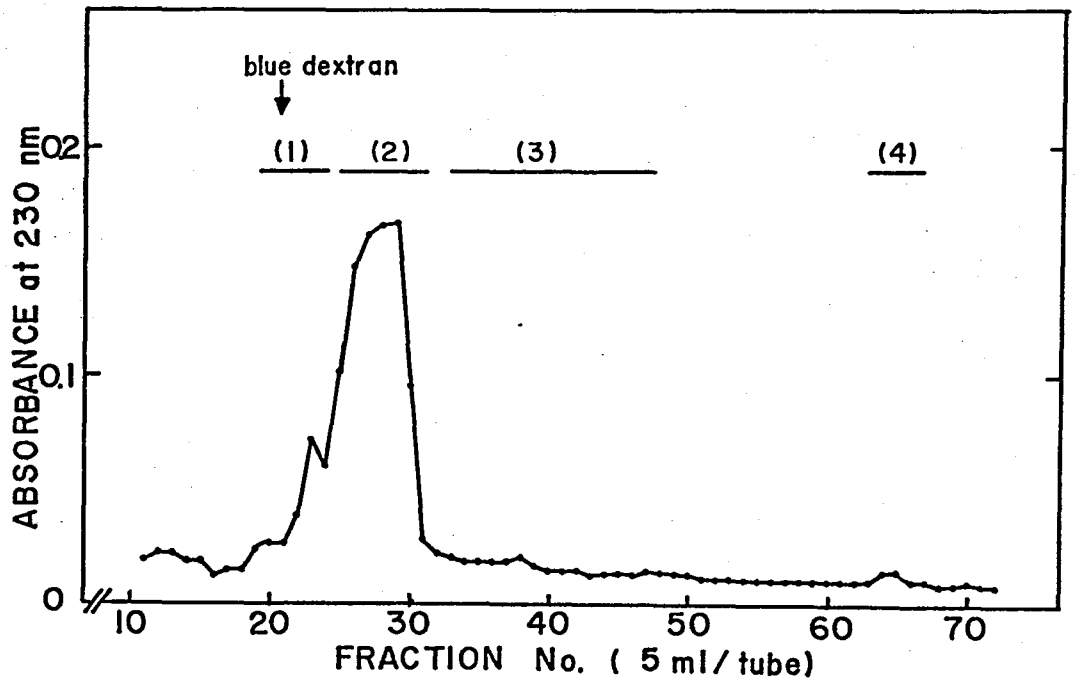

Fig. 5. Sephadex G-15 gel chromatography of the small peptide fraction produced from the cytochrome $\underline{b}_5$-liposome complex by tryptic digestion. The isolation of the small peptide fraction and its Sephadex G-75 (or G-100) gel chromatography were conducted as described in Materials and Methods. Four portions of the 230 nm-absorbing eluates, indicated as (1) through (4) in the figure, were collected separately, lyophilized, and subjected to high-voltage paper electrophoresis.

nm-absorbing eluates called Fraction 1 through 4 were separately pooled as indicated in Fig. 5, lyophilized, and subjected to high-voltage paper electrophoresis. Only Fractions 1 and 2 contained significant amounts of peptides; only a trace amount was detected in Fraction 3 and none in Fraction 4. Fraction 2 contained three ninhydrin- and fluorescamin-positive peptides called Peptide I, II and III, whereas only one peptide identical with Peptide III was present in Fraction 1. Among these peptides, Peptide I was the major component; the yield of Peptide II and III was less than 20% of that of the major peptide. Amino acid analysis indicated that Peptide I was a dipeptide consisting of serine and lysine, as shown in Table II. Peptide II contained one residue each of leucine, serine, and lysine, whereas 8 different amino acids were contained in Peptide III.

Peptide Bonds Cleaved by Tryptic Digestion. From the results described above it was possible to identify the peptide bonds which had been cleaved during tryptic digestion of the cytochrome b_5-liposome complex. One of the main products formed was a hydrophilic, heme-containing fragment of the cytochrome consisting of 88 amino acid residues (Table I), and its NH_2-terminal residue was blocked as in the case of the intact molecule (4). It could, therefore, be reasonably concluded that this fragment corresponds to the NH_2-terminal portion of the intact cytochrome extending from the masked NH_2-terminus to residue 88 which has been shown to be arginine (18). Another main product was the tail-liposome complex, the tail moiety of which had the same COOH-terminal structure as the intact molecule (-Asp-COOH, cf. Fig. 4). Most of the tail protein was found to possess an NH_2-terminal sequence

Table II. Amino acid compositions of small peptides isolated from tryptic digest of the cytochrome $\underline{b}_5$-liposome complex. Peptides were isolated as described in Materials and Methods. Acid hydrolysis of the samples was carried out only for 24 h. The data for Peptide I and II are expressed assuming that each peptide contained 1 residue of lysine, and for Peptide III the presence of 1 residue of serine is assumed. Figures in parentheses represent the nearest integers.

Amino acid	Peptide I	Peptide II	Peptide III
Lysine	1.0 (1)	1.0 (1)	0.6
Aspartic acid	-	-	2.0 (2)
Serine	0.8 (1)	0.9 (1)	1.0 (1)
Glycine	-	-	0.6
Alanine	-	-	1.4 (1)
Methionine	-	-	1.1 (1)
Leucine	-	0.9 (1)	0.8 (1)
Tyrosine	-	-	0.8 (1)

of NH_2-Leu-Ser-Lys-Pro-Met-, a sequence which is identical with that from residues 91 to 95 reported for a hydrophilic fragment of rabbit cytochrome $\underline{b}_5$ prepared after solubilization with crude pancreatic lipase (18). It was thus clear that the main peptide component of the tail-liposome complex fraction is a hydrophobic segment which spans residue 91 (leucine) to the COOH-terminal residue (aspartic acid, residue 133). These considerations led us to conclude that trypsin hydrolized rather specifically the peptide bonds between residues 88 and 89 and between residues 90 and 91 of the liposome-bound cytochrome $\underline{b}_5$ molecule. If this is so, then it is expected that a dipeptide derived from residues 89 and 90 (serine and lysine, respectively, according to ref. 18) must also be formed as a main product. Such a peptide could actually be detected in the tryptic digest in a relatively large quantity (Table II).

Although the two bonds, 88-89 and 90-91, were the main sites of tryptic attack on the cytochrome $\underline{b}_5$-liposome complex, the results presented above indicated that at least two other bonds were also cleaved to lesser extents. A small fraction of the hydrophobic peptide in the tail-liposome complex was found to have an NH_2-terminal sequence of NH_2-Pro-Met- instead of NH_2-Leu-Ser-Lys-Pro-Met-, suggesting that this minor component had been formed from the main component by removal of the NH_2-terminal tripeptide, Leu-Ser-Lys. This was actually supported by the detection of a small amount of a tripeptide consisting these three amino acids in the digest (Table II). It could, therefore, be concluded that the bond between residues 93 (lysine) and 94 (proline) had also been cleaved to a slight extent. Another

minor peptide detected (Peptide III) contained two residues of aspartic acid, one residue each of serine, alanine, methionine, leucine, and tyrosine, and smaller amounts of glycine and lysine (Table II). Assuming that the presence of glycine and lysine was insignificant, the composition of this peptide was similar to that of the COOH-terminal portion of the cytochrome released by carboxypeptidase Y (cf. Fig. 3), and also to the expected COOH-terminal sequence, -Met-Tyr-Arg-Leu-Tyr-Met-Ala-Asp-Asp-COOH, which was estimated from amino acid compositions of two peptides produced by cyanogen bromide cleavage of hydrophobic fragment (46). The possibility was, therefore, suggested that Peptide III had been derived from the COOH-terminal end of the cytochrome molecule. However, another possibility was that Peptide III was still a mixture of two or more peptides, because it remained near the origin upon high-voltage paper electrophoresis.

Products Formed from Free, Unbound Cytochrome b_5 by Tryptic Digestion. In the experiments so far described, cytochrome b_5 complex with liposomes was subjected to tryptic digestion to protect the hydrophobic tail of the cytochrome from tryptic attack. To see if the hydrophobic tail would be further cleaved in the unprotected form, free cytochrome b_5 was digested with trypsin under similar conditions. In this case, too, the main products formed were a hydrophilic, heme-containing fragment and a hydrophobic peptide (not in association with liposomes). The amino acid compositions of these products were found to be identical with those of the corresponding fragments obtained from the cytochrome-liposome complex (data not shown). Furtheremore,

the COOH-terminal sequence of the hydrophobic peptide was estimated to be -Met-Ala-Asp-Asp-COOH (46). Edman degradation of the hydrophobic peptide yielded NH_2-terminal sequence, NH_2-Leu-Ser-Lys-Pro-Met- (46). These results offered rather conclusive evidence that the same peptide bonds in free cytochrome b_5 had been hydrolyzed by trypsin as in the case of the cytochrome b_5-liposome complex, although no attempts were made to isolate small peptides produced from the free cytochrome.

Far Ultraviolet CD Spectra of Cytochrome b_5 and Its Tryptic Products. Fig. 6 shows the far ultraviolet CD spectra of intact cytochrome b_5 and its fragments produced by tryptic digestion. The spectra were measured in the presence of 0.4% sodium deoxycholate to facilitate the dispersion of the hydrophobic fragment. The shapes of the CD spectra obtained were essentially similar to those reported by Visser et al. (22) for similar preparations of porcine cytochrome b_5. As shown in Fig. 6A, the magnitude of mean residue ellipticity of the hydrophobic fragment at the CD trough around 220 nm was much larger than that of the hydrophilic fragment. Fig. 6B shows, on the other hand, that the shape and magnitude of CD spectrum of intact cytochrome b_5, expressed in terms of molar ellipticity, was similar to, though not identical with, the sum of spectra of the hydrophilic and hydrophobic fragments as well as the spectrum of the whole tryptic digest of cytochrome b_5. This indicated that the hydrophilic and hydrophobic regions of the cytochrome maintained their respective three-dimensional structures even after tryptic cleavage of the molecule and thus provided further evidence for the view that the two regions of the cytochrome molecule exist as discrete domains

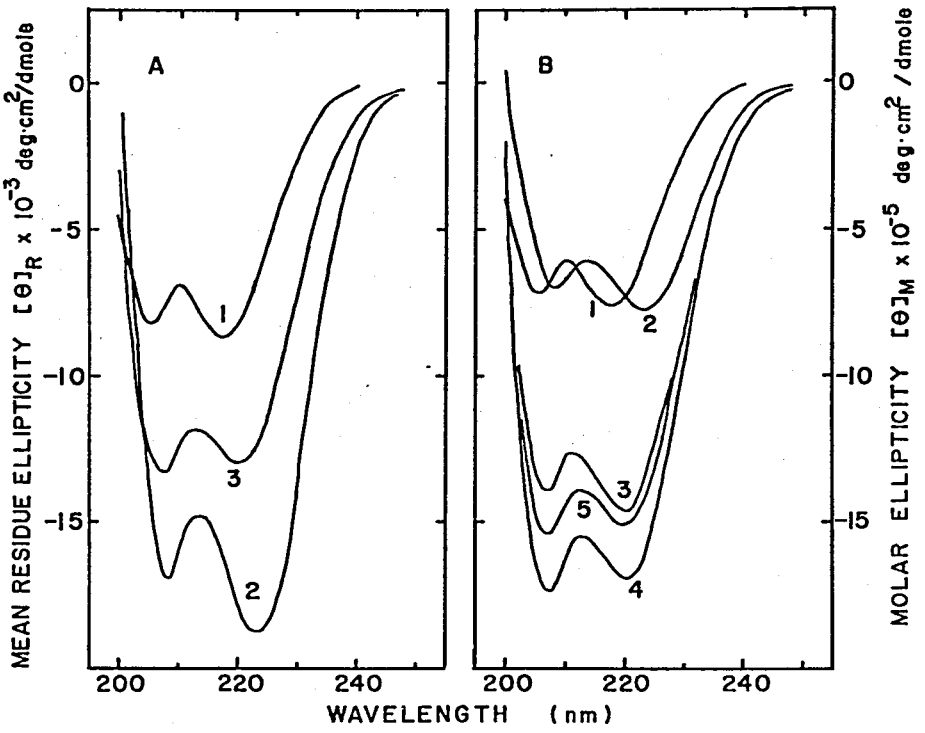

Fig. 6. Far ultraviolet CD spectra of cytochrome $\underline{b}_5$ and its tryptic fragments. Measurements were performed at 20-25° in a medium containing 20 mM Tris-acetate buffer (pH 8.1), 0.2 mM EDTA, and 0.4% (w/v) sodium deoxycholate. The concentrations of cytochrome $\underline{b}_5$, the hydrophilic fragment, and the hydrophobic fragment were 7.6, 11.3, and 14.2 μM, respectively. Values of mean residue ellipticities ($[\theta]_R$) and molar ellipticities ($[\theta]$) were calculated and plotted against wavelength. A, plots of $[\theta]_R$; Curve 1, hydrophilic fragment; Curve 2, hydrophobic fragment; Curve 3, intact cytochrome $\underline{b}_5$. B, plots of $[\theta]$: Curve 1, hydrophilic fragment; Curve 2, hydrophobic fragment; Curve 3, sum of the spectra of hydrophilic and hydrophobic fragments; Curve 4, intact cytochrome $\underline{b}_5$; Curve 5, whole tryptic digest of cytochrome $\underline{b}_5$.

having almost independent conformations (22). The small differences noted between the spectrum of intact cytochrome $\underline{b}_5$ and the sum of spectra of the fragments, however, suggested that a slight structural change had been induced by tryptic cleavage in either of the fragments or in both. The results of similar CD studies on the cytochrome $\underline{b}_5$-liposome complex and its tryptic fragments are shown in Fig. 7. The CD spectra of both the cytochrome $\underline{b}_5$- and tail-liposome complexes, but not the hydrophilic fragment, were significantly reduced in magnitude (especially in the wavelength region near 200 nm) as compared with those of cytochrome $\underline{b}_5$ and its hydrophobic tail (Fig. 6). It was likely that this reduction of CD intensity was due to the scattering and absorption statistics effects caused by liposomes (47); phosphatidylcholine liposomes themselves showed practically no CD spectrum in this wavelength region. In any way, the CD spectrum of the cytochrome $\underline{b}_5$-liposome complex, expressed in terms of molar ellipticity, was again similar to the sum of CD spectra of the hydrophilic fragment and tail-liposome complex (Fig. 7B). It also resembled the spectrum of the whole tryptic digest of the cytochrome $\underline{b}_5$-liposome complex. The two domains of the cytochrome, therefore, seemed to retain their independent three-dimensional structures even when the cytochrome was bound by liposomes.

