The Two-Domain Structure of Cytochrome b_5 in Deoxycholate Solution†

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ABSTRACT: The membrane protein cytochrome b_5 and the polar and hydrophobic fragments into which it is cleaved by trypsin have been investigated, with major emphasis on the deoxycholate-solubilized form of the protein. Molecular weight measurements show that both the intact protein and the fragments are in a monomeric state in deoxycholate and that a small peptide of perhaps 15 residues is excised when the fragments are formed. Measurements of Stokes radius show that the major fragments are globular, but that intact cytochrome b_5 has an asymmetric shape, consistent with a structure composed of two globular domains joined by a link region that may be as long as 30 to 40 Å. Circular dichroism measurements were made both in the far-ultraviolet and in the Soret region, and they add to previously existing data to make it virtually certain that the polar heme-containing domain is unaffected by proteolysis or by removal of deoxycholate. A significant change in the ultraviolet circular dichroism is, however, observed when proteolysis occurs and it is likely that it arises from the link between the domains, which appears to be highly structured (perhaps helical) in the intact protein, but randomly coiled after it is excised. The binding studies reported previously from this laboratory suggest that these inferences about the structure of cytochrome b_5 in deoxycholate solution apply also to the protein as solubilized by detergent micelles, by phospholipid vesicles, or by the microsomal membrane.

The membrane protein cytochrome b_5 belongs to a class of proteins possessing functionally distinct and independent domains that are formed by different sections of the same polypeptide chain. They are recognized by the effect of mild proteolysis, which results in the formation of fragments that separately retain the original functional properties. The first protein assigned to this class was immunoglobulin G (Porter, 1959); Spatz and Strittmatter (1971, 1973) have shown that both cytochrome b_5 and the reductase that acts upon it in the microsomal electron transport chain are proteins of this type.

In cytochrome b_5 one of the domains carries the electron transport site and the function of the other domain is to attach the molecule to the membrane. Spatz and Strittmatter (1971) showed that the latter domain has an abnormally high content of hydrophobic amino acids and suggested that this property is responsible for insertion into the membrane (Strittmatter et al., 1972). This suggestion was supported by a previous paper from this laboratory, showing that the hydrophobic domain (or the corresponding fragment after proteolysis) quite generally associates with micellar aggregates formed by a variety of amphiphilic molecules or ions (Robinson and Tanford, 1975).

In this paper we report the results of physical studies that describe the general structural features of intact cytochrome b_5 and its fragments, similar to the experiments we carried out with immunoglobulin G some years ago (Noelken et al., 1965), which led to the suggestion that the domains of that molecule are joined by a flexible hinge region. Since cytochrome b_5 is a membrane protein, a simple aqueous medium does not represent its native environment. On the other hand the membrane itself or vesicles formed by membrane lipids are not suitable for at least some conformational studies because the protein would tend to be a minor component of the system or would exist in the system in multiple copies. A reasonable compromise is to carry out the studies in a nondenaturing detergent or detergent-like amphiphile in which the major features of the native lipid environment can be simulated in a relatively small soluble particle. We have chosen deoxycholate micelles for this purpose, because the micelles are very small and their complexes with cytochrome b_5 are correspondingly small, leading to a particle in which the protein is the dominant component. One cannot say with certainty that the state of the protein under these conditions is identical with the native state in the membrane, but the binding studies done earlier do suggest that the gross conformational features are retained (Robinson and Tanford, 1975).

Experimental Section

Materials. Porcine cytochrome b_5 and its tryptic fragments were prepared and purified as described previously (Robinson and Tanford, 1975). Sperm whale myoglobin was purchased from Schwarz/Mann and chlorohemin from Sigma Chemical Co. Other proteins and reagents were as described in earlier papers (Fish et al., 1969; Reynolds and Tanford, 1970; Makino et al., 1973).

Methods. Measurements were carried out in 0.02 *M* Tris-acetate–0.2 m*M* EDTA–0.09 *M* NaCl (pH 8.1), with other reagents (e.g., deoxycholate) present in addition where stated. Protein concentrations were measured spectrophotometrically using $\epsilon_{412.5} = 1.17 \times 10^5\ M^{-1}\ cm^{-1}$ for

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Table I: Molecular Weight Measurements.

	Polypeptide Chain in 6 *M* Gdn·HCl		Protein Moiety in Complexes with DOC, Sed. Eq.	Protein in Aq Soln in Absence of DOC, Sed. Eq.
	Sed. Eq.	Gel Chromat.		
Cytochrome b_5 (heme included)			16,200[a]	~100,000
Apocytochrome b_5	15,600	15,700	(15,600)[b]	
Polar fragment (heme included)				10,500
Apo fragment		9,500		(9,900)[b]
Hydrophobic fragment		4,800	4,500[a]	Aggregated
Link peptide (by difference)		1,400	1,200	

[a] Excluding bound detergent as measured by equilibrium dialysis. Molecular weight measurements for the intact protein were made at $\bar{v}$ = 20 and $\bar{v}$ = 31, for the hydrophobic fragment at $\bar{v}$ = 19. [b] Molecular weight of heme (616) has been subtracted from the actual result for the heme-containing protein.

intact cytochrome b_5 or its polar fragment (Strittmatter and Velick, 1956; Spatz and Strittmatter, 1971) and ϵ_{280} = $1.93 \times 10^4\ M^{-1}\ cm^{-1}$ for the hydrophobic fragment (Robinson and Tanford, 1975). All measurements were made near room temperature (20–25°).

Molecular Weight. Polypeptide chain molecular weights were determined in 6 *M* Gdn · HCl,[1] both by sedimentation equilibrium and by the gel chromatographic method of Fish et al. (1969). Molecular weights in aqueous buffer (with or without DOC[1]) were determined by sedimentation equilibrium, with allowance for bound DOC, where present, as described by Tanford et al. (1974). A Model E Beckman-Spinco analytical ultracentrifuge equipped with photoelectric scanner was used for the sedimentation results, as described by Robinson and Tanford (1975). When gel chromatography was used, the Stokes radius (R_s) was determined first and molecular weights were obtained from a logarithmic plot of R_s vs. molecular weight for standard proteins.

Gel Chromatography. Sephadex G-200 (0.9 × 55 cm), G-100 (0.9 × 55 cm), and G-75 (1.5 × 90 cm) were used in aqueous buffer with and without DOC. The G-75 column was used for measurements in Gdn · HCl, the small pore size being ideal for the most important measurement in this solvent, which was the R_s value of the small hydrophobic fragment. The columns were calibrated using standard proteins (Fish et al., 1969; Tanford et al., 1974). The data were plotted as suggested by Ackers (1967), with the results shown in Figure 1. As our previous studies have indicated (Fish et al., 1970; Tanford et al., 1974) the solvent medium did not significantly affect the calibration. The two calibration points in DOC are for proteins known not to combine with DOC, and their R_s values were taken to be those applicable to simple aqueous buffers. Measured R_s values for cytochrome b_5 and its fragments are averages of at least three independent determinations, employing (except in Gdn · HCl) at least two different columns.

Circular Dichroism. A Cary 60 recording spectropolarimeter with circular dichroic (CD) attachment was used. Ellipticities were calculated on a molar or mean residue basis and were not corrected for refractive index. A 1-cm light path was used for the visible region, a 1-mm light path in the ultraviolet. Protein solutions used in the ultraviolet region had optical densities of 0.02 to 0.09 at 280 nm. Visible CD spectra in the presence of SDS were recorded 30 min after preparation of the solutions to allow for equilibration with the detergent. Optical densities at 412 nm were between 2 and 3. Small artifacts due to these rather high absorbances (Dorman et al., 1973) were corrected by determining the base lines with optically inactive solutions of chlorohemin in SDS with the same optical densities as the protein solutions being measured.

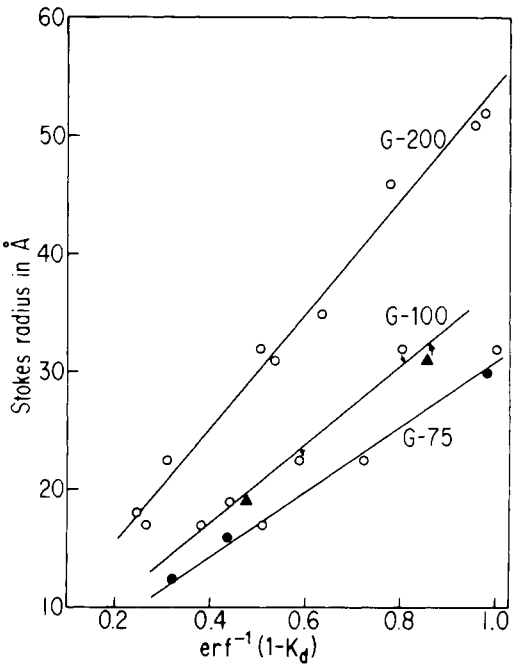

FIGURE 1: Calibration curves for gel chromatography. Standard proteins in aqueous buffer (○) were cytochrome *c*, ribonuclease, myoglobin, chymotrypsinogen, ovalbumin, hemoglobin, serum albumin, aldolase, immunoglobulin G, and catalase; those in DOC (▲) were myoglobin and ovalbumin. Standard polypeptide chains in Gdn · HCl (●) were alkylated A and B chains of insulin and cytochrome *c*. R_s values for the insulin polypeptides were calculated from the established relation between R_s and molecular weight in Gdn · HCl.

Results

Molecular Weights. Molecular weights determined as part of this investigation are shown in Table I. The molecular weights of the protein moieties in DOC were determined by sedimentation equilibrium, under conditions where accurate values for the amount of bound DOC were available

[1] Abbreviations used are: DOC, sodium deoxycholate: SDS, sodium dodecyl sulfate; cmc, critical micelle concentration; Gdn · HCl, guanidine hydrochloride.

Table II: Stokes Radii (Å)

	In Aq. Buffer	In DOC	R_{min}[a]
Cytochrome b_5	60[b]	31	20.8
Polar fragment	19.5	20	14.6
Hydrophobic fragment	>60[b]	20	17.4

[a] R_{min} is the smallest possible radius for a perfectly spherical particle containing bound DOC where appropriate but no bound water. [b] Cytochrome b_5 and the hydrophobic fragment are aggregated in the absence of DOC.

from equilibrium dialysis measurements reported in our preceding paper (Robinson and Tanford, 1975). These experiments are quite independent of the sedimentation equilibrium results cited in the previous paper, which were obtained at higher DOC concentrations to determine DOC binding. The agreement between the molecular weights in DOC and the polypeptide chain weights shows that the DOC complexes of both cytochrome b_5 and its hydrophobic fragment contain single polypeptide chains. In aqueous buffer in the absence of detergent the intact protein and the hydrophobic fragment are both aggregated; only the polar fragment is monomeric under these conditions.

Table I shows that the sum of the molecular weights of protein in the two principal fragments fails to account for the molecular weight of the intact polypeptide chain by about 1000 to 1500 daltons, indicating that a peptide linking the two fragments has been excised during proteolysis.

Our results are consistent with the available data on the amino acid composition and sequence (Spatz and Strittmatter, 1971; Ozols, 1974). The polar fragments obtained when rabbit or bovine cytochrome b_5 is extracted with pancreatic lipase are larger than those obtained when trypsin is used (Strittmatter and Ozols, 1966b), and Spatz and Strittmatter (1971) observed a discrepancy of only four residues between the intact polypeptide chain (for the rabbit) and the two major fragments. It is, however, evident from the three-dimensional structure obtained (for the bovine protein) by X-ray crystallography (Mathews et al., 1971a,b) that the extra residues retained in the lipase-extracted polar fragment are not part of the ordered structure of the polar domain, i.e., it is reasonable to conclude that the number of residues in the link between the domains is about the same in all species, even though the cleavage position may differ depending on the species and on whether lipase or trypsin is used. Our molecular weight for the hydrophobic fragment agrees well with that deduced by Spatz and Strittmatter (1971) from amino acid analysis of the isolated fragment of the rabbit protein. Ozols (1974) has reported a considerably larger size for the hydrophobic fragment of the porcine protein, but his result was based only on the difference between the number of residues in the intact chain and in the polar fragment, assuming that only a single cleavage had occurred. Since the size of the polar fragment observed by him is smaller by 10–15 residues than the polar fragments of lipase-extracted rabbit or bovine protein, it is likely that cleavage at more than one point took place in his experiments, as evidently occurred in our proteolysis employing trypsin.

Stokes Radii. Table II shows Stokes radii in ångströms measured by gel chromatography using calibrated Sephadex columns (Figure 1). The observed values are compared with the minimal possible values (R_{min}) for spherical unsolvated particles, including bound DOC and heme where applicable, calculated by use of eq 10 of Tanford et al. (1974). The results show that the polar fragment in aqueous solution and the hydrophobic fragment in the form of its DOC complex both behave as globular particles with $R_s/R_{min} \simeq 1.25$. Intact cytochrome b_5 in its DOC-solubilized form, on the other hand, has $R_s/R_{min} \simeq 1.5$, indicating that it is significantly more extended. (The alternative explanation that it is much more highly solvated is not reasonable here.) Both the spectral measurements described below and the binding studies of our previous paper (Robinson and Tanford, 1975) suggest that the polar and hydrophobic *domains* of the intact protein in its DOC complex have essentially the same conformation as the corresponding *fragments* after proteolysis. For the polar domain the same conclusion follows from measurement of biological activity (Spatz and Strittmatter, 1971). It is thus reasonable to propose that the small peptide region linking the two domains is responsible for the relatively large R_s of the intact molecule, i.e., this peptide appears to keep the domains separated from each other by a considerable distance.

To obtain some idea of the spacing between domains in intact cytochrome b_5 we have used two approaches.

(1) The model of Bloