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Reduced Nicotinamide Adenine Dinucleotide-Cytochrome b_5 Reductase: Location of the Hydrophobic, Membrane-Binding Region at the Carboxyl-Terminal End and the Masked Amino Terminus†

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ABSTRACT: Microsomal NADH-cytochrome b_5 reductase is an amphiphilic protein consisting of a hydrophilic (catalytic) region and a hydrophobic (membrane-binding) segment. Digestion of the reductase purified from rabbit liver microsomes with carboxypeptidase Y (CPY), but not with aminopeptidases, resulted in the abolishment of the capacities of the reductase to bind to phosphatidylcholine liposomes and to reconstitute an active NADH-cytochrome *c* reductase system upon mixing with cytochrome b_5. The NADH-ferricyanide reductase activity of the flavoprotein was, however, inactivated only slightly by the CPY digestion. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analyses indicated that the CPY treatment removed about 30 amino acid residues from the COOH terminus of the reductase and that about 70% of the amino acids released were hydrophobic. It is concluded that the hydrophobic region of the reductase, responsible for both membrane binding and effective reconstitution of NADH-cytochrome *c* reductase activity, is located at the COOH-terminal portion of the molecule. No NH_2-terminal residue could be detected in the intact and CPY-modified reductase preparations. The location of the hydrophobic, membrane-binding segment at the COOH-terminal end and the masked NH_2 terminus have also been reported for cytochrome b_5, another microsomal membrane protein.

The hepatic microsomal flavoprotein, NADH-cytochrome b_5 reductase, is an amphiphilic protein consisting of a hydrophilic moiety carrying the FAD prosthetic group and a hydrophobic segment (Spatz & Strittmatter, 1973; Mihara & Sato, 1975). It is composed of a single peptide chain having a molecular weight of about 33 000 and exists in aqueous solution as an oligomeric aggregate possessing an apparent molecular weight of about 360 000 (Mihara & Sato, 1975). The hydrophobic segment has been shown to be required for the tight binding of the reductase to various natural and artificial lipid bilayer membranes (Rogers & Strittmatter, 1974; Mihara & Sato, 1975) and for effective reconstitution of NADH-cytochrome *c* reductase activity upon mixing with the intact form of cytochrome b_5 (Mihara & Sato, 1972, 1975; Okuda et al., 1972). Digestion of liver microsomes with lysosomes (Takesue & Omura, 1970a) or a lysosomal acid protease (St. Louis et al., 1970) leads to solubilization of a hydrophilic, FAD-containing fragment of the reductase. This fragment has a molecular weight of about 27 000 and retains a high NADH-ferricyanide reductase activity (Takesue & Omura, 1970b). However, nothing is known of the location of the hydrophobic segment having the aforementioned properties in the structure of the reductase. In this paper, we report evidence obtained by the use of CPY[1] as modifying agent that the COOH-terminal portion of the reductase molecule constitutes the hydrophobic segment. We also report that the NH_2-terminal residue of the reductase is chemically masked. These observations are of special interest in view of the fact reported by Ozols (1974) that cytochrome b_5, another microsomal amphiphilic protein (Ito & Sato, 1968; Spatz & Strittmatter, 1971), also has its hydrophobic, membrane-binding segment at the COOH-terminal end and a masked NH_2-terminal residue.

Experimental Procedure

Materials. The intact forms of NADH-cytochrome b_5 reductase and cytochrome b_5 were purified from rabbit liver microsomes to homogeneity as described by Mihara & Sato (1975) and Spatz and Strittmatter (1973), respectively. The purified preparations were free from detergents and phospholipids. A hydrophilic, FAD-containing fragment of NADH-cytochrome b_5 reductase ("cathepsin-solubilized reductase") was purified also from rabbit liver microsomes as follows. Microsomes (36.7 g of protein) were digested with partially purified cathepsin D (1.96 g of protein) in 2.36 L of 0.1 M Tris–maleate buffer (pH 5.6) containing 1 mM EDTA at 37 °C for 3 h and then the mixture was centrifuged at 65 000*g* for 1 h. The reductase thus solubilized and recovered in the supernatant fluid was purified by ammonium sulfate fractionation, Sephadex G-100 gel chromatography, and DEAE-cellulose column chromatography as described by Takesue & Omura (1970b). The concentrated reductase preparation thus obtained (in 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl) was diluted 10-fold with distilled water and applied to a CM-Sephadex C-50 column equilibrated with 10 mM sodium phosphate buffer (pH 6.5). After

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[1] Abbreviations used are: CPY, carboxypeptidase Y; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism.

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extensive washing of the column with the same buffer, the reductase was eluted with 60 mM sodium phosphate buffer (pH 7.5). The fractions containing NADH-ferricyanide reductase activity were pooled and concentrated by means of a collodion bag. The reductase preparation thus obtained was homogeneous on $NaDodSO_4$–polyacrylamide gel electrophoresis and had a NADH-ferricyanide reductase activity of 2280 units per mg of protein. Its molecular weight was estimated to be about 27 000 by $NaDodSO_4$–polyacrylamide gel electrophoresis. Cathepsin D was partially purified from 2.5 kg of fresh porcine livers up to the second acetone fractionation step of the method of Barret (1967). CPY from bakers' yeast was purchased from Oriental Yeast Co., Tokyo. Hog kidney leucine aminopeptidase (type III-PC) was obtained from Sigma and used after treatment with phenylmethanesulfonyl fluoride as described by Potts (1967). Aminopeptidase M was purchased from Protein Research Foundation, Osaka. Multilamellar liposomes of egg-yolk phosphatidylcholine were prepared as described (Mihara & Sato, 1975).

Enzyme Assays. NADH-cytochrome b_5 reductase was determined by measuring its NADH-ferricyanide reductase activity (Mihara & Sato, 1972) or from the intensity of the absorption peak of the oxidized flavoprotein at 461 nm, assuming a millimolar extinction coefficient of 11.3. NADH-cytochrome *c* reductase activity of the reconstituted system was assayed by the method of Okuda et al. (1972). One unit of both activities was defined as the amount catalyzing the reduction of 1 μmol of ferricyanide or cytochrome *c* per min. The reconstitution of NADH-cytochrome *c* reductase activity from NADH-cytochrome b_5 reductase and cytochrome b_5 was conducted in the presence of 0.002% Triton X-100 as described by Mihara & Sato (1975). The efficiency of reconstitution was expressed in terms of the ratio of NADH-cytochrome *c* reductase activity (V_c) to NADH-ferricyanide reductase activity (V_f) of the reconstituted system, as described by Mihara & Sato (1975). All the enzyme assays were performed at room temperature.

Analytical Methods. Protein was determined by the method of Lowry et al. (1951). Amino acid analyses of the intact and cathepsin-solubilized forms of NADH-cytochrome b_5 reductase were performed using a JEOL JIC-5AH amino acid analyzer after acid hydrolysis of the sample for 24, 48, and 72 h according to the method of Spackman et al. (1958). Half-cystine was determined on the 24-h hydrolyzate of the performic acid oxidized sample as described by Hirs (1967), and tryptophan was estimated by the fluorimetric method of Pajot (1976). For analysis of amino acids released from the reductase by the action of CPY, an aliquot of the digestion mixture (containing 5 to 20 nmol of the reductase) was withdrawn, boiled for 5 min, and centrifuged. The supernatant fluid containing the released amino acids was lyophilized and subjected to amino acid analysis in a Hitachi KLA-3B analyzer by the method of Spackman et al. (1958). The method of Benson et al. (1967) was, however, used for determination of serine, threonine, asparagine, and glutamine. The dansyl–Edman method described by Weiner et al. (1972) was employed to determine NH_2-terminal residue. The intact and cathepsin-solubilized forms of the reductase were directly subjected to dansyl–Edman degradation. In the case of CPY-digested reductase, the digested mixture was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and the band corresponding to the digested reductase was extracted from the gel with 5 mM sodium phosphate (pH 9.8) containing 0.05% $NaDodSO_4$ (Weiner et al., 1972). The extracted protein was then analyzed for the NH_2-terminal residue. Identification of dansylated amino acids was performed by two-dimensional ascending chromatography on polyamide sheets as described by Hartley (1973); NH_3:H_2O (1:100, v/v) and formic acid:l-butanol:heptane (1:10:10, v/v/v) were used as solvents for the first and second dimensions, respectively. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out in the presence of 1.0% $NaDodSO_4$ according to the procedure of Hinman & Philips (1970); gels were stained with Coomassie blue R-250. Far-ultraviolet CD spectra of the reductase preparations were measured in a JASCO J-20 spectropolarimeter equipped with CD attachment (calibrated with *d*-10-camphorsulfonic acid). The concentration of the reductase preparations was 1.95–2.24 μM and the solvent was composed of 20 mM Tris-HCl buffer (pH 8.0), 0.1 M KCl, 1 mM EDTA, and 0.5% Emulgen 109P (a nonionic detergent, Kao-Atlas Co., Tokyo). A quartz cell of 1.0-mm optical path was used and measurements were performed at 20 °C. The contents of secondary structures in the proteins were estimated from the CD data by using the values given by Chen et al. (1972). The fractions of α helix, β, and unordered forms were analyzed by the least-square method using the ellipticity values at 31 points between 215 and 245 nm, and the calculations were made by using an Acos Series-77 NEAC system 700 computer of Osaka University.

Digestion of Reductase with Exopeptidases. A standard reaction mixture for CPY digestion contained, in a final volume of 0.36 mL, 6 μM NADH-cytochrome b_5 reductase, 6 μg of CPY (corresponding to 8.3% of the reductase protein), and 60 mM sodium acetate buffer (pH 5.6). In some experiments, 0.009% Triton X-100 was also included. The digestion was carried out at 37 °C and at intervals 60 μL of the reaction mixture (containing 0.36 nmol of the reductase) was withdrawn and used for measuring the ability of the reductase to bind to phosphatidylcholine liposomes. A 10-μL portion of the mixture was also withdrawn and diluted 11-fold with 0.1 M potassium phosphate buffer (pH 7.5), and 10 μL each of the diluted mixture (containing 0.0054 nmol of the reductase) was used for assay of NADH-ferricyanide reductase activity and for reconstitution of NADH-cytochrome *c* reductase activity. For digestion with leucine aminopeptidase, a mixture (0.36 mL) consisting of 0.46 μM NADH-cytochrome b_5 reductase, 12 μg of phenylmethanesulfonyl fluoride treated leucine aminopeptidase (corresponding to 67% of the reductase protein), 2 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 8.0) was incubated at 37 °C. At intervals, 25 μL of the mixture was assayed for NADH-ferricyanide reductase activity and another 10 μL (containing 0.0046 nmol of the reductase) was used for the reconstitution of NADH-cytochrome *c* reductase activity.

Binding of Reductase to Liposomes. The capacity of NADH-cytochrome b_5 reductase to bind to multilamellar liposomes of phosphatidylcholine was measured as follows. A mixture (2.56 mL) containing 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 0.3–0.4 nmol of the reductase, and 14 mg (dry weight) of liposomes was incubated at 37 °C for 15 min and then centrifuged at 105 000*g* for 30 min. The precipitated liposomes were resuspended to a volume of 1.5 mL in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The NADH-ferricyanide reductase activity of the suspension was taken as a measure of the reductase bound.

Isolation of CPY-Digested Reductase. A mixture (5.15 mL) containing 2.84 μM NADH-cytochrome b_5 reductase, 330 μg of CPY, and 0.1 M sodium acetate buffer (pH 5.6) was incubated at 37 °C for 120 min. The pH of the mixture was then adjusted to 8.0 with Tris base and Emulgen 109P was added to a concentration of 0.5%. This was applied to a Sephadex G-200 column (2.2 × 65 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl, 1 mM EDTA, and 0.5% Emulgen 109P. Elution was conducted with the same

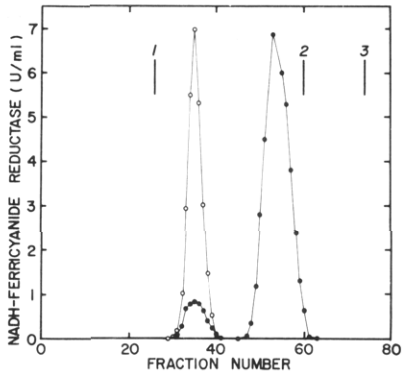

FIGURE 1: Sephadex G-200 gel chromatography of intact and CPY-digested NADH-cytochrome b_5 reductase. The reductase was digested with CPY and subjected to gel chromatography as described in text. The intact (undigested) reductase was also subjected to chromatography under the same conditions. Each fraction was assayed for NADH-ferricyanide reductase activity. (○) Intact reductase; (●) CPY-digested reductase. 1, 2, and 3 indicate the elution positions of blue dextran, cytochrome *c*, and FAD, respectively.

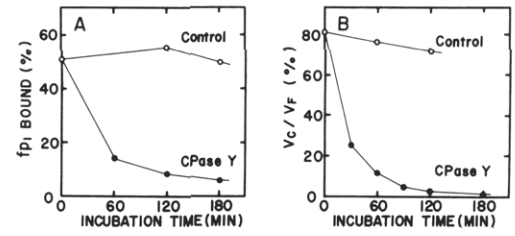

FIGURE 2: Effects of CPY digestion on the abilities of NADH-cytochrome b_5 reductase to bind to phosphatidylcholine liposomes and to reconstitute active NADH-cytochrome *c* reductase system upon mixing with intact cytochrome b_5 and 0.002% Triton X-100. The incubation and assays were made as described in Experimental Procedure. (●) Incubation with CPY; (○) incubation without CPY. (A) Changes in binding capacity (fp_1 stands for NADH-cytochrome b_5 reductase); (B) changes in reconstitution capacity (reconstitution efficiency is expressed in terms of V_c/V_f).

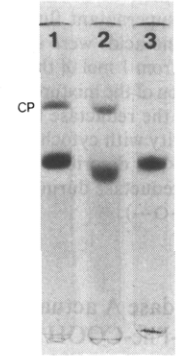

FIGURE 3: Modification of NADH-cytochrome b_5 reductase by CPY digestion as revealed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The digestion was done as in Figure 2, except that 4.77 μM reductase and 20 μg of CPY were used. The mixture (0.12 mL) was subjected to gel electrophoresis. Gel, 7.5%, was used: (gel 1) 0 incubation; (gel 2) 120-min incubation; (gel 3) 120-min incubation in the absence of CPY. The minor bands (marked "CP") in gels 1 and 2 were due to CPY added to the incubation mixture.

buffer at a rate of 10 mL per h, and 3-g fractions were collected. The NADH-ferricyanide reductase activity was eluted in two peaks, as shown in Figure 1. The fractions corresponding to the main peak were collected and concentrated by means of a collodion bag. The minor peak corresponded to the undigested reductase.

Results

CPY is an exopeptidase having a very wide substrate specificity and releases even proline from the COOH terminus of various proteins (Hayashi et al., 1973). As shown in Figure 2A, incubation of rabbit NADH-cytochrome b_5 reductase with CPY resulted in a rapid decline of the ability of the flavoprotein to bind to phosphatidylcholine liposomes, though no appreciable loss of the binding capacity was seen when incubated in the absence of CPY. The incubation both in the presence and absence of CPY also caused a slow decrease in the NADH-ferricyanide reductase activity of the flavoprotein (about 30% inactivation after incubation for 120 min), probably because of the instability of the reductase to the incubation temperature (37 °C) and the acidic medium (pH 5.6). However, the extent of this inactivation was much less than that of the binding activity. Figure 2B shows further that the CPY treatment also caused a sharp decrease in the ability of the flavoprotein to reconstitute an active NADH-cytochrome *c* reductase system upon mixing with the intact form of cytochrome b_5 and 0.002% Triton X-100 (Mihara & Sato, 1975). The time course of the decrease of the reconstitution activity nearly paralleled that of the binding activity. It was, therefore, suggested that a modification of the reductase at the COOH-terminal portion was responsible for the loss of both binding and reconstitution activities.

To obtain information concerning the nature of the modification, the reductase preparation was examined by $NaDodSO_4$–polyacrylamide gel electrophoresis before and after incubation with CPY. As shown in Figure 3, the protein band due to the reductase was slightly broadened and its mobility was somewhat increased after incubation. By using several molecular weight markers, it was estimated that the molecular weight of the reductase was decreased by 3000–4000 after incubation for 120 min, a period sufficient for almost complete loss of the reconstitution activity (cf. Figure 2B). No decrease in the molecular weight was detectable in the absence of CPY even after extensive incubation. Figure 3 also shows that the CPY treatment produced no peptide fragments other than the modified reductase, indicating that the CPY preparation used was free from contamination with endopeptidases. When the period of CPY digestion was extended to 270 min, no further decrease in the molecular weight was evident (data not shown), suggesting that the action of CPY became limited when a COOH-terminal segment of 3000 to 4000 daltons had been removed. It could be concluded that the abolishment of the binding and reconstitution activities was caused by the removal of this COOH-terminal peptide segment by the stepwise action of CPY.

Figure 4 shows the time course of CPY-induced release of amino acids from the reductase. In this experiment, 0.009% Triton X-100 was included in the digestion mixture, because the detergent was found to facilitate the attack of CPY on the reductase which exists in aqueous media as oligomeric aggregates. Actually, under this condition almost complete loss of the reconstitution activity was attained within 60 min, compared with 120 min observed in Figure 2B. It should be noted that the release of serine, threonine, asparagine, and glutamine is not illustrated in Figure 4, because these amino acids were not well resolved under the conditions employed in amino acid analysis and, therefore, could not be determined. Histidine, arginine, aspartate, glutamate, and cysteine were not released at all by the action of CPY. Among the amino acids released, phenylalanine seemed to be the first residue released, followed by alanine or leucine. Preliminary experi-

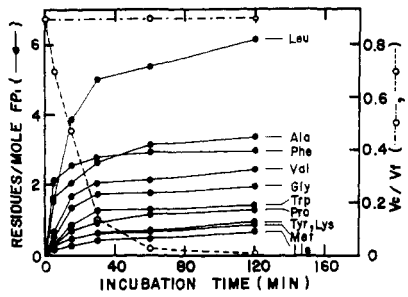

FIGURE 4: Time course of amino acid liberation from intact NADH-cytochrome b_5 reductase by the action of CPY. A mixture (11.3 mL) containing 5.4 μM reductase, 179 μg of CPY (8.8% of the reductase protein), 0.009% Triton X-100, and 0.1 M sodium acetate buffer (pH 5.6) was incubated at 37 °C. After incubation for 5, 15, 30, 60, and 120 min, 4, 3, 2, 1, and 1 mL of the mixture, respectively, were withdrawn and boiled for 5 min, and the supernatant fluids were analyzed for amino acids. As a time zero control, 47.5 μg of heat-inactivated CPY was added to 3 mL of the above mixture from which CPY was omitted. The mixture was immediately boiled and the supernatant fluid was analyzed for amino acids, but practically no amino acids were detectable. The numbers of amino acid residues liberated from 1 mol of the reductase (fp_1) are plotted (closed circles). A 10-μL portion of the mixture was also withdrawn at each time point and the ability of the reductase to reconstitute the NADH-cytochrome c reductase activity with cytochrome b_5 was determined. It is expressed in terms of V_c/V_f as described in text (- - -O- - -). The reconstitution capacity of the reductase during incubation in the absence of CPY was also followed (---O---).

ments with carboxypeptidase A actually indicated a COOH-terminal sequence of Ala-Phe-COOH for the reductase. It can be seen that the release of amino acids, except for leucine, was completed 30 to 60 min after the start of incubation under the experimental conditions employed. The slow release of leucine seemed to be proceeding even 120 min after the initiation of incubation.

The numbers of amino acids released per molecule of the reductase after incubation for 120 min are summarized in Table I; the data for serine, threonine, asparagine, and glutamine, obtained by the method of Benson et al. (1967), are now included. For comparison, the amino acid compositions of the intact and cathepsin-solubilized forms of NADH-cytochrome b_5 reductase are also shown in Table I. As can be seen, the intact reductase is composed of 290 residues (M_r = 32 800), whereas the cathepsin-solubilized reductase consists of 236 residues (M_r = 26 500), indicating that cathepsin D has removed a peptide segment or segments composed of 54 residues. On the other hand, only 30 to 31 residues were released from the reductase by the action of CPY. A remarkable finding is that 70% of the amino acids released by CPY are hydrophobic ones.[2] The percentages of hydrophobic residues in the intact reductase, cathepsin-solubilized reductase, and the peptide segment(s) removed by cathepsin D are 51, 49, and 61%, respectively. These data indicate that the COOH-terminal portion of the reductase is significantly richer in hydrophobic residues than the rest of the molecule. It may, therefore, be concluded that the COOH-terminal portion of the reductase constitutes a hydrophobic region and its removal leads to the abolishment of the membrane-binding and reconstitution activities.

There is the possibility that the loss of these activities was due to a profound conformational change of the reductase induced by the removal of the COOH-terminal peptide segment. However, this seems to be unlikely in view of the fact that the NADH-ferricyanide reductase activity was essentially unaffected by the CPY treatment. In an attempt to eliminate this possibility further, the reductase from which the COOH-terminal segment had been removed was isolated by Sephadex G-200 chromatography. As shown in Figure 1, most of the NADH-ferricyanide reductase activity of the CPY digest was eluted as a symmetrical peak after a small peak due to the unattacked reductase. The CPY-modified reductase thus separated gave a single protein band upon NaDodSO$_4$-polyacrylamide gel electrophoresis and its molecular weight was clearly larger than that of the cathepsin-solubilized reductase (data not shown).

Figure 5 shows the CD spectra of the intact, CPY-modified, and cathepsin-solubilized reductase preparations in the far-ultraviolet region. All the spectra showed a negative CD peak at about 220 nm and a shoulder at about 210 nm. The contents of α helix, β, and unordered structures in the three preparations were estimated from the CD data by the method of Chen et al. (1972) and the results are summarized in Table II. The α-helix content decreased and that of unordered structure increased slightly upon CPY digestion and cathepsin D treatment. However, these data do not give any conclusive information about the conformational changes induced by the enzyme treatments, because considerable decreases in ellipticity were induced by the treatments and it is impossible to say that these decreases represent the secondary structure of the peptide segment(s) removed by the enzyme treatments. Nevertheless, the relatively small changes in the contents of three secondary

TABLE I: Amino Acid Compositions of Intact and Cathepsin-Solubilized NADH-Cytochrome b_5 Reductase and Amino Acids Released from the Intact Reductase by the Action of CPY.[a]

amino acid	intact reductase	cathepsin-solubilized reductase	Δ[b]	amino acids released by CPY[c]
Lys	19	16	3	1
His	9	8	1	0
Arg	15	14	1	0
Asp, Asn	24	22	2	0
Glu, Gln	27	22	5	2
Thr	12	9	3	2
Ser	15	13	2	3
Gly	21	17	4	2
Pro	25	22	3	1
Ala	16	13	3	3
½-cystine	4	3	1	0
Val	21	15	6	3
Met	9	7	2	1
Ile	15	13	2	1
Leu	30	21	9	6
Tyr	9	7	2	1
Phe	14	11	3	3
Trp	5	3	2	1 ~ 2
total residues	290	236	54	~30-31
mol wt	32 800	26 500	6050	~3300-3500

[a] The values given are in moles of amino acid residue per mole of protein and are nearest integers. They are expressed relative to the alanine content. [b] Difference between intact and cathepsin-solubilized reductases. [c] Moles of amino acids released from 1 mol of intact reductase.

[2] In this calculation, it was assumed that hydrophobic amino acids are proline, alanine, half cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, and tryptophan (cf. Tanford, 1973).

TABLE II: Contents of α-Helix, β, and Unordered Structures in Intact, CPY-Modified, and Cathepsin-Solubilized NADH-Cytochrome b_5 Reductase Preparations Estimated from Far-Ultraviolet CD Spectra.[a]

reductase preparation	α helix (%)	β structure (%)	unordered structure (%)
intact	39	7.7	54
CPY-modified	32	9.2	56
cathepsin-solubilized	33	3.7	64

[a] Estimation was carried out by using a computer by the method of Chen et al. (1972).

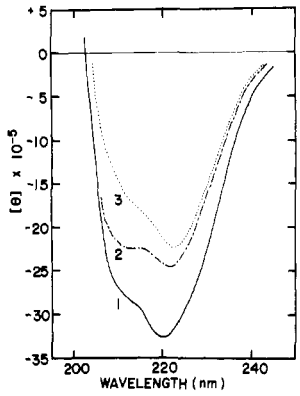

FIGURE 5: Far-ultraviolet CD spectra of intact, CPY-digested, and cathepsin-solubilized NADH-cytochrome b_5 reductase preparations. The CD measurements were performed as described in Experimental Procedure and the results are expressed in terms of molar ellipticity. (Curve 1) Intact reductase; (curve 2) CPY-digested reductase; (curve 3) cathepsin-solubilized reductase.

structures might suggest that no profound conformational changes were provoked in the reductase molecule by the removal of the COOH-terminal segment by CPY and by modification with cathepsin D. If it could be assumed that no conformational changes took place in the residual portion of the reductase molecule, it can be calculated from the data in Table II that the contents of helix in the peptide segment(s) removed by CPY and cathepsin D are 99% and 65%, respectively.

Digestion of the reductase with leucine aminopeptidase caused no effect on the capacity of the flavoprotein to reconstitute an active NADH-cytochrome c reductase system with cytochrome b_5 and Triton X-100, even though a large amount of the aminopeptidase (67% of the reductase protein) was used. Furthermore, no amino acids were released even after incubation for 150 min, suggesting that the NH_2 terminus of the reductase is resistant to the aminopeptidase. The NH_2 terminus of the reductase was also resistant to the attack of aminopeptidase M, which has been shown to have a wider substrate specificity than leucine aminopeptidase (Wachsmuth et al., 1966). NH_2-terminal end group determinations were, therefore, performed on the intact, CPY-modified, and cathepsin-solubilized reductase preparations by two cycles of dansyl-Edman degradation. However, no NH_2-terminal residue could be detected in the intact and CPY-modified flavoproteins, indicating that the terminus is masked.[3] On the other hand, serine was detected as the NH_2-terminal residue of the cathepsin-solubilized reductase. This indicated that cathepsin D removed not only a COOH-terminal peptide segment but also a peptide from the NH_2 terminus.

Discussion

As mentioned above, it is now established that the capacity of microsomal NADH-cytochrome b_5 reductase to bind to phospholipid bilayer membranes and to reconstitute an active NADH-cytochrome c reductase system with cytochrome b_5 depends on the presence of a hydrophobic moiety in its molecule (Mihara & Sato, 1972, 1975; Okuda et al., 1972; Rogers & Strittmatter, 1974). The results described above now provide evidence that the COOH-terminal segment consisting of some 30 amino acid residues (about 3500 daltons) constitutes the hydrophobic moiety which is responsible for the membrane-binding and reconstitution activities of the reductase. This region is in fact hydrophobic; out of 30 to 31 residues present, 20 to 21 can be classified as hydrophobic. CD studies give preliminary evidence that this region is very rich in α-helix (99%). It has been reported that the reductase purified after solubilization with lysosomes has a molecular weight which is about 6000 daltons less than that of the intact reductase (Takesue & Omura, 1970b) and that this fragment has lost both the membrane-binding and reconstitution activities (Mihara & Sato, 1975). Therefore, it is certain that lysosomal digestion has removed a COOH-terminal region including the hydrophobic segment mentioned above. In view of the finding that cathepsin D, a lysosomal acid protease, removes an unknown number of NH_2-terminal residues, it is highly likely that the lysosomally solubilized reductase has also lost the NH_2-terminal residues.

Another structural feature of intact NADH-cytochrome b_5 reductase is that its NH_2 terminus is chemically blocked, although the nature of this masking is not yet clear. Because of this masking, the reconstitution activity of the reductase is not affected by severe attacks by leucine aminopeptidase and aminopeptidase M. It is, therefore, not possible to examine if the NH_2-terminal peptide segment is involved in the membrane-binding and reconstitution activities, though circumstantial evidence suggests that this is not the case. The two structural features of the intact reductase just discussed, i.e., the location of the membrane-binding, hydrophobic region at the COOH-terminal end and the possession of the masked NH_2 terminus, are of special interest in view of the fact that these two features are also shared by intact cytochrome b_5 (Ozols, 1974). Our preliminary experiments have further shown that NADPH-cytochrome c (P-450) reductase, another amphiphilic protein functional in the microsomal electron transport system, also has its membrane-binding, hydrophobic segment at the COOH-terminal portion and its NH_2 terminus is masked. The similarity among the three microsomal amphiphilic proteins, therefore, does not seem to be fortuitous, but the two structural features appear to be shared by a certain group of membrane-bound, amphiphilic proteins. At present, nothing is known of the significance of these structural features, but it is likely that the location of the membrane-binding segment at the COOH-terminal end is somehow related to the mechanism by which these proteins, when newly synthesized, become assembled into microsomal membranes.

It should be noted that not all integral membrane proteins possess these structural features. For instance, Haugen et al. (1977) have recently reported that two species of cytochrome P-450 purified from rabbit liver microsomes have open NH_2 termini. They have further shown that 17 out of 20 NH_2-terminal residues of one of the cytochromes P-450 are hydrophobic and the sequence of this region is similar to those of the

[3] J. Ozols (personal communication) has also shown that the NH_2 terminus of the intact form of human HADH-cytochrome b_5 reductase is masked.

short-lived hydrophobic NH_2-terminal precursor segments, so-called signal sequences (Milstein et al., 1973; Blobel & Dobberstein, 1975), of certain other proteins such as pancreatic zymogens (Devillers-Thiery et al., 1975). It is, however, not yet known whether or not the COOH-terminal portion of the cytochromes P-450 is hydrophobic.

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