DISCUSSION

The results reported above indicate, at least under the digestion conditions employed in this study, trypsin hydrolyzes rather specifically the peptide bonds between residues 88 (arginine)

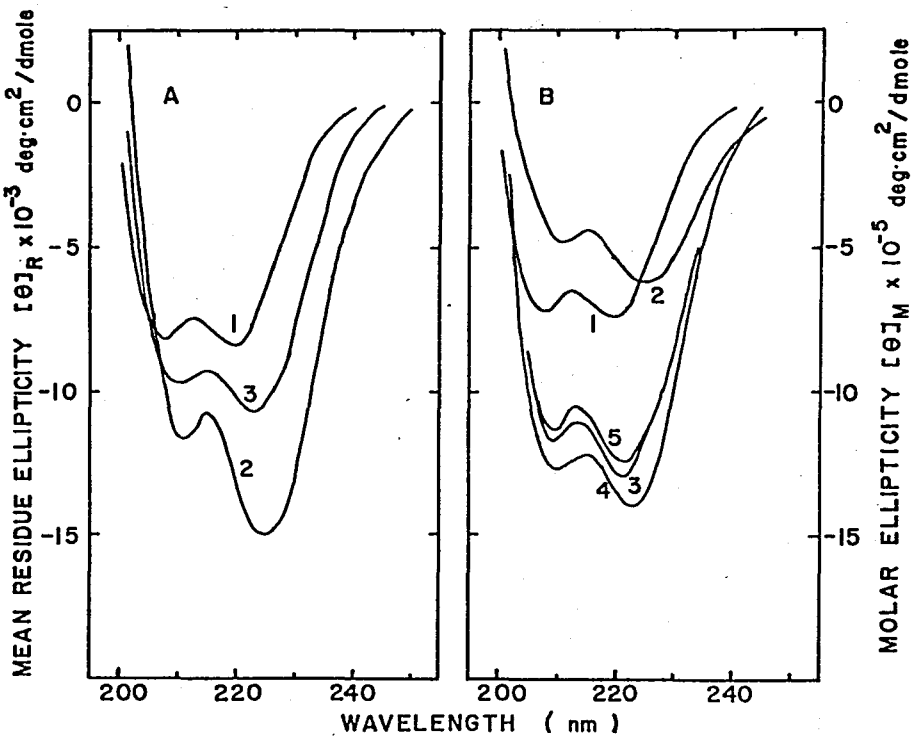

Fig. 7. Far ultraviolet CD spectra of the cytochrome b_5-liposome complex and its tryptic fragments. Measurements were performed as described in Fig. 6, except that 10 mM sodium phosphate buffer (pH 7.5) was used as the medium and the concentrations of the cytochrome b_5-liposome complex, hydrophilic fragment, and tail-liposome complex were 7.2, 9.7, and 11.3 μM, respectively. A, plots of $[\theta]_R$: Curve 1, hydrophilic fragment; Curve 2, tail-liposome complex; Curve 3, cytochrome b_5-liposome complex. B, plots of $[\theta]$: Curve 1, hydrophilic fragment; Curve 2, tail-liposome complex; Curve 3, sum of the spectra of hydrophilic fragment and tail-liposome complex; Curve 4, cytochrome b_5-liposome complex; Curve 5, whole tryptic digest of cytochrome b_5-liposome complex.

and 89 (serine) and between residues 90 (lysine) and 91 (leucine) of rabbit cytochrome $\underline{b}_5$ both in its free state or in its complex with phosphatidylcholine liposomes. The main products formed from the cytochrome are, therefore, 1) a hydrophilic, heme-containing fragment corresponding to the sequence from the masked NH_2-terminus to residue 88, 2) a hydrophobic fragment consisting of the COOH-terminal sequence starting from residue 91, and 3) a dipeptide, Ser-Lys, derived from residues 89 and 90. A small portion of the hydrophobic fragment is further convertible to a somewhat shorter peptide by hydrolysis of the bond between residues 93 (lysine) and 94 (proline), resulting in the liberation of a tripeptide, Leu-Ser-Lys, from the NH_2-terminus of the fragment. At least in the case of tryptic digestion of cytochrome $\underline{b}_5$ complex with liposomes, a small amount of a peptide (Peptide III in Table II) is also formed and it is suggested, though not proved, that this peptide results from the hydrolysis of a certain bond near the COOH-terminal end of the cytochrome molecule. The sites of tryptic attack on rabbit cytochrome $\underline{b}_5$ can be illustrated as shown in Fig. 8.

As already mentioned, Visser *et al.* (22) reported that a helix-rich peptide consisting of some 15 residues is excised from porcine cytochrome $\underline{b}_5$ by tryptic digestion and suggested that this peptide segment constitutes a link between the two domains of the cytochrome molecule. Our results, however, indicate that no such a long peptide is present in the tryptic digest of rabbit cytochrome $\underline{b}_5$; instead a dipeptide, Ser-Lys, is formed as a main product in addition to the hydrophilic and hydrophobic fragments.

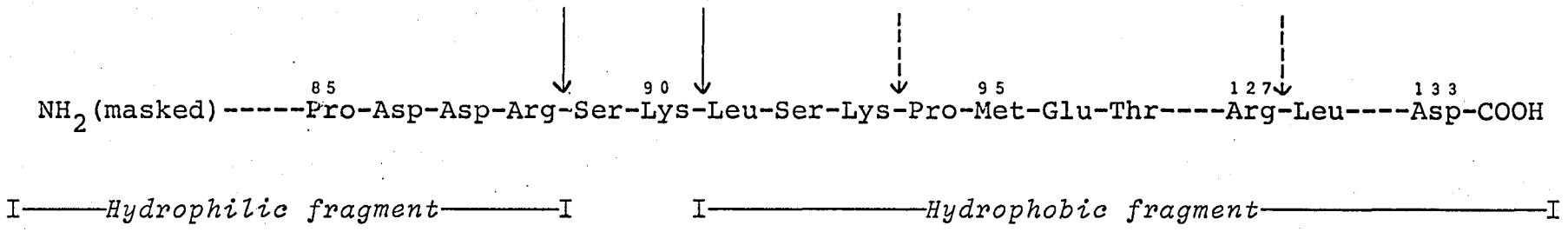

Fig. 8. Schematic illustration of the sites of tryptic attack on cytochrome b_5. The major (⟶) and minor (⇢) sites of attack are shown. The sequence from residue 85 to 97 are adopted from the data reported by Ozols (18), 127 and 128 residues, Arg and Leu, respectively, are from the personal communication by Kondo (46), and the COOH-terminal amino acid, Asp-COOH, was determined in this study (Fig. 4).

Although we have not studied porcine cytochrome b_5, the complete primary structure of this protein recently determined by Ozols and Gerard (20) shows that the porcine and rabbit proteins are very homologous to each other in the region located between the two domains. Both have an arginine residue at the 88th and lysine residues at the 90 and 93 positions. It is, therefore, highly likely that trypsin hydrolyzes the two proteins at the same sites. Moreover, a survey of the complete sequence of porcine cytochrome b_5 (18,20) indicates that no such a long peptide can be cut out with trypsin from the region linking the two domains. Therefore, the proposal by Visser *et al.* (22) has to be reexamined. However, it should be noted that the failure to detect the postulated peptide does not necessarily rule out the presence of a link peptide. The fact that tryptic attack on rabbit cytochrome b_5 takes place primarily in a very narrow portion of the sequence (between residues 88 and 93) (Fig. 8) suggests that this portion is not built in any rigid structures and therefore constitutes a link between the two regions. However, no data are available to estimate the length of the link segment.

Cytochrome b_5 has been shown to bind to various membranes by inserting its hydrophobic tail into the apolar interior of the membranes (1-3,5-10,41-43). Therefore, the finding that both free and liposome-bound cytochrome b_5 are cleaved at the same site with trypsin suggests that the susceptible bonds are exposed outside the vesicular membrane in the liposome-cytochrome b_5 complex. It is also suggested that the hydrophobic region of the cytochrome molecule is rather resistant to tryptic attack even if it is not protected by binding to liposomes. Another

finding of interst is that a peptide having an amino acid composition very similar to that of the COOH-terminal end of the cytochrome (Peptide III) can be isolated in a small amount from the tryptic digest of the cytochrome $\underline{b}_5$-liposome complex. If this peptide is actually derived from the COOH-terminus of the cytochrome, then it should be concluded that the COOH-terminal portion of the liposome-bound cytochrome is at least partly exposed to the aqueous phase outside the liposomal vesicle, because the cytochrome $\underline{b}_5$-liposome complex is a closed system which is impermeable to macromolecules such as trypsin, as will be reported in Part IV of this thesis. In Part IV data will also be presented to indicate that this is actually the case.

Measurements of CD spectra in the far-ultraviolet region have shown that the CD spectrum of intact cytochrome $\underline{b}_5$ in 0.4% deoxycholate is closely similar to the sum of the spectra of the spectra of the hydrophilic and hydrophobic fragments of the molecule in the same solution. This indicates that the cleavage of the cytochrome with trypsin does not significantly affect the conformations of the two domains and thus provides further evidence that the two domains of the cytochrome molecule are folded almost independently from each other. This finding is, however, in contrast to the CD data reported by Visser *et al.* (22), who reported that the intensity of ultraviolet CD spectrum of pocine cytochrome $\underline{b}_5$ is significantly higher than the sum spectrum of the two products. The reason for this discrepancy is unclear, but our data seems more reasonable because only a dipeptide is produced by tryptic digestion in addition to the

two fragments. The CD spectrum of the cytochrome b_5-liposome complex is again similar to the sum of the spectra of the hydrophilic fragment and the tail-liposome complex, indicating that the two independently folded domain structure is retained even when the cytochrome is bound by liposomes. Table III compares the molecular weights, mean residue ellipticities, and molar ellipticities at 222 nm of the hydrophilic and hydrophobic fragments of the cytochrome. The mean residue ellipticity of the hydrophobic fragment is more than twice as high as that of the hydrophilic fragment, suggesting that the former has a considerably higher helix content than the latter. Huntley and Strittmatter (48) and Visser et al.(22) have shown that the apparent helix content in the hydrophilic fragment estimated from the CD data is less than that calculated from the X-ray crystallographic data (49,50), but the reason for this difference has not yet been explained. The helix content in the hydrophobic fragment can be estimated from the CD data to be 40-50%, a value which is not particularly high compared with many water-soluble globular proteins.

Table III. Molecular weights (M_r), mean residue ellipticities ($[\theta]_R$), and molar ellipticities ($[\theta]$) at 222 nm of the hydrophilic and hydrophobic fragments of rabbit cytochrome $\underline{b}_5$. The molecular weights were calculated from the amino acid compositions listed in Table I, and ellipticities were obtained from Fig. 6.

Fragment	M_r	$[\theta]_R$	$[\theta]$
Hydrophilic	10,107	-7.6×10^3	-6.8×10^5
Hydrophobic	4,919	-18.6×10^3	-7.7×10^5

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PART III

DENATURATION OF NADH-CYTOCHROME b_5 REDUCTASE BY GUANIDINE HYDROCHLORIDE: ANOTHER EXAMPLE OF TWO-DOMAIN STRUCTURE IN INTEGRAL MEMBRANE PROTEINS

SUMMARY

Microsomal NADH-cytochrome $\underline{b}_5$ reductase is an amphiphilic protein consisting of a hydrophilic (FAD-carrying) moiety and a hydrophobic (membrane-binding) segment and exists in aqueous media as a micelle. Circular dichroism studies show that denaturation of the reductase by guanidine hydrochloride in the presence of Emulgen 109P, a nonionic detergent, is a two-stage process as a function of the denaturant concentration. The first transition occurs at the denaturant concentration of about 1 M and the second one at much higher concentrations of guanidine hydrochloride. The denaturant concentration causing the second-stage denaturation depends on the concentration of Emulgen 109P added. A hydrophilic fragment of the reductase lacking the hydrophobic segment, on the other hand, undergoes one-stage denaturation at the denaturant concentration of about 1 M regardless of the presence and absence of Emulgen 109P. Abolishment of NADH-ferricyanide reductase activity of and detachment of FAD from the reductase as well as its hydrophilic fragment also take place at about 1 M guanidine hydrochloride in the presence of the detergent. These findings are consistent with the view that the first-stage denaturation of the reductase represents the unfolding of the hydrophilic moiety and the second one that of the hydrophobic segment. Gel chromatography experiments suggest that in the presence of Emulgen 109P the reductase exists as a mixed micelle with the detergent and this aggregation state persists even after the first-stage denaturation (unfolding of the hydrophilic moiety). The destruction of the mixed micelle structure seems to take place concomitant with the second-stage denaturation.

It is concluded that the two moieties of the reductase molecule exist as relatively independent domains undergoing unfolding separately, at least in the presence of Emulgen 109P. This structural feature of the reductase is similar to that of cytochrome $\underline{b}_5$ reported by us. The reductase is, therefore, the second example of amphiphilic membrane proteins having two independent structural domains in the molecule.

INTRODUCTION

NADH-cytochrome $\underline{b}_5$ reductase (abbreviated here as fp_1) is a flavoprotein which is tightly bound to microsomal membranes in liver and other cells and has been purified in a homogeneous state from liver microsomes after solubilization with detergents (1,2). The flavoprotein thus purified, called d-fp_1 (detergent-solubilized fp_1), has a molecular weight of about 33,000 and is composed of a single peptide chain (2). Like cytochrome $\underline{b}_5$, another microsomal membrane protein, d-fp_1 is an amphiphilic protein consisting of a hydrophilic moiety to which FAD is non-covalently attached (catalytic segment) and a hydrophobic region by which it binds to microsomal membranes (membrane-binding segment) (1,2). Because of this amphiphilic nature, purified d-fp_1 exists in aqueous media in the form of a micelle or an oligomeric aggregate (1,2). Digestion of liver microsomes with liver lysosomal fraction or with cathepsin D at an acidic pH's leads to the cleavage of the fp_1 molecule between the two segments and liberates a hydrophilic, FAD-carrying fragment (2-8). This fragment, referred to as l-fp_1 (lysosome-solubilized fp_1), has a molecular weight of

about 28,000 (5,8) and exhibits the same absorption spectra as d-fp_1 in the visible and Soret regions (1,2). Because of the lack of the membrane-binding segment, l-fp_1 is incapable of binding to microsomal and other membranes (1,2), although it still retains the NADH-ferricyanide reductase activity of the parent molecule (1-8). Recent studies have further shown that the NH_2-terminus of d-fp_1 is blocked and its hydrophobic segment is located in COOH-terminal end of the molecule, as in the case of cytochrome $\underline{b}_5$ (9). All these findings indicate that the structural features of d-fp_1 are very similar to those of cytochrome $\underline{b}_5$, despite the differences in molecular weight and the prosthetic group contained.

In Parts I and II of this thesis, we described evidence that the hydrophilic and hydrophobic segment of cytochrome $\underline{b}_5$ exist as discrete domains, the three-dimensional structures of which are almost independent from each other (10). In view of the structural similarities between cytochrome $\underline{b}_5$ and d-fp_1, it seemed likely that the d-fp_1 molecule also possess a similar two-domain structure. To obtain information concerning this point, we studied the denaturation of both d-fp_1 and l-fp_1 in guanidine hydrochloride (GuHCl) by measuring both circular dichroism (CD) and fluorescence. The results obtained indicate that the hydrophilic and hydrophobic segments of the d-fp_1 molecule are folded almost independently from each other at least in a medium containing low concentration of a nonionic detergent, Emulgen 109P (a polyoxyethylene lauryl ether). Thus the unique two-domain structure is shared by at least two integral proteins of microsomal membranes.

MATERIALS AND METHODS

Enzymes and Chemicals. NADH-cytochrome b_5 reductase (d-fp_1) and its hydrophilic fragment (l-fp_1) were purified from rabbit liver microsomes by the method of Mihara and Sato (2) and a modification (2) of the method of Takesue and Omura (5), respectively. The purified d-fp_1 and l-fp_1 preparations had NADH-linked ferricyanide reducing activities of about 1200 and 2300 units per mg of protein, respectively. Polyacrylamide gel electrophoresis (7.5% gel) in the presence of urea and sodium dodecyl sulfate (11) showed that l-fp_1preparation was homogeneous, whereas a small band due to an impurity was detected in the d-fp_1 preparation (Fig. 1). However, no attempts were made to further purify the d-fp_1 preparation. FAD was purchased from Boehringer-Mannheim and purified further by Sephadex G-15 gel chromatography, and FMN was obtained from Nakarai Chemical Co. Guanidine hydrochloride (specially prepared reagent grade) was obtained from Nakarai Chemical Co., Kyoto. Bovine serum albumin (Cohn Fraction V) and horse heart cytochrome c (Type II-A) were purchased from Sigma, and NADH from Oriental Yeast Co., Tokyo. Emulgen 109P was a gift from Kao-Atlas Co., Tokyo. Sephadex gels were purchased from Pharmacia.

Analytical Methods. The concentrations of d-fp_1 and l-fp_1 were estimated from the intensity of the absorption peak at 461 nm in the oxidized form, by using a molar extinction coefficient of 1.13×10^4 (12). CD measurements were performed at room temperature (20-25°) in a JASCO J-20 recording spectropolarimeter equipped with a CD attachment; quartz cells of 1 mm path were

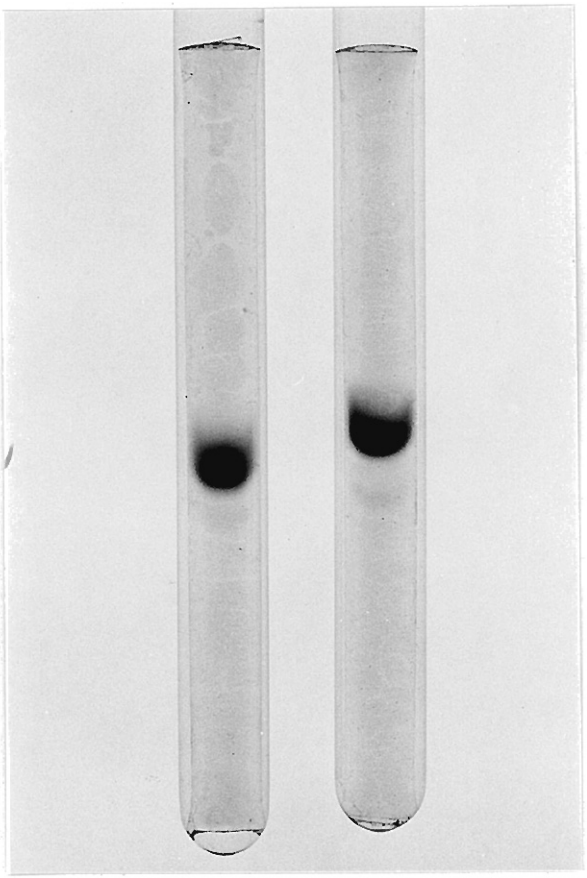

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified l-fp_1 and d-fp_1 preparations. Left, l-fp_1; right, d-fp_1. The samples (6 μg and 8 μg of protein for l-fp_1 and d-fp_1, respectively) were dissolved by heating in 10 mM Tris-acetate buffer (pH 9.0), containing 1% dodecyl sulfate, 0.1% β-mercaptoethanol and 2 M urea, and applied to 7.5% cross-linked gel disks. Electrophoresis was performed in 0.1 M Tris-acetate (pH 9.0), containing 1% dodecyl sulfate, 0.1% β-mercaptoethanol and 0.1% EDTA for 4 h at current of 1.25 mA per tube. The gel was stained with Coomassie blue.

used and the concentration of d-fp_1 and l-fp_1 was usually 6-10 μM. Fluorescence spectra of FAD and fp_1 preparations were recorded at room temperature in a Hitachi MPF-4 fluorospectrophotometer using quartz cells of 1.0 cm path; excitation was at 450 nm and the concentration of FAD and fp_1 was 0.3-1.2 μM. For both CD and fluorescence measurements, the sample was dissolved in 20 mM or 50 mM sodium phosphate (pH 7.5) or 20 mM Tris-acetate-0.2 mM EDTA (pH 8.1) containing 0-1.8% Emulgen 109P and a desired concentration of GuHCl; if necessary, the pH of the mixture was further adjusted between 7 and 8 with a NaOH solution. Prior to CD and fluorescence measurements, each solution was allowed to stand at 25° for more than 16 h to ensure the establishment of denaturation equilibrium. For fluorescence measurements, the incubation was carried out in the dark to prevent the light-induced destruction of FAD in the presence of GuHCl. The results of CD measurements were expressed in terms of molar ellipticity, [θ]. NADH-ferricyanide reductase activity was assayed as described by Mihara and Sato (8).

Sephadex G-200 Gel Chromatography. A Sephadex G-200 column (1.9 x 100 cm) was used to obtain information concerning the apparent size of mixed micelles of d-fp_1 and Emulgen 109P in the presence and absence of GuHCl. The column was equilibrated with 50 mM sodium phosphate buffer (pH 7.5) containing 0.1% Emulgen 109P with or without a desired concentration of GuHCl, and elution was conducted at room temperature with the same solution. For each GuHCl concentration to be studied, the column was calibrated by applying 1.0 ml of the elution buffer containing blue dextran (for determination of the void volume), FMN (for

determination of the internal volume), bovine serum albumin and cytochrome c (as molecular weight markers). Elution of these substances was monitored by measuring the absorbance at 280 nm; blue dextran, FMN, and cytochrome c were also detected by measuring the absorbance at 630, 460, and 409 nm, respectively. A solution containing d-fp_1 and small amounts of blue dextran and FMN in 1.0 ml of the elution buffer was applied to the calibrated column and elution of d-fp_1 was monitored by measuring the absorbance at 280 nm. When no GuHCl was used, d-fp_1 was also detected by measuring its NADH-ferricyanide reductase activity. From the elution data obtained, K_d values were calculated by using the equation of Gelette (13).

RESULTS

CD Studies of Denaturation of NADH-cytochrome b_5 Reductase. Fig. 2 shows the far-ultraviolet CD spectra of l-fp_1 and d-fp_1 measured in the presence of 0.1% Emulgen 109P (a polyoxyethylene lauryl ether having a HLB value of 13.8), a nonionic detergent which shows no CD in this wavelength region. Both CD spectra exhibit a distinct trough at about 222 nm and their shapes are characteristic of the so-clled "polyglutamic acid type" CD curve (14), suggesting that both proteins contain considerable amounts of helical structure. By using the molar ellipticity at 222 nm ($[\theta]_{222}$) as a marker, the denaturation of l-fp_1 and d-fp_1 by GuHCl was then studied. For this purpose, the proteins were incubated at 25° for more than 16 h with various concentrations of GuHCl and the magnitude of the CD trough at 222 nm was determined.

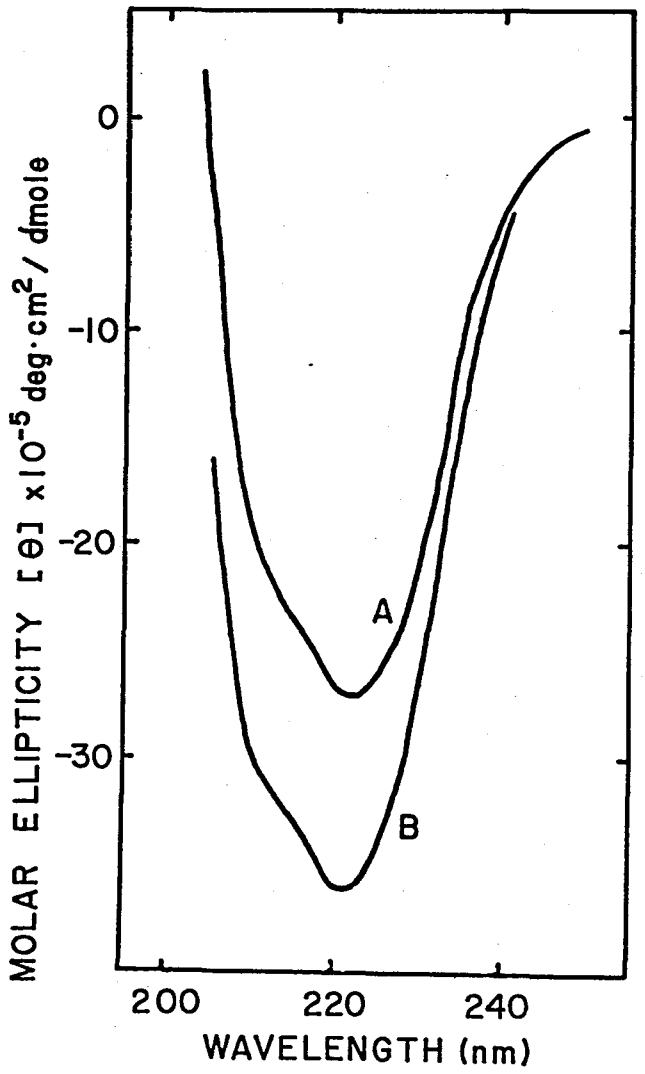

Fig. 2. Far ultraviolet CD spectra of l-fp_1 and d-fp_1 in the presence of 0.1% Emulgen 109P. (A) Spectrum of l-fp_1; (B) spectrum of d-fp_1. Measurements were made after incubation of the samples at 25° in 20 mM Tris buffer containing 0.1 M NaCl and 0.1% Emulgen 109P. The concentrations were about 6.4 and 7.2 μM for l-fp_1 and d-fp_1, respectively.

In Fig. 3A is plotted the $[\theta]_{222}$ value of l-fp_1 attained after incubation for more than 16 h against the GuHCl concentration in the incubation medium. As can be seen, the destruction of the secondary structure of l-fp_1 proceeded in one stage as a function of the denaturant concentration, and the transition midpoint was seen at a GuHCl concentration of about 1 M. It was also found that the denaturation behavior of l-fp_1 was not affected significantly by the presence of 0.1-0.9% Emulgen 109P. In the case of d-fp_1, on the other hand, a precipitate was formed in the presence of 1 to 2 M GuHCl if the incubation medium contained no detergent. Since l-fp_1 formed no precipitate under the same conditions, it seemed that the hydrophobic moiety of d-fp_1 was responsible for the sedimentation observed. A likely possibility was that the salting-out effect of GuHCl was one of the causes of the precipitation phenomenon. Although this phenomenon made it impossible to obtain the denaturation curve for d-fp_1 in the entire GuHCl concentration range, the portion of the curve obtained suggested that the denaturation was not a two-stage process, as shown in Fig. 3B. It was found that the precipitation of d-fp_1 in the presence of 1-2 M GuHCl was prevented if Emulgen 109P was added to the incubation medium at concentrations higher than 0.45%. Moreover, in the presence of 0.45% Emulgen 109P the denaturation of d-fp_1 was clearly a two-stage process, having the first transition midpoint at about 1 M GuHCl and the second one at about 8.3 M GuHCl (Fig. 3B). Although 0.1 and 0.2% Emulgen 109P could not prevent the precipitation of d-fp_1 in the presence of 1-2 M GuHCl, these concentrations of the detergent also elicited the second-stage denaturation having the transition midpoint

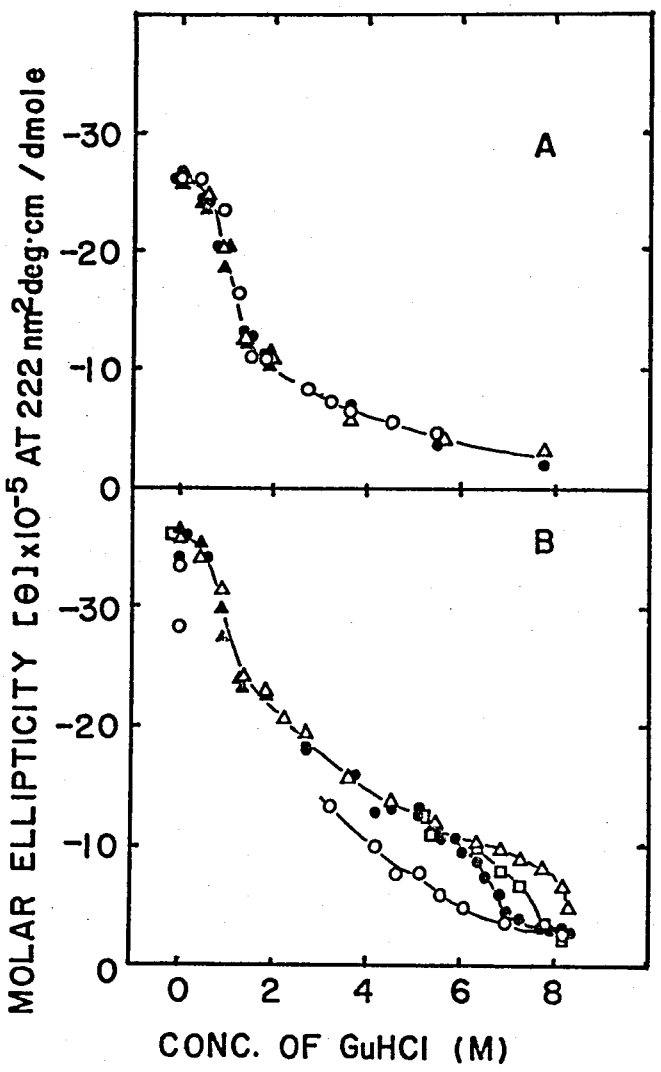

Fig. 3. Effects of GuHCl concentration on molar ellipticities at 222 nm of l-fp_1 and d-fp_1. (A) l-fp_1; (B) d-fp_1. Measurements were made after incubation of the sample at 25° in 20 mM or 50 mM sodium phosphate buffer or in 20 mM Tris buffer containing 0% (0), 0.1% (●), 0.2% (□), 0.45% (Δ), and 0.9% (▲) Emulgen 109P and indicated concentration of GuHCl. The concentrations of both l-fp_1 and d-fp_1 were about 6-10 μM.

at about 6.5 and 7.5 M GuHCl, respectively. At concentrations lower than 0.01%, however, the detergent did not elicit the second-stage denaturation. Although the transition midpoint of the second-stage denaturation increased as the concentration of Emulgen 109P was increased, that of the first-stage denaturation did not change when the detergent concentration was raised from 0.45 to 0.9%.

Fig. 4 illustrates the effects of the concentration of Emulgen 109P on the molar ellipticities at 222 nm of both d-fp_1 and l-fp_1 in the absence of GuHCl. As can be seen, the $[\theta]_{222}$ value of d-fp_1 increased somewhat on addition of 0.1% Emulgen 109P, but was kept constant thereafter upon further addition of the detergent up to a concentration of 1.8%. The $[\theta]_{222}$ values of d-fp_1 measured after incubation at 0° overnight in the presence of 0.1-1.8% Emulgen 109P were almost the same as those shown in Fig. 4, which were determined after incubation at 25° for about 16 h. The fact that addition of the detergent increases the $[\theta]_{222}$ value suggests that the hydrophobic moiety of d-fp_1 is in a somewhat loose conformation in the absence of the detergent and is converted to a more rigid structure on addition of Emulgen 109P. In fact, it has been reported that d-fp_1 is more stable in the presence of nonionic detergents (2). The $[\theta]_{222}$ value of l-fp_1 was considerably lower than that of d-fp_1 and was essentially unaffected by the addition of Emulgen 109P.

Nature of First-Stage Denaturation. Since the destruction of the secondary structure of l-fp_1 took place at the same GuHCl concentration (about 1 M) as causing the first-stage denaturation of d-fp_1, it was suggested that the first-stage denaturation

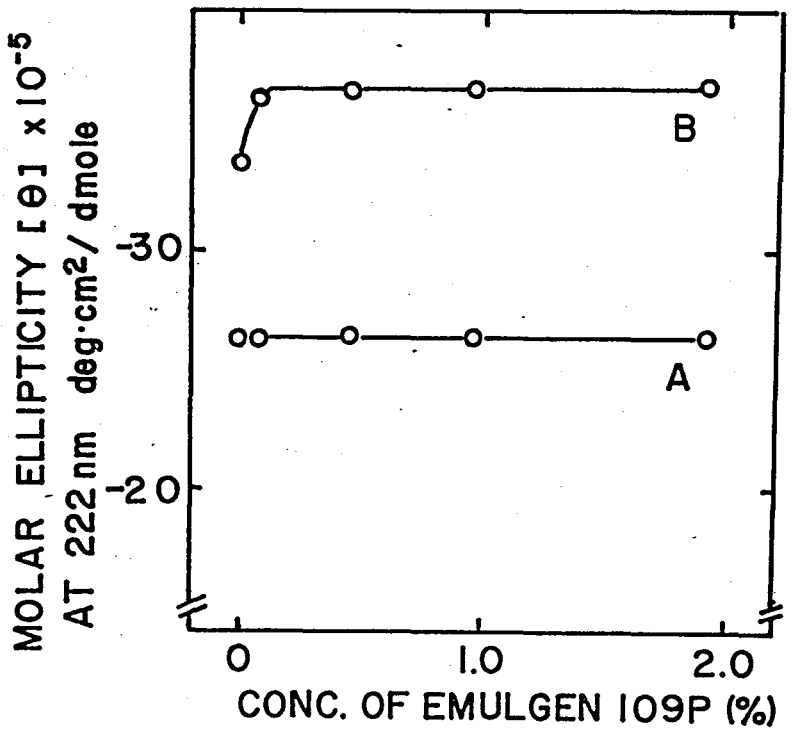

Fig. 4. Effects of Emulgen 109P concentration on molar ellipticities at 222 nm of l-fp_1 and d-fp_1. (A) l-fp_1; (B) d-fp_1. Measurements were made after incubation of the samples at 25° in 20 mM Tris buffer, containing indicated concentration of Emulgen 109P. The concentrations of both l-fp_1 and d-fp_1 were about 6.2 and 8.2 μM, respectively.

represented the unfolding of the hydrophilic, FAD-containing moiety of d-fp_1. This suggestion could be confirmed by studying the effect of GuHCl on the NADH-ferricyanide reductase activity, which is associated with l-fp_1 and the hydrophilic moiety of d-fp_1. As shown in Fig. 5, incubation of both l-fp_1 and d-fp_1 with GuHCl resulted in inactivation of the reductase activity, and half maximal inactivation was observed when the incubation was carried out in the presence of about 1 M GuHCl in both cases. It was, therefore, concluded that the inactivation of NADH-ferricyanide reductase activity was accompanied by the destruction of whole structure of l-fp_1 and the unfolding of the hydrophilic moiety of d-fp_1. The NADH-ferricyanide reductase activities of l-fp_1 and d-fp_1 after incubation in the presence of Emalgen 109P, but in the absence of GuHCl, at 25° for about 24 h were about 60 units per nmole of the flavoprotein. This activity is similar to those of freshly prepared l-fp_1 and d-fp_1. If no Emulgen 109P was present during the incubation, the activity of d-fp_1 fell to about half of the original value. Since no such a drastic decrease of the activity of l-fp_1 was observed, it was likely that the fall of activity was due to aggregation of d-fp_1 by its hydrophobic moiety.

Although free FAD shows an intense fluorescence emission peak at 530 nm, this fluorescence is quenched when it is attached to l-fp_1 and to the hydrophilic moiety of d-fp_1. If the structures of l-fp_1 or the hydrophilic moiety of d-fp_1 to which FAD is noncovalently bound are destroyed as a result of unfolding, the FAD should be detached from the protein, causing a marked increase in fluorescence at 530 nm. We took advantage of this

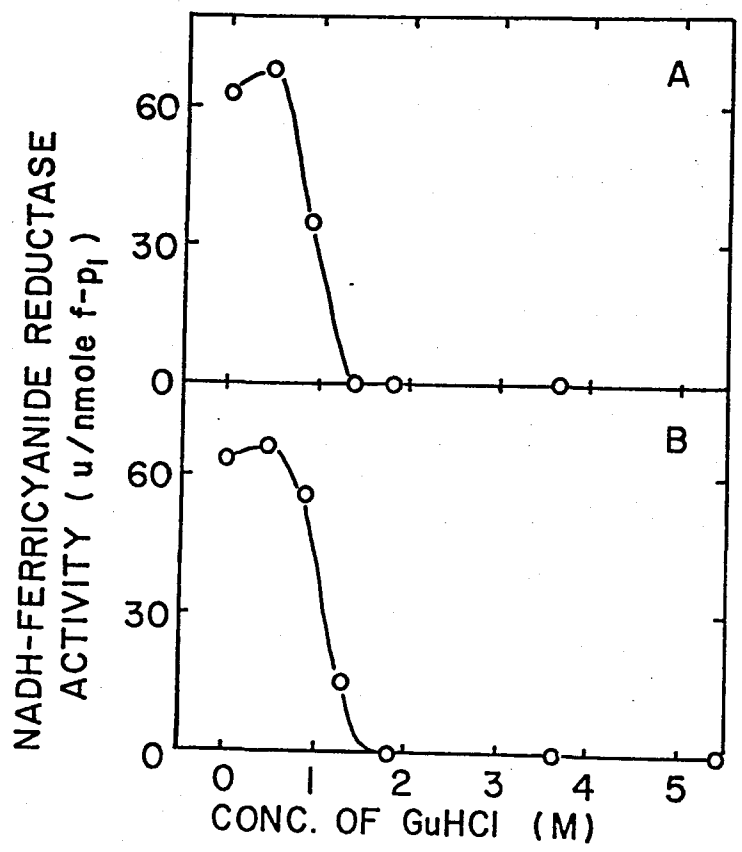

Fig. 5. Effects of GuHCl concentration on NADH linked ferricyanide reducing activities of l-fp_1 and d-fp_1. (A) l-fp_1; (B) d-fp_1. The samples were dissolved in 20 mM Tris buffer, containing 0.45% Emulgen 109P and indicated concentration of GuHCl. The d-fp_1, incubated in 0.87 and 1.30 M GuHCl concentrations, contained 0.9% Emalgen 109P, differed from other samples. The activities of both l-fp_1 and d-fp_1 were expressed as units per mole fp_1.

fact to further confirm the nature of the first-stage denaturation of d-fp_1. As shown in Fig. 6, the fluorescence of free FAD increased gradually as the GuHCl concentration was increased for unknown reasons. The quenched fluorescence of both l-fp_1 and d-fp_1 in a medium containing Emulgen 109P increased markedly when the preparations were incubated in the presence of GuHCl, and the most steep increase in fluorescence took place at about 1 M GuHCl. These findings indicated that the detachment of FAD from l-fp_1 was accompanied by destruction of the conformation of the whole molecule and that from d-fp_1 was concomitant with the first-stage denaturation. At higher GuHCl concentrations than those causing the liberation of FAD, the fluorescence intensities of both l-fp_1 and d-fp_1 increased gradually as a function of the GuHCl concentration in the case of free FAD. These results, together with the CD data and the inactivation of NADH-ferricyanide reductase activity described above, supported the view that the first-stage denaturation of d-fp_1 represented the unfolding of the hydrophilic, FAD-containing moiety of the molecule. Consequently, the second-stage denaturation of d-fp_1 observable in the presence of Emulgen 109P seemed to correspond to the destruction of the hydrophobic segment. It can be concluded that at least in the presence of appropriate concentrations of Emulgen 109P the hydrophilic and hydrophobic moieties of the d-fp_1 molecule, though linked to each other covalently, constitute separate domains that can undergo unfolding independently, as in the case of cytochrome b_5 (10).

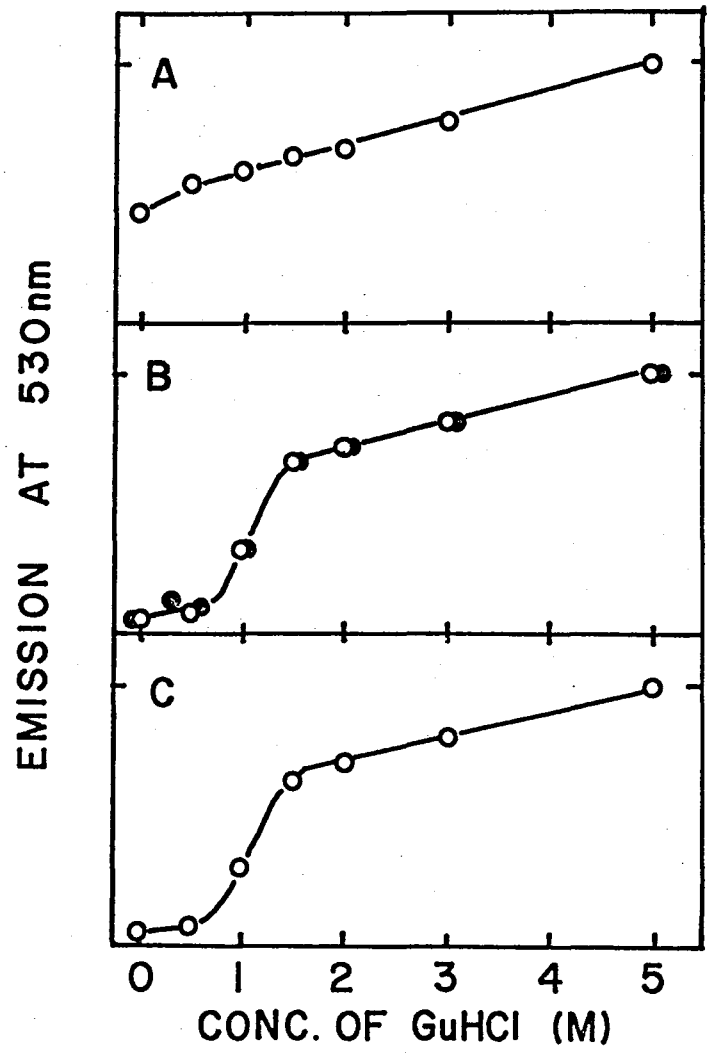

Fig. 6. Detachment of FAD from l-fp_1 and d-fp_1 as a function of GuHCl concentration. (A) FAD; (B) l-fp_1; (C) d-fp_1. The sample dissolved in 20 mM Tris buffer containing 0.5% Emulgen 109P and indicated concentration of GuHCl was incubated at 25° in the dark. The d-fp_1 incubated in 0.1 and 1.5 M GuHCl concentrations contained 0.9% Emulgen 109P, different from other samples. The l-fp_1, dissolved in 20 mM sodium phosphate buffer containing no detergent and indicated concentration of GuHCl (●), was also measured after incubation at 25° in the dark. The sample solution was excited at 450 nm and the emission at 530 nm was measured. The values shown as % emission were normalized by the fluorescence intensity of the sample in 5 M GuHCl concentration of each series. The treatment of the samples were performed under dim light. The concentrations of FAD, l-fp_1 and d-fp_1 were between 0.3-1.2 μM.

Aggregation State of the Reductase in the Presence of GuHCl. Cytochrome b_5 exists in aqueous solution as a micelle or an oligomeric aggregation because of its amphiphilic nature (16-18) and, as described in Part I, the micelle can be dissociated into the monomers concomitant with the second-stage denaturation by GuHCl. Being an amphiphilic protein, d-fp_1 also forms a micell or an oligomeric aggregate in aqueous media (1,2) and the apparent molecular weight of the micelle has been reported to be about 360,000 in 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (2). By analogy with the case of cytochrome b_5 (10), it was expected that the aggregation state of d-fp_1 would be maintained during the first-stage denaturation and degraded concomitant with the second-stage denaturation. However, the two-stage denaturation of d-fp_1 takes place only in the presence of Emulgen 109P, which appears to have a great influence on the aggregation state of the system. To obtain information concerning the change in aggregation state of d-fp_1 induced by GuHCl and the detergent, we estimated the apparent molecular weight of the system in the presence of 0.1% Emulgen 109P and 0, 4.5, and 8 M GuHCl. The estimation was made by Sephadex G-200 gel chromatography, using bovine serum albumin and cytochrome c as reference standards, and the results obtained are summarized in Table I. From the K_d value it was estimated that the apparent molecular weight of d-fp_1 falls in the order of 100,000 daltons. This value is much smaller than 360,000 daltons for the apparent molecular weight of the d-fp_1 micelle in the absence of the detergent. Although further studies are needed, it was suggested that the detergent dissociated the

Table I

K_d values of d-fp_1, bovine serum alubumin and cytochrome *c* in gel chromatography on a Sephadex G-200 column at several GuHCl concentrations in the presence of 0.1% Emulgen 109P.

GuHCl concentration (M)	K_d		
	d-fp_1	Bovine serum albumin	Cytochrome *c*
0	0.190	0.287	0.743
4.5	0.116	0.143	0.564
8	0.250	0.144	0.551

d-fp_1 micelle to a dimer or monomer state to which a considerable amount of the detergent were tightly associated; the association site being most probably the hydrophobic moiety of the d-fp_1 molecule. Table I also indicates that the K_d values for both bovine serum albumin and cytochrome c were considerably smaller in 4.5 and 8 M GuHCl than the absence of the denaturant, because of considerable increase in Stokes radius of the proteins caused by unfolding (19). A similar reduction of K_d value was also observed for d-fp_1 in 4.5 M GuHCl, indicating that the effective size of the d-fp_1-Emulgen 109P mixed micelle had been increased upon exposure to this GuHCl concentration because of the unfolding of the hydrophilic moiety. Thus, it seemed that the first-stage denaturation of d-fp_1 at about 1 M did not affect the micellar state of the system. In 8 M GuHCl the K_d value of the system became even higher than that obtained in the absence of GuHCl. It was, therefore, suggested that under this condition the mixed micelle structure was seriously destroyed and the destruction had taken place concomitant with the second-stage denaturation at 6.5 M in the presence of 0.1% Emulgen 109P.

DISCUSSION

As described above, the denaturation of d-fp_1 by GuHCl is a two-stage event as a function of the denaturant concentration if Emulgen 109P is present in the medium. Thus, a large portion of the d-fp_1 molecule undergoes unfolding at the denaturant concentration of about 1 M, but a significant amount of ordered

structure still remains even after this first-stage denaturation. A much higher concentration of GuHCl is required to destroy this remaining structure; the GuHCl concentration required for this second-stage denaturation increases as the concentration of Emulgen 109P in the medium is increased. It is clear that the first-stage denaturation represents the unfolding of the hydrophilic, FAD-carrying moiety (catalytic segment) of the d-fp_1 molecule, because this denaturation is accompanied by the loss of NADH-ferricyanide reductase activity and detachment of FAD from the protein. Another support to this view is the finding that the destruction of the whole structure of l-fp_1, a hydrophilic fragment of the reductase lacking the hydrophobic segment, is effected also at about 1 M GuHCl and this process is also accompanied by the loss of the catalytic activity and detachment of FAD. This latter observation further confirms that the second-stage denaturation of d-fp_1 corresponds to the unfolding of the hydrophobic region of the reductase molecule. These findings are consistent with the view that at least in the presence of Emulgen 109P the hydrophilic and hydrophobic moieties of the d-fp_1 molecule exist as relatively independent structural domains differing from each other in susceptibility to the denaturing action of GuHCl. Thus, this feature of the structure of d-fp_1 is similar to that of cytochrome $\underline{b}_5$ reported previously (10). However, the situation is more complicated in d-fp_1 than in cytochrome $\underline{b}_5$, because the two-stage denaturation of the former can be clearly demonstrated only in the presence of Emulgen 109P and the transition midpoint for the second-

stage denaturation is dependent on the detergent concentration. It is, therefore, necessary to consider how the detergent interacts with d-fp_1 and how this interaction affects the denaturation behavior of the protein.

The K_d value determined by gel chromatography for d-fp_1 in the presence of 0.1% Emulgen 109P (Table I) suggests that under this condition d-fp_1 exists as a mixed micelle with the detergent and each micelle contains one or two d-fp_1 molecules and a considerable amount of the detergent attached tightly to the hydrophobic moiety of the protein. The formation of similar micelles from cytochrome b_5 and detergents such as Triton X-100 and deoxycholate has been reported (18,20). The binding of Emulgen 109P to the hydrophobic domain seems to convert the conformation of d-fp_1 to a somewhat more rigid one (cf. Fig. 4) and thus stabilize the protein. This is consistent with the reported stabilizing effect of nonionic detergents on d-fp_1 (2). In the present study it has been demonstrated that the drastic decrease in NADH-ferricyanide reductase activity of d-fp_1 after incubation at 25° can be prevented by Emulgen 109P. It should be mentioned in this connection that the conformation of d-fp_1 in the absence of detergents, as monitored by the $[\theta]_{222}$ value, is not constant but varies considerably depending on the lot of preparation (data not shown) and probably also on other conditions. It is, therefore, likely that the "native" conformation of d-fp_1 can be maintained only when the protein is in association with detergents and possibly also with phospholipids. However, egg phosphatidylcholine liposomes to which d-fp_1 molecules hve been incorporated in vitro has been

reported to be unstable at room temperature forming in short period of time (21,22). At any rate, once the "native" conformation of d-fp_1 has been assumed in the presence of a low concentration (0.1%) of Emalgen 109P, this conformation is not affected by increasing the detergent concentration (Fig. 4).

The instability of d-fp_1 micelles in the absence of detergents is in contrast to the behavior of cytochrome $\underline{b}_5$ micelles which remain stable even in the absence of detergents. This differnce may be an intrinsic one arising from the difference in the primary structures of the hydrophobic regions of the two proteins. Another possibility is that the relative size of the hydrophilic moiety to that of the hydrophobic domain is responsible for the difference in stability. The size of the hydrophilic moiety of cytochrome $\underline{b}_5$ (about 11,000 daltons) may be small enough to permit its hydrophobic domain to interact strongly with each other and thus form very stable micelles. The hydrophilic domain of d-fp_1 (about 28,000 daltons in size), on the other hand, may be too large as compared with the hydrophobic domain (about 5,000 daltons or less, see below) so that the d-fp_1 molecules cannot form sufficiently stable micelles by hydrophobic interactions of the steric hindrance caused by the large hydrophilic domain. It might be suggested that the somewhat loose and variable conformation of d-fp_1 in the absence of detergents is due to such an inadequate micelle state. It is also conceivable that such a steric hindrance is abolished by the formation of mixed micelles with detergents.

The results reported in this paper, especially those shown in Fig. 3B, suggest that the unfolding of the hydrophobic

domain of d-fp_1 in the presence of Emulgen 109P takes place at the same time with the dissociation of the mixed micelle into its component molecules. It is, therefore, likely that the rigid structure of the hydrophobic domain is maintained by its tight interaction with the detergent and this structure is destroyed only when the domain is freed from the detergent. If this is so, then it is conceivable that the unfolding of the hydrophobic domain is dependent on the strength by which the detergent is attached. As a matter of fact the GuHCl concentration required for the second-stage denaturation of d-fp_1 increases as the concentration of Emulgen 109P in the medium is increased (Fig. 3B). Since it is unlikely that the quality of interactions of the detergent with the hydrophobic domain is altered depending on the detergent concentration, it is more reasonable to assume that the strength of detergent binding is related to the quantity of the detergent bound. Thus, at higher concentrations of Emulgen 109P the amount of the detergent bound by the hydrophobic domain increases and renders the domain more resistant to denaturation. It should be noted that binding of increasing amounts of the detergent does not cause any change in the conformation of the protein molecule as descussed above. This can also be concluded from the fact that the magnitude of $[\theta]_{222}$, which is affected by the second-stage denaturation of d-fp_1, is almost constant regardless of the concentration of Emulgen 109P added (Fig. 4). The binding, therefore, seems to stabilize the hydrophobic domain of d-fp_1 without changing its conformation. Whatever reason can be conceived of for this phenomenon, it is certain

that in the state of mixed micelle with the detergent the hydrophilic and hydrophobic moieties of the d-fp_1 molecule are folded independently from each other and the two domains undergo unfolding separately. The denaturation process of the d-fp_1-detergent mixed micelle and that of l-fp_1 may be illustrated schematically as shown in Fig. 7. Since phospholipids are amphiphilic like detergents, it is not unreasonable to assume that d-fp_1 also assume the two-domain structure when it is bound to phospholipid liposomes and microsomal membrane, but experimental confirmation of this view is not possible at least by the denaturation method, because the d-fp_1-liposome complex forms turbid precipitate at room temperature as described above.

Finally, it is of interest to point out that d-fp_1 is similar to cytochrome $\underline{b}_5$ not only in the two-domain structure and other properties mentioned earlier in this paper but also in the nature of their hydrophobic domains. In Table II are compared the molecular weight and $[\theta]_{222}$ values of the hydrophilic and hydrophobic domains of d-fp_1 and cytochrome $\underline{b}_5$. Although the hydrophilic domains of the two proteins are significantly different from each other, their hydrophobic domains have similar molecular weights and $[\theta]_{222}$ values. Moreover, Mihara *et al.* (9) have recently reported evidence that the hydrophobic domain of d-fp_1 is located at the COOH-terminal end of the molecule, as has been shown to be the case for cytochrome $\underline{b}_5$ (23). These similarities may, therefore, be also shared by some other membrane proteins, although its significance is not known at present.

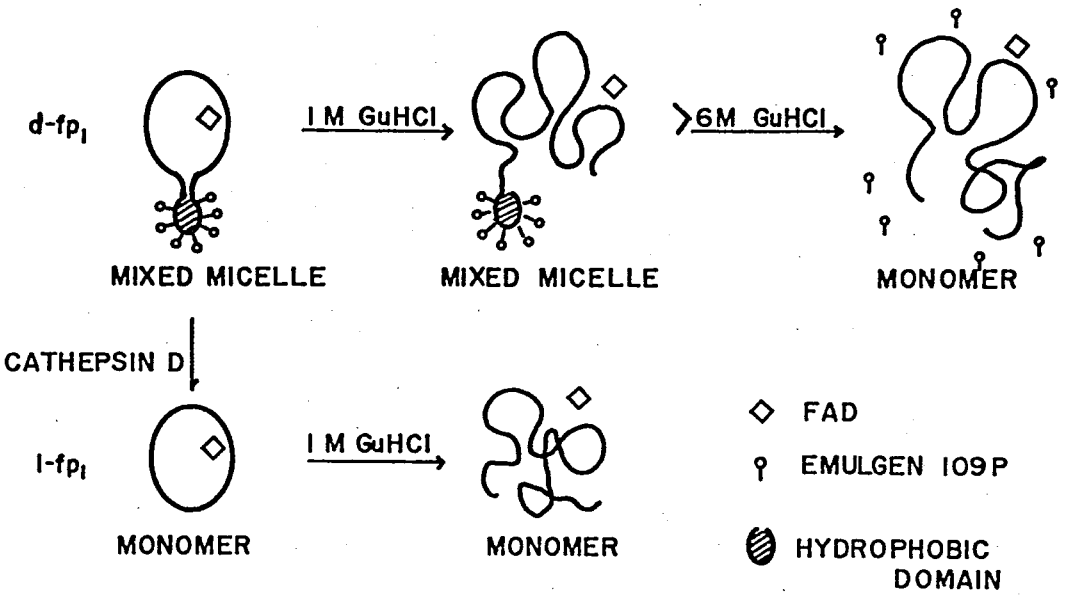

Fig. 7. Schematic illustration of structural transitions of l-fp$_l$ and d-fp$_l$ induced by GuHCl in the presence of Emulgen 109P.

Table II

Molecular weights and molar ellipticites at 222 nm of hydrophilic and hydrophobic segments of cytochrome $\underline{b}_5$ and d-fp_1.

	Cytochrome $\underline{b}_5$		d-fp_1	
	MW	$[\theta] \times 10^{-5}$	MW	$[\theta] \times 10^{-5}$
Hydrophilic moiety	10,107	-6.8	28,000	-26
Hydrophobic moiety	4,919	-7.7	5,000	-9

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PART IV

MODE OF BINDING OF CYTOCHROME $\underline{b}_5$ TO PHOSPHATIDYLCHOLINE LIPOSOMES

SUMMAY

Treatment of cytochrome $\underline{b}_5$ embedded in egg-yolk phosphatidylcholine liposomes with yeast carboxypeptidase Y resulted in the liberation of 25 amino acids from the COOH-terminal portion of the cytochrome molecule. Since density gradient centrifugation experiments indicated that the cytochrome $\underline{b}_5$-liposome complex existed as closed vesicles which were impermeable to macromolecules such as Dextran T-40, the above observation strongly suggested that the COOH-terminal end of the liposome-bound cytochrome was exposed to the outer surface of the lipid vesicle. There was, however, the possibility that a small amount of cytochrome $\underline{b}_5$ was present in the aqueous phase in equilibrium with the liposome-bound molecules and the carboxypeptidase attacked the free cytochrome molecules. This possibility could be excluded by the observation that the exchange of the bound cytochrome with the free molecules was much slower than the carboxypeptidase action. The COOH-terminus of cytochrome $\underline{b}_5$ embedded in dipalmitoyl phosphatidylcholine liposomes could also be attacked below the transition temperature of the lipid. This latter cytochrome $\underline{b}_5$-liposome complex was also impermeable to Dextran T-40. It was further found that cytochrome $\underline{b}_5$ was released from the liposomes as a result of the attack of carboxypeptidase Y on the cytochrome $\underline{b}_5$-liposome complex. The cytochrome $\underline{b}_5$ thus released could neither rebind to liposomes nor form micelles (oligomeric aggregates), suggesting that the carboxypeptidase Y hydrolyzed all the membrane-binding residues from the hydrophobic domain of the cytochrome $\underline{b}_5$ molecule. The size of the peptide segment which are directly involved in the interaction of the

cytochrome with the lipid bilayer is discussed.

INTRODUCTION

In Parts I, II, and III, we have shown that cytochrome b_5 (1) and NADH-cytochrome b_5 reductase, which are amphiphilic membrane proteins, consist of two independently folded domains, i.e. a hydrophilic moiety which bears the prosthetic group and a hydrophobic moiety by which they are tightly attached to microsomal membranes (2-5). In microsomal vesicles the hydrophilic domains of these proteins are extruded to the aqueous phase from the outer surface of the vesicular membrane, as evidence by the inhibition of their catalytic activities by their respective antibodies added exogenously (6,7) and by the liberation of their hydrophilic moieties upon proteolytic digestion of microsomes (8,9). Several lines of evidence indicate that the same topological situation holds for these proteins incorporated into sonicated phosphatidylcholine (PC) liposomes (10-12). Little is, however, known of the topology of their hydrophobic domains in microsomes and artificial phospholipid vesicles. To obtain information concerning the topology of the hydrophobic domain of cytochrome b_5 incorporated into PC liposomes, we used yeast carboxypeptidase Y as a tool, because it is known that the COOH-terminal portion of the cytochrome molecule constitutes the hydrophobic domain (13).

In this paper, we report that the COOH-terminal region of cytochrome b_5 embedded in PC liposomes can be attacked by exogenously added carboxypeptidase Y and present evidence that the

COOH-terminus of the cytochrome molecule is exposed to the outer space of the liposomal vesicle. The nature of the peptide segment of the cytochrome which is in direct interaction with the lipid bilayer is also discussed.

MATERIALS AND METHODS

Enzymes and Chemicals. Cytochrome b_5 was purified from rabbit liver microsomes by the method of Spatz and Strittmatter (3) and was found to be homogeneous upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and urea. The heme-containing hydrophilic fragment (abbriviated as t-b_5), produced from cytochrome b_5 by tryptic digestion, was separated from the other fragments by using a Sephadex G-100 column (3). Egg-yolk PC was prepared as described by Singleton et al. (14); its purity was confirmed by thin-layer chromatography. D,L-α-dipalmitoyl phosphatidylcholine (DPPC) was obtained from Sigma. Sephadex gels, Sepharose 4B, Dextran T-40 and blue dextran 2000 were obtained from Pharmacia and $Na^{125}I$ was from Radio Chemical Centre, England. Yeast carboxypeptidase Y was purchased from Oriental Yeast Co., Tokyo. The other chemicals were of the highest quality available.

Preparation of Cytochrome b_5-Liposome Complex. Phospholipid liposome were prepared by sonication in a Branson sonifier model M 185. Single-walled liposomes of egg PC were prepared as described by Huang (15). DPPC (about 60 mg) was suspended in 4-8 ml of 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and sonicated for about 30 min at 40-50°. The sonicated suspension

was centrifuged at 100,000 x g for 40 min and the supernatant fluid was used as DPPC liposomes. The complexes of cytochrome b_5 with egg PC liposomes were prepared as described in Part II. The cytochrome b_5-DPPC liposome complex was also prepared similarly, except that the mixture was incubated for 1-3 h at 45° after overnight incubation at 4° or for 48 h at 4°. The molar ratio of cytochrome b_5 to DPPC phosphorus in the complex was about 1:34, which was different from that in the cytochrome b_5-egg PC liposome complex (1:10-11). The complex thus prepared was diluted with or dialyzed against 0.1 M acetate buffer (pH 5.6) and concentrated to about 200 μM with respect to cytochrome b_5 by centrifugation at 100,000 x g for 3 h or in an Amicon model microultrafiltration system using XM 50 membrane. The pH of the final complex solution were between 5.6-5.9.

Yeast Carboxypeptidase Y Treatment. To a solution containing the complex in 0.1 M acetate buffer (pH 5.6-5.9) was added yeast carboxypeptidase Y (CPase Y) to a CPase Y to cytochrome b_5 ratio of 1:20 to 1:200 (w/w) and the mixture was usually allowed to stand at 30° for 1-3 h.

Dextran T-40 Density Gradient Centrifugation. About 0.15 ml of cytochrome b_5-egg PC or DPPC liposome complex in 0.1 M acetate buffer (pH 5.6-5.9) containing Dextran T-40 ($d>1.12$) was placed at the bottom of the tube, and over the sample was layered 5 ml of a linear gradient of Dextran T-40 in 0.1 M acetate buffer (pH 5.6) ranging in density value from about 1.12 to 1.05. This was then centrifuged at 50,000 rpm for 50 h at 2° in an SW 65K rotor of a Beckman L-2 centrifuge or in an RPS 50-II rotor of a Hitachi 55P centrifuge. After

centrifugation, three-drop fractions were collected from the bottom of the tube and assayed for cytochrome $\underline{b}_5$ by measuring the absorbance at 413 nm. The density of each fraction was determined by weighing a small volume of sample taken with a calibrated micropipette.

Sucrose Density Gradient Centrifugation. For sucrose density gradient centrifugation 0.1 to 0.15 ml of suspension was layered over 5 ml of a linear sucrose concentration gradient containing 50 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA and centrifugd at 2° in an SW 65K rotor of a Beckman L-2 or L-5 centrifuge, or in an RPS 50-II rotor of a Hitachi 55P centrifuge. To determine the density of cytochrome $\underline{b}_5$-egg PC or DPPC liposome complex, a sucrose gradient from 10 to 45% (w/w) was employed and centrifugation was carried out at 50,000 rpm for 50 h. For studies of the cytochrome $\underline{b}_5$ exchange reaction, a sucrose gradient from 5 to 40% (w/v) was used and centrifugation was at 28,000 rpm for 18 h. These latter conditions could separate the cytochrome $\underline{b}_5$ micelles from the cytochrome $\underline{b}_5$-egg PC liposome complex. To separate the hydrophilic fragment of cytochrome $\underline{b}_5$ (monomeric) from the intact cytochrome (oligomeric) micelles or its complex with PC liposomes, a sucrose gradient from 5 to 45% (w/v) was used and centrifugation was performed usually at 50,000 rpm for 20 h. After centrifugation, three-drop fractions were collected from the bottom of the centrifuge tube and assayed for cytochrome $\underline{b}_5$. The density of each fraction was determined with the aid of an Abbe refractometer. In studies of the cytochrome $\underline{b}_5$ exchange reaction, ^{125}I radioactivities of each fraction was determined in an Aloka auto well gamma system JDC-751.

Labeling of Cytochrome b_5 with ^{125}I. A mixture containing 200 μM cytochrome b_5, 20 μCi $Na^{125}I$, 0.5 mM chloramine T, and 50 mM Tris-HCl buffer (pH 8.0) was incubated at 4° for 20 min. After stopping the reaction by adding 100 μl of 1.5 mM $Na_2S_2O_5$, the whole mixture was dialyzed at 4° for 5 days against 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The cytochrome b_5 thus labeled was directly used for preparation of the complex with egg PC liposomes which was used for the exchange study after displacing the buffer with 0.1 M acetate buffer (pH 5.6-5.9).

Analytical Methods. Cytochrome b_5 and its hydrophilic fragment were estimated from the intensity of the oxidized Soret peak at 413 nm by using a molar extinction coefficient of 1.17×10^4 (3,16). Phospholipid phosphorous was determined by the method of Bartlette (17) after digestion of the phospholipid sample. For digestion, 0.5 ml of 10 N H_2SO_4 was added to 0.1 ml of sample and the mixture was heated at 220° for 30 min. Then a drop of 30% H_2O_2 solution was added to the mixture and was heated at 220° for another 30 min. Amino acid analysis were performed by the method of Spackman et al.(18).

RESULTS

The cytochrome b_5-egg PC liposome complex used in this study contained 10-11 moles of phospholipid phosphorous per mole of the cytochrome b_5 and consisted of small vesicles having a diameter of 300-600 Å, as described in Part II. To a suspension of the complex (about 200 μM with respect to cytochrome b_5) in 0.1 M acetate buffer (pH 5.6-5.9) was added

Cpase Y to make the weight ratio of CPase Y to cytochrome 1:50 to 1:100, and the mixture was incubated at 30° for 1 or 2 h. The mixture was then heated for 5 min in a boiling water bath and lyophilized. The free amino acids contained in the lyophilized sample were analyzed and the results obtained are shown in Table I, which shows that about 25 amino acid residues were released from 1 mole of cytochrome b_5 in the complex by the action of CPase Y. The results obtained in three digestion experiments, in which the CPase Y to cytochrome ratio and incubation time were varied, were essentially the same with one another, suggesting that the action of CPase Y became limited when about 25 residues had removed from the COOH-terminal end of the cytochrome. The yield of aspartic acid, the COOH-terminal residue of rabbit cytochrome b_5 (see Part II), was about 2 moles per mole of the cytochrome. It was thus certain that the COOH-termini of all the cytochrome molecules in the complex had been attacked by CPase Y. These observations suggested that the COOH-terminal portion of cytochrome b_5 in the complex was extruded to the aqueous phase from the outer surface of the liposomal membrane, because CPase Y is a macromolecule having a molecular weight of 61,000 (19) and therefore does not seem to penetrate the phospholipid membrane.

To confirm the impermeability of the vesicles to macromolecules, the cytochrome b_5-liposome complex was subjected to isopycnic centrifugation in both sucrose and Dextran T-40 (average molecular weight, 40,000) density gradients, essentially

Table I. Amino Acids Released from Cytochrome b_5-Egg PC Liposome Complex by CPase Y. The digestion and analysis of released amino acids were performed as described in Materials and Methods, and the average of three determinations are shown.

Amino Acid	residue/mole cytochrome b_5	
Aspartic acid	2.0	(2)
Serine	1.5	(2)
Threonine	1.2	(1)
Glutamic acid	0.0	
Proline	1.0	(1)
Glycine	0.0	
Alanine	3.8	(4)
Valine	2.0	(2)
Methionine	1.5	(2)
Isoleucine	2.8	(3)
Leucine	2.8	(3)
Tyrosine	1.9	(2)
Phenylalanine	0.0	
Lysine	0.0	
Histidine	0.0	
Arginine	0.9	(1)
Tryptophan	1.9	(2)
Total	25	

as described by Ito and Sato (8). As can be seen in Figs. 1A and 1B, the buoyant density of the complex in the dextran density gradient (d=1.094) was definitely lower than that in the sucrose gradient (d=1.151). As pointed out by Kamat and Wallach (20), this observation is consistent with the view that the vesicles were impermeable to Dextran T-40, though they permitted the entry of sucrose. It could be concluded that CPase Y, being a macromolecule, could not enter the inside of the vesicles and attacked cytochrome $\underline{b}_5$ only from outside the membrane.

When the complex that had been digested with CPase Y was subjected to isopycnic centrifugation in the sucrose gradient under the same conditions above, the cytochrome $\underline{b}_5$ chromophore formed a broad band at an apparent buoyant density of 1.09 (Fig. 1C), a value which is lower than that for the untreated complex (d=1.151). Isopycnic centrifugation of the complex that had been incubated in the acidic medium (pH 5.6) for 3 h gave the same pattern as that for the unincubated complex (data not shown). Under these centrifugation conditions, it is expected that the micellar aggregate of cytochrome $\underline{b}_5$ would form a band at a density higher than that for the complex, and the hydrophilic fragment of cytochrome $\underline{b}_5$ would move much slower than the complex and cytochrome $\underline{b}_5$ micelles because of its monomeric state and therefore would not reach the density equilibrium. Since the result shown in Fig. 1C corresponded to that expected for the hydrophilic fragment, it was suggested that CPase Y treatment caused the detachment of the cytochrome from the complex and that the detached molecules existed in the monomeric state.

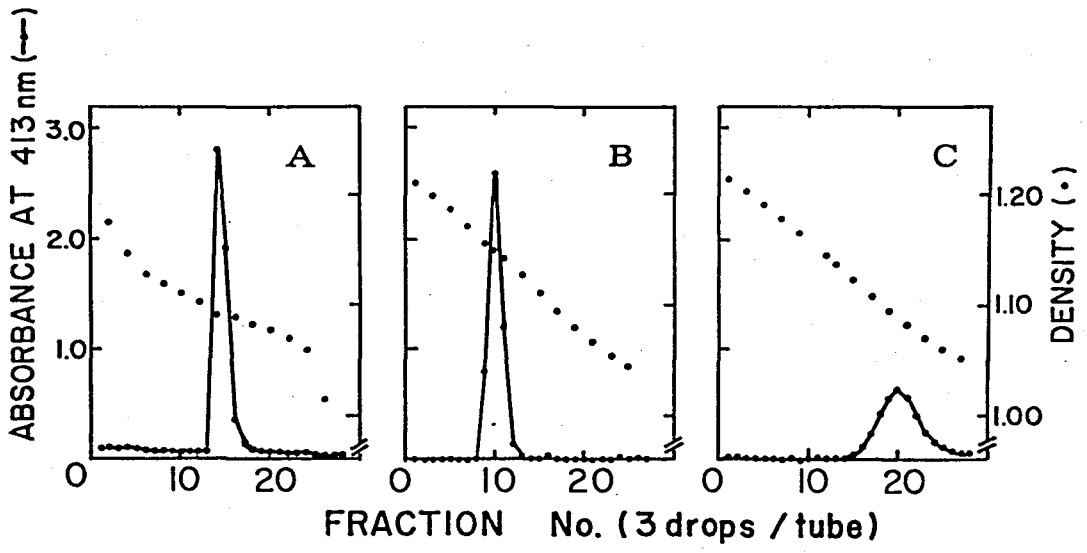

Fig. 1 Dextran and sucrose density gradient centrifugation of cytochrome $\underline{b}_5$-egg PC liposome complex. (A) about 0.15 ml of a suspension of the complex containing about 15 nmoles cytochrome $\underline{b}_5$ and 0.1 M acetate buffer (pH 5.6-5.9), the density of which had been adjusted to more than 1.12 with dextran, was placed at the bottom of a centrifuge tube, and 5 ml of a continuous density gradient of Dextran T-40 containing 0.1 M acetate buffer (pH 5.6) was layered over the sample. (B) About 0.15 ml of a suspension of the complex containing about 15 nmoles cytochrome $\underline{b}_5$ and 0.1 M acetate buffer (pH 5.6) was layered over 5 ml of a linear gradient of sucrose containing 50 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA. (C) About 0.1 ml of a suspension about 10 nmoles cytochrome $\underline{b}_5$ and 0.1 M acetate buffer (pH 5.6-5.9) which had been treated by CPase Y (1/50 w/w) at 30° for 2 h was layered over 5 ml of the same sucrose gradient. The tubes were centrifuged at 50,000 rpm for 50 h at 2° in an SW 65K rotor of a Beckman L-2 centrifuge. Three-drop fractions were collected from the bottom of the tubes and absorbance at 413 nm was measured.

It was, therefore, of interst to examine if the cytochrome in the CPase Y digest of the complex could rebind to PC liposomes. Thus, the CPase Y digest of the complex was delipidated with 90% acetone and passed through a Sephadex G-25 column. The cytochrome $\underline{b}_5$ prepration thus obtained was incubated with egg-PC liposomes at 0° overnight or at 37° for 30 min. Fig. 2 shows the result of sucrose density gradient centrifugation of the mixture incubated at 0°. As can be seen, almost all the material absorbing at 413 nm (Soret peak of oxidized cytochrome $\underline{b}_5$) showed a sedimentation rate which was identical with that of the hydrophilic fragment of cytochrome $\underline{b}_5$ (t-$\underline{b}_5$), indicating that the CPase Y-treated cytochrome $\underline{b}_5$ was unable to rebind to PC liposomes. Practically the same result was obtained with the mixture incubated at 37° (data not shown). Fig. 3 shows the elution pattern obtained when the concentrated CPase Y digest of the complex was subjected to gel chromatography on a Sephadex G-100 column (1.5 x 52 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. Almost all the 413 nm-absorbing material was eluted at a position corresponding to a molecular weight which is somewhat higher than that of t-$\underline{b}_5$. The small amount of the 413 nm-absorbing substance eluted at the void volume may be a mixture of PC and cytochrome $\underline{b}_5$ unattacked by the CPase Y. From these results it could be concluded that exogenously added CPase Y attacked the COOH-terminal portion of cytochrome $\underline{b}_5$ embedded in egg PC liposomes and liberated the cytochrome into the aqueous medium by excising about 25 amino acid residues and that the liberated cytochrome had lost the membrane-binding and micelle-forming capacities

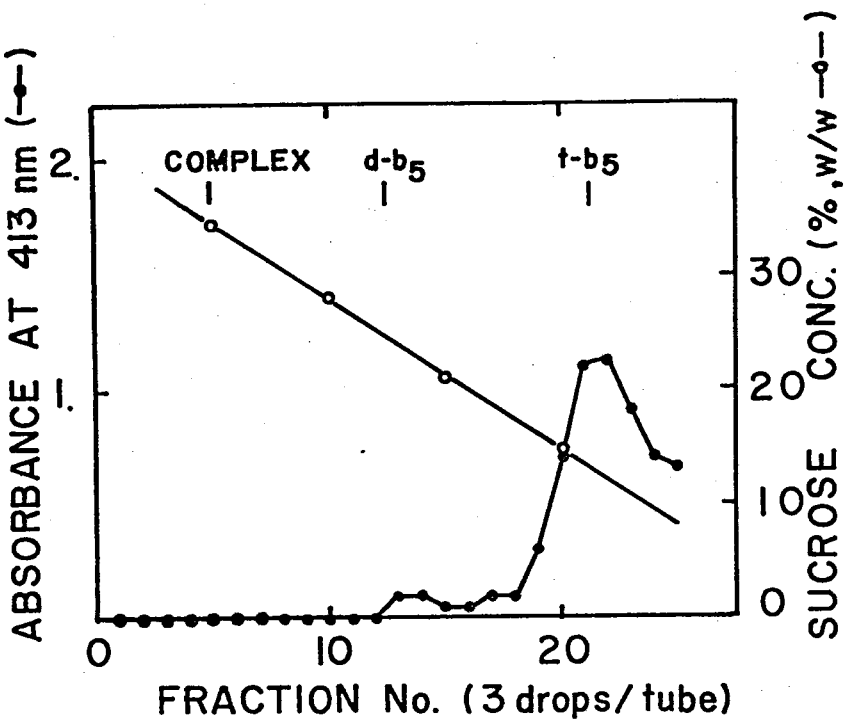

Fig. 2 Sucrose density gradient centrifugation of analysis of liposome-binding capacity of CPase Y-treated cytochrome $\underline{b}_5$. After the cytochrome $\underline{b}_5$-egg PC liposome complex (about 190 μM cytochrome $\underline{b}_5$) was treated with CPase Y (1/200 w/w)at 30° in 0.1 M acetate buffer (pH 5.6-5.9) for 1 h, the mixture was delipidated with 90% acetone and passed through a Sephadex G-25 column equilibrated with 50 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA. The cytochrome $\underline{b}_5$ thus obtained was concentrated with a Sartorius membrane filter. To the concentrated solution was added egg PC liposome (molar ratio, 1 cytochrome to 36 PC) and the mixture was allowed to stand at 0° overnight. About 0.1 ml of this mixture (about 20 nmoles cytochrome $\underline{b}_5$, 50 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA) was layered over a 5 ml of 5-45% (w/v) sucrose linear density gradient and centrifuged at 50,000 rpm for 20 h at 2° in an SW 65K rotor of a Beckman L-2 centrifuge. After centrifugation, three-drop fractions were collected from the bottom of the tube and absorbance at 413 nm was measured.

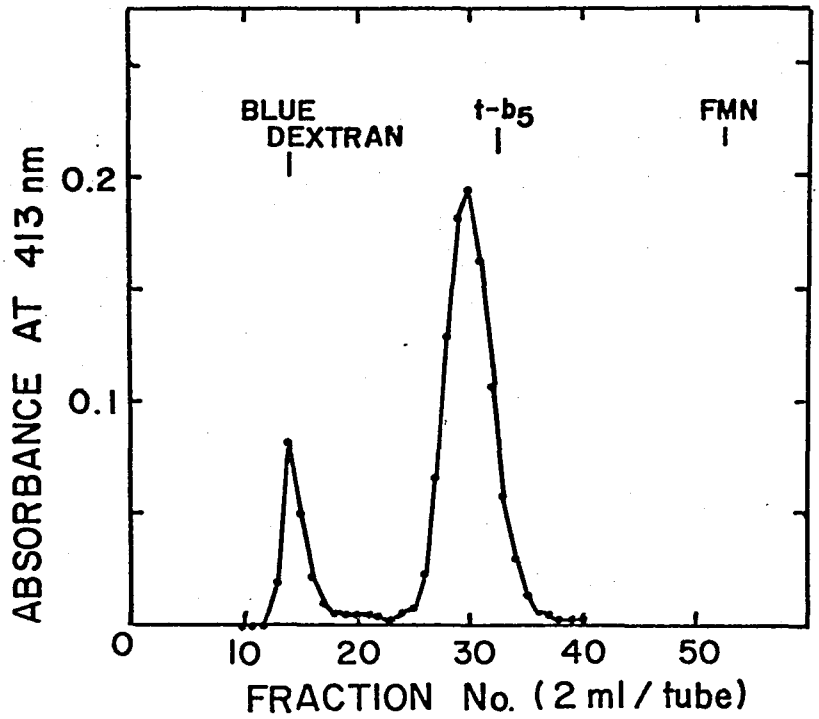

Fig. 3 Sephadex G-100 gel chromatography of CPase Y treated cytochrome $\underline{b}_5$(about 0.5 ml, 40 nmoles cytochrome $\underline{b}_5$) obtained after sucrose density gradient centrifugation in Fig. 2. was concentrated with a Sartorius membrane filter and directrly subjected to a Sephadex G-100 column (1.5 x 52 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA without treatment with 90% acetone. Fraction of 2 ml were collected and absorbance at 413 nm was measured. The column was calibrated by blue dextran 2000 (for determination of void volume), FMN (for determination of internal volume) and t-$\underline{b}_5$ (as molecular weight standard).

of the parent molecule.

How can CPase Y added exogenously attack the COOH-terminal portion of cytochrome $\underline{b}_5$ bound to egg PC vesicles which are impermeable to macromolecule? Three possibilities are conceivable and they are schematically illustrated in Fig. 4. The first possibility is that CPase Y attacks a small amount of unbound cytochrome $\underline{b}_5$ which is in equilibrium with that bound by the liposomes. In the second possibility it is assumed that the liposome-bound cytochrome exists in two different topological states which are in a dynamic equilibrium, i.e. one facing its COOH-terminus outside and the other inside the liposomal vesicle, and CPase Y attacks those molecules exposing the COOH-termini outside the membrane. Finally, it may be assumed as the third possibility that all the bound cytochrome molecules expose their COOH-termini outside the vesicles so that they are readily susceptible to the attack of exogenous CPase Y.

Among these possibilities, the first one deserves special attention, because it has been reported that exchange of cytochrome $\underline{b}_5$ can occur between phospholipid vesicles (21) and between the cytochrome $\underline{b}_5$-liposome complex and the micellar form of cytochrome $\underline{b}_5$ (11) in neutral pH's. To evaluate the validity of the first possibility, an attempt was made to measure the velocity of cytochrome $\underline{b}_5$ exchange between the cytochrome $\underline{b}_5$-liposome complex and cytochrome $\underline{b}_5$ micelles at pH 5.6-5.9 where the CPase Y digestion was conducted. For this purpose, ^{125}I-labeled cytochrome $\underline{b}_5$ and its complex with egg PC liposomes were prepared. Unlabeled cytochrome $\underline{b}_5$ micelles were mixed with an equivalent amount (with respect to the

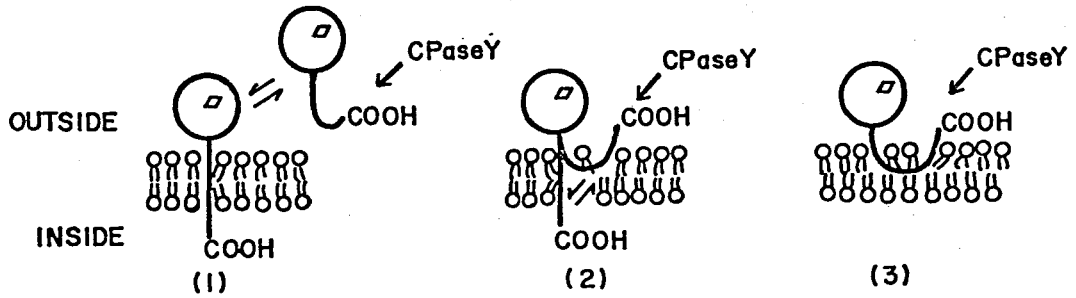

Fig. 4. Schematic illustration of three possible mechanisms by which exogenously added CPase Y attacks the COOH-terminus of cytochrome b_5 embedded in the membrane of PC liposomes.

cytochrome) of the labeled complex, and the mixture was incubated at pH 5.6-5.9 for 1 h at 30°. The cytochrome micelles were then separated from the complex by sucrose density gradient centrifugation, and ^{125}I radioactivities of the fractions obtained were counted. As shown in Fig. 5A, a significant radioactivity was detected in the cytochrome $\underline{b}_5$ micelle fraction, indicating that transfer of labeled cytochrome $\underline{b}_5$ from the complex to the micelles had occurred. Similarly, when labeled cytochrome $\underline{b}_5$ micelles were incubated with the unlabeled complex, there was a transfer of the label from the micelles to the complex (Fig. 5B). These results indicated that the exchange of cytochrome $\underline{b}_5$ did occur even at the acidic pH value and the rate of transfer from the micelles to the complex seemed to be apparently faster than that of the opposite direction. It was likely that this difference in apparent exchange rate was due to rapid binding of cytochrome $\underline{b}_5$ to the cytochrome—unsaturated complex present in the preparation. In support of this view the amount of liposome-bound cytochrome $\underline{b}_5$ was larger than that in the micellar state after incubation for 1 h (Fig. 5).

To estimate the velocity of exchange, equivalent amount of labeled cytochrome $\underline{b}_5$ and unlabeled liposome-bound cytochrome $\underline{b}_5$ were incubated at 30° and after appropriate time intervals the radioactivities in the two fractions were determined. In Fig. 6 are plotted the ratio of radioactivity (cpm) to the absorbance at 413 nm at the peak fractions of the micelles and the complex against the incubation time. From these results it was estimated that the time required for 50% exchange was about 10 h. The

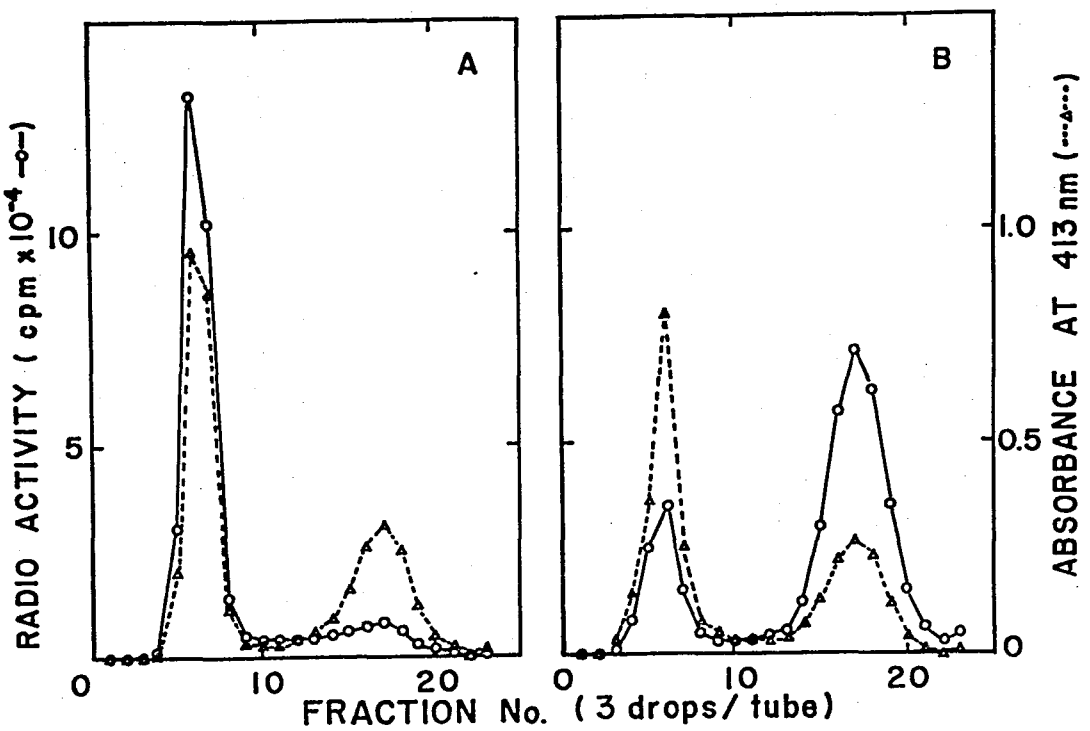

Fig. 5. Exchange of cytochrome $\underline{b}_5$ between egg PC liposomes and micelle state. Transfer of cytochrome $\underline{b}_5$ from liposomes to micelle state (A), and from micelle state to liposomes (B). About 0.15 ml of the sample containing the [^{125}I] cytochrome $\underline{b}_5$-egg PC liposome complex (120 μM with respect to cytochrome $\underline{b}_5$) and unlabeled cytochrome $\underline{b}_5$ (120 μM) (A) or cold cytochrome $\underline{b}_5$-egg PC liposome complex (120 μM with respect to cytochrome $\underline{b}_5$) and [^{125}I] cytochrome $\underline{b}_5$ (120 μM) (B) were incubated at 30° for 1 h in 0.1 M acetate buffer (pH 5.6-5.9). The suspensions were layered over 5 ml of sucrose linear density gradient (5-40%, w/v) and centrifuged at 28,000 rpm for 18 h at 2° in an RPS 50-II rotor of a Hitachi 55P centrifuge. After centrifugations three-drop fractions were collected from the bottom of the tube and absorbance at 413 nm and radioactivitiy were mesured.

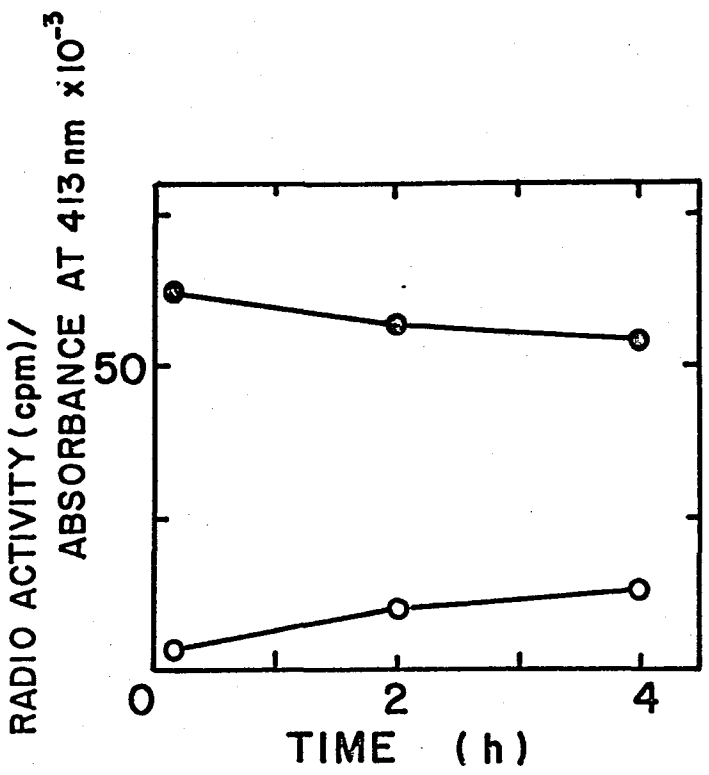

Fig. 6. Velocity of cytochrome b_5 exchange between egg PC liposome and micelle state. Incubation and sucrose density gradient centrifugation was performed as described in Fig. 5B, except for the incubation time was varied as indicated. The ratio of radioactivity (cpm) to absorbance at 413 nm of the peak fraction of micellar cytochrome b_5 (-●-) and that of the complex (-O-) were plotted against the incubation time at 30°.

maximal effect of CPase Y on the complex, on the other hand, was achieved less than 1 h at the same pH and temperature. Thus, the first possibility of CPase Y action on liposome-bound cytochrome $\underline{b}_5$ seems to be unlikely, if we assume the followings: 1) ^{125}I-labeled cytochrome $\underline{b}_5$ behaves identically with unlabeled one in binding, exchange, and other respects; 2) CPase Y hydrolyzes quickly the COOH-terminus of cytochrome $\underline{b}_5$ when it is released from the vesicles; 3) affinity of cytochrome $\underline{b}_5$ remains constant even if the binding density of cytochrome $\underline{b}_5$ to the vesicles is changed; and 4) Cpase Y cannot attack the cytochrome undergoing the inter-vesicle transfer. These assumptions will be examined in the Discussion section.

In the experiments described so far we used egg PC liposomes which are in "fluid" state at 30°. However, it seemed advantageous to use DPPC liposomes, which have the transition temperature at 41° and therefore exist in a "solid" state at 30°, to study the mechanism of CPase Y attack on liposome-bound cytochrome $\underline{b}_5$, because it has been reported that the transfer of NADH-cytochrome $\underline{b}_5$ reductase, another microsomal amphiphilic protein, embedded in DPPC liposomes to other vesicles can hardly occur within 2 h at 30° in a neutral medium (22). At 30°, therefore, it is expected that the velocity of cytochrome $\underline{b}_5$ detachment from DPPC liposome as postulated in Fig. 4 (1) would be extremely slow. Furthermore, in DPPC liposomes at 30° the achievement of the topological equilibrium assumed in the second possibility (fig. 4 (2)) would also be an extremely slow process. If this is so, then the attack of CPase Y on DPPC liposome-bound cytochrome $\underline{b}_5$ by the mechanisms postulated in the first and second

possibilities should be much slower at 30° than that on egg PC liposome-bound cytochrome $\underline{b}_5$.

Prior to conducting CPase Y digestion, we examined if the vesicles of DPPC-cytochrome $\underline{b}_5$ complex was permeable to macromolecules by isopycnic centrifugation in sucrose and Dextran T-40 density gradients. As can be seen in Fig. 7, the buoyant densities of the complex obtained in the sucrose and dextran gradients were 1.133 and 1.904, respectively. The difference in buoyant densities in the two gradients also indicated that the vesicles were impermeable to macromolecules. The density of the cytochrome $\underline{b}_5$-DPPC liposome complex obtained in the sucrose gradient (d=1.133) was lower than the corresponding value for the cytochrome $\underline{b}_5$-egg PC liposome complex (d=1.151), in agreement with the higher molar ratio of the cytochrome to phospholipid (1:34) in the former than that (1:10-11) in the latter. Fig. 7B shows that the cytochrome $\underline{b}_5$-DPPC liposome complex formed a rather broad peak with a small shoulder. It was thus likely that the complex was somewhat heterogeneous with respect the molar ratio of the cytochrome to DPPC.

When the cytochrome $\underline{b}_5$-DPPC complex prepared at 4° was digested with CPase Y (1/20 of the cytochrome by weight) at 10° for 8.5 h and then subjected to sucrose density gradient centrifugation, a peak formed at the position corresponding to that of the intact complex, indicating that no liberation of cytochrome $\underline{b}_5$ from the complex took place under these conditions (Fig. 8A). Upon digestion of the same sample at 30° for 3 h, however, all the cytochrome was detached from the complex and sedimented much slower than cytochrome $\underline{b}_5$ micelles (d-$\underline{b}_5$),

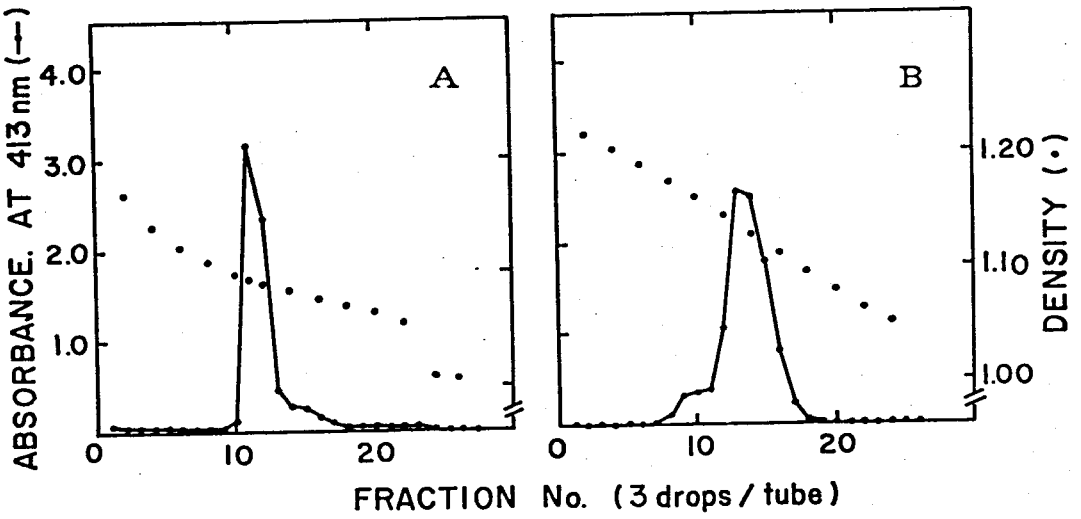

Fig. 7. Dextran and sucrose density gradient centrifugations of cytochrome $\underline{b}_5$-DPPC liposome complex. Density gradient centrifugation was performed as described in Fig. 1, except for the cytochrome $\underline{b}_5$-DPPC liposome complex containing about 20 nmoles of cytochrome was used and centrifugation was carried out in an RPS 50-II rotor of a Hitachi 55P centrifuge. (A), in Dextran T-40 gradient; (B) in sucrose gradient.

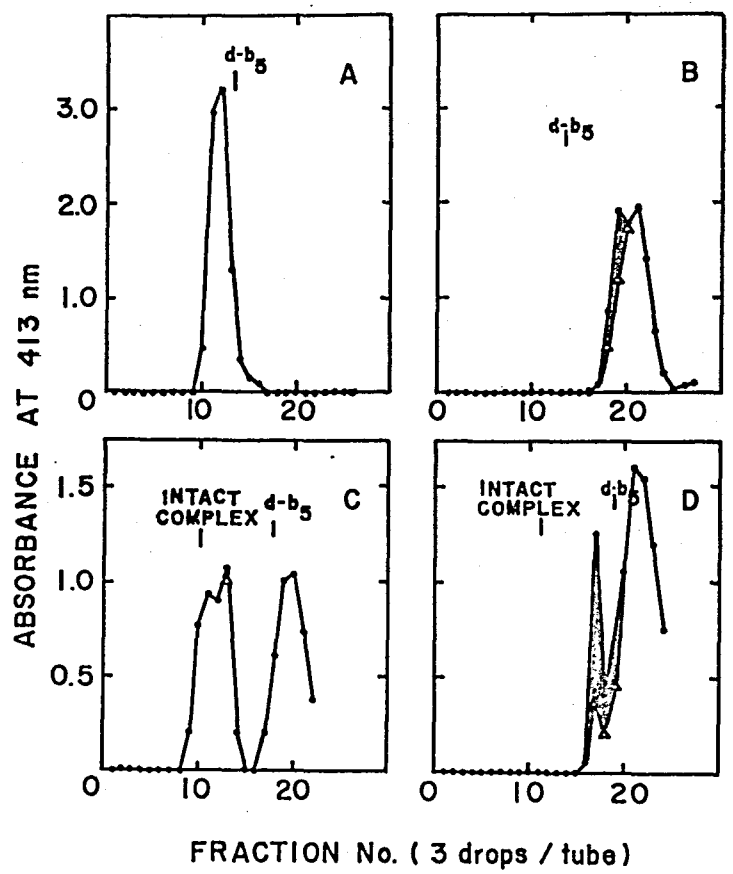

Fig. 8. Sucrose density gradient centrifugation analysis of the effects of CPase Y treatment of the cytochrome $\underline{b}_5$-DPPC liposome complex. The binding of cytochrome $\underline{b}_5$ to the liposomes was carried out either at 4° for 48 h (for A and B) or at 45° for 1 h after incubation at 4° overnight (for C and D). The complex thus formed containing 20 μM (for A and B) or 200 μM cytochrome $\underline{b}_5$ (for C and D) was treated with CPase Y (1/20, w/w) in 0.1 M acetate buffer (pH 5.6-5.9) at 10° for 8.5 h (A), at 30° for 2 h(B), at 13° for 6.5 h (C), and at 30° for 3 h (D). After the digestion, about 0.1 ml of the digest containing 2-8 nmoles of cytochrome $\underline{b}_5$ was layered over 5 ml of linear sucrose density gradient from 5 to 45% (w/v) and centrifuged at 50,000 rpm for 20 h (A and B) or at 40,000 rpm for 14 h (C and D) in an RPS 50-II rotor of a Hitachi 55P centrifuge. After centrifugation, three-drop fractions were collected from the bottom of the tube and their absorbance at 413 nm were measured after appropriate dilution. The absorbance at 413 nm of turbid fractions was measured before (-●-) and after (-Δ-) clarification with 10 μl of 10% Triton X-100.

suggesting that it was in the monomeric state (Fig. 8B). At the same time a turbid band, presumably due to DPPC liposomes which had lost bound cytochrome $\underline{b}_5$, was observed just beneath the cytochrome $\underline{b}_5$ band. The cytochrome $\underline{b}_5$-DPPC complex was also prepared by incubating the components at 4° overnight followed by further incubation at 45° for 1 h. When the complex thus prepared was treated with CPase Y at 13° for 6.5 h, about half of the cytochrome was detached and converted to the monomeric state, and the remaining complex showed a certain degree of heterogeneity (Fig. 8C). This heterogeneity of the vesicles may have been caused by different extents of cytochrome $\underline{b}_5$ excision. Despite the fact that cytochrome $\underline{b}_5$ had been partially excised under these conditions, no turbidity appeared, suggesting that the turbid band seen in Fig. 8B could be formed after all the cytochrome was detached from the vesicles. The different behavior of the complex between Figs. 8A and 8C seemed to be due to the lower concentration (20 μM) of the complex used in Fig. 8A than in Fig. 8C (200 μM) rather than to the temperature at which the complex was prepared. When the same complex was digested at 30° for 3 h, almost all the cytochrome was released from the complex and the formation of a turbid band was observed as in the case of Fig. 8B. In this respect, no difference was noticed between the complex prepared at 4°, a temperature lower than the transition temperature of DPPC, and that prepared at 45°, which is higher than the transition temperature. This suggested that the mode of binding of cytochrome $\underline{b}_5$ to DPPC liposomes may be the same whether the fatty acyl chains in the

bilayer are in a "fluid" state or in a "solid" (semicrystalline) state. When the cytochrome $\underline{b}_5$-DPPC liposome complex was incubated at 30° for 3 h in 0.1 M acetate buffer (pH 5.6-5.9) in the absence of CPase Y, the sample showed a single peak in sucrose density gradient centrifugation and its density corresponded to that of the unincubated complex. At any rate, the finding that cytochrome $\underline{b}_5$ complex with DPPC liposomes could be readily released from the complex (Fig. 8B and 8D) were in contrast to the expectation described above and seemed to exclude the mechanisms of CPase Y action postulated in the first and second possibilities (Fig. 4 (1) and (2)). This leads to the conclusion that the third possibility (Fig. 4 (3)) is most likely as the mechanism accounting for the attack of CPase Y on liposome-bound cytochrome $\underline{b}_5$

DISCUSSION

The results reported in this paper indicate that CPaseY added exogenously can release about 25 amino acid residues from the COOH-terminal end of rabbit cytochrome $\underline{b}_5$ which is incorporated into PC liposomes and this attack results in the detachment from the liposomal membrane of a form of cytochrome $\underline{b}_5$ which is incapable of rebinding to the membrane. Since the vesicles of cytochrome $\underline{b}_5$-PC liposome complex is impermeable to macromolecules (Fig. 1), it is clear that CPase Y can attack the COOH-terminus of cytochrome $\underline{b}_5$ only from outside the vesicles. As schematically illustrated in Fig. 4, three possibilities are conceivable

about the mechanism by which this attack can be effected. In the first possibility it is assumed that a small amount of cytochrome $\underline{b}_5$ which exists in the aqueous phase in equilibrium with the liposome-bound cytochrome is attacked by CPase Y. This possibility can be excluded if the rate of detachment of cytochrome $\underline{b}_5$ from the vesicle is much slower than that of CPase Y attack. We have estimated this rate by measuring the velosity of cytochrome $\underline{b}_5$ exchange between liposome-bound cytochrome $\underline{b}_5$ and cytochrome $\underline{b}_5$ micelles and shown that the exchange rate is actually much slower than that of CPase Y-induced liberation of cytochrome $\underline{b}_5$ from the vesicles (Fig. 5). If the four assumptions described in the Results section are tentable, this observation shows that the first possibility is not valid. In what follows these four assumptions will be examined in some details.

Assumption 1. In the exchange experiments described ^{125}I-labeled cytochrome $\underline{b}_5$ was used to measure the exchange velosity. However, if the exchange rate of the labeled cytochrome is slower than that of the unlabeled one, the results of measurement will be of no use for the purpose. Since labeled cytochrome $\underline{b}_5$ can move from the micelles to the vesicles as well as to the oppsite direction (Fig. 5), it seems that affinity of labeled cytochrome $\underline{b}_5$ for the vesicles is not very much different from that of the unlabeled one. It is also shown that the binding properties of labeled cytochrome $\underline{b}_5$ to egg PC liposomes is similar to that of the unlabeled cytochrome. Assumption 2. If CPase Y does not always attack the cytochrome detached from the vesicle,

the velosity of cytochrome $\underline{b}_5$ transfer from the vesicle to the micelle must be faster than 1 h, a period which is sufficient for CPase Y to liberate all the cytochrome molecules from the vesicle, to suppose the mechanism assumed in the first possibility. Assumption 3. In the binding experiment the double reciprocal plot of the concentration of cytochrome $\underline{b}_5$ added in the mixture against the amount of cytochrome $\underline{b}_5$ bound by microsomes is linear giving a dissociation constant of 2×10^{-5} (23). This suggests that the affinity of cytochrome $\underline{b}_5$ for microsomes remains constant regardless of the amount of the cytochrome bound. Although the binding behavior of cytochrome $\underline{b}_5$ to egg PC liposomes is rather complicated (24), it is difficult to assume that the affinity of cytochrome $\underline{b}_5$ becomes lower when the amount of bound cytochrome $\underline{b}_5$ becomes smaller and that detachment of the cytochrome is accelerated when the amount of liposome-bound cytochrome $\underline{b}_5$ becomes smaller. Assumption 4. It seems unlikely that cytochrome $\underline{b}_5$ undergoes specific transfer between vesicles rather than between a vesicle and a micelle. However, if the vesicle-to-vesicle transfer does exist and its velocity is slow enough to permit CPase Y to attack the cytochrome during the transfer, it is possible to explain the experimental results mainly by the mechanism postulated in the first possibility. As a matter of fact, the occurrence of such a vesicle-to-vesicle transfer has been reported in neutral pH's (21), though its velosity has not yet been measured. If this transfer takes place by direct collision of vesicles, it seems difficult for CPase Y to attack the

cytochrome during the transfer. On the other hand, if this transfer occurs via a pool of monomeric or micellar cytochrome $\underline{b}_5$, the transfer velocity should be reflected in the exchange velocity between the vesicle and micelle.

From these considerations, it may be concluded that the mechanism postulated in the first possibility (Fig. 4 (1)) is not operating, but CPase Y attacks the liposome-bound cytochrome directly. It has been reported that the exchange of NADH-cytochrome $\underline{b}_5$ reductase between DPPC liposome is extremely slow below the transition temperature of the phospholipid (22). As shown in Fig. 8, however, CPase Y can attack and liberate cytochrome $\underline{b}_5$ from the cytochrome-DPPC liposome complex at sufficient rates even below the transition temperature of DPPC. This observation excludes not only the mechanism assumed in the first possibility but also that in the second possibility, as already discussed. Furthermore, the COOH-terminal residue of rabbit cytochrome $\underline{b}_5$ is aspartic acid which is hydrophilic in nature, and the seventh residue from the COOH-terminus seems to be arginine by analogy with the sequences determined for horse and porcine cytochrome $\underline{b}_5$ (25,26). It is,therefore, unlikely that the COOH-terminus of liposome-bound cytochrome $\underline{b}_5$ is in a dynamic equilibrium between the two states, i.e. one exposed to outside and the other to inside the membrane, as postulated in the second possibility. In conclusion, it can be said that the COOH-termini of all the cytochrome $\underline{b}_5$ molecules embedded in PC liposomes are exposed to the outer aqueous phase surrounding the vesicle and are attacked by CPase Y directly.

The liberation of cytochrome $\underline{b}_5$ from the cytochrome-liposome complex by the action of CPase Y is accompanied by the removal of about 25 amino acid residues from the COOH-terminal end of the cytochrome molecule (Table I), and the cytochrome thus liberated is capable of neither forming a micelle in aqueous solution nor rebinding to phospholipid liposomes. These observations indicate that the CPase Y can excise the entire segment of the cytochrome which are directly interacting with the liposomal membrane. This implies that CPase Y can attack the hydrophobic residues which are embedded within the membrane matrix. Although the mechanism by which this can be achieved is not clear, one possibility is to assume that the cleavage of the hydrophilic residues at the COOH-terminal end leads to the exposure of the COOH group of the embedded hydrophobic residue to the membrane surface and thus makes it possible for CPase Y to hydrolyze this residue resulting in the exposure of the COOH group of the next residue. It has also been shown that the attack of CPase Y becomes limited when about 25 amino acid residues have been removed from the COOH-terminus for unknown reasons. Amino acid analysis of the liberated cytochrome also indicates that its peptide length is shorter than that of intact cytochrome $\underline{b}_5$ by about 25 residues (data not shown).

It is of interest to consider which portion of the COOH-terminal segment composed of 25 amino acid residues is actually embedded in the liposomal membrane. Recent partial elucidation of the primary structure of the hydrophobic tail of rabbit cytochrome $\underline{b}_5$ indicates that its COOH-terminal sequence is

-Arg-Leu-Tyr-Met-Ala-Asp-Asp-COOH (unpublished results). Because of the hydrophilic nature of the arginine residue, it does not seem unreasonable to assume that this COOH-terminal segment consisting of 7 residues is exposed to the outerside of the lipid bilayer. If this is so, then it has to be concluded that a segment consisting of 18 residues is responsible for direct interaction with the hydrophobic interior of the membrane. According to the unpublished study mentioned above, this 18-residue segment is highly hydrophobic except for the presence of one serine residue, the polarity of which may be greatly reduced by hydrogen bond formation. The length of a peptide composed of 18 residues is not sufficient to return to the outer surface after penetrating the hydrocarbon region of the lipid bilayer membrane (30-40 Å) even if its conformation is stretched and has no helical region. As a matter of fact, it is more likely that this segment contains helical region(s) in view of the finding reported in Part II that the helical content of the hydrophobic tail is about 40-50%. It is, therefore, concluded that the hydrophobic segment of cytochrome $\underline{b}_5$ does not span the lipid bilayer membrane. This conclusion is consistent with the report of Dehlinger *et al.* (27) that the hydrophobic segment of cytochrome $\underline{b}_5$ is not so hydrophobic as the hydrocarbon chain region of the lipid bilayer. It is also consistent with the finding by Enomoto and Sato (28) that asymmetric binding of cytochrome $\underline{b}_5$ to human erythrocyte ghost is due to the preferential localization of cholesterol in the outer leaflet of the bilayer of the ghost membrane, suggesting that

cytochrome $\underline{b}_5$ can interact only with the inner leaflet of the membrane.

From all the considerations described above, it can be concluded that upon binding of cytochrome $\underline{b}_5$ to PC liposomes its COOH-terminal portion, like the heme-containing, hydrophilic head, is extruded to the aqueous phase from the outer surface of the liposomal vesicle, and its membrane-interacting segment does not span the membrane. NADH-cytochrome $\underline{b}_5$ reductase, another amphiphilic microsomal protein, also binds to liposomes in a similar way (29). Since newly synthesized molecules of cytochrome $\underline{b}_5$ and NADH-cytochrome $\underline{b}_5$ reductase released from ribosomes are inserted into the membrane of endoplasmic reticulum from the cytosolic side, it is likely that the mode of cytochrome $\underline{b}_5$ binding to PC liposomes reflect the native binding state in microsomes. The mechanism by which cytochrome $\underline{b}_5$ is liberated from the liposomal membrane by the action of CPase Y is unknown. But it is very interesting that a hydrophilic exopeptidase, CPase Y, can liberate a membrane protein from membranes. This type of event may also occur *in vivo* in lysosomes or in other organelles.

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ACKNOWLEDGMENTS

I am greatly obliged to Prof. R. Sato for his guidance and encouragement throughout this work and indebted to Drs. K. Mihara and K. Enomoto for their valuable discussions and help in various phases of this study. It is also a pleasure for me to acknowledge the numerous cooperations and kindness extended by many other people, without whose help this work would not be accomplished. Thus, I would like to thank Drs. T. Takagi and K. Yutani for instructions and discussions on CD measurements, Drs. K. Hamaguchi and T. Azuma for discussions on two-domain structures of proteins, Dr. S. Iwanaga, Mr. Y. Hokama, and Mr. K. Kondo for instructions on and cooperations in sequence studies, Dr. A. Asano for performing negative-staining electron microscopic experiments, and Mrs. K. Shimizu for performing gas-liquid chromatography. Amino acid analyses reported in this study were kindly conducted by Mrs. M. Matsuyama, Miss K. Fukunishi, and Miss Y. Yagi, to whom I am obliged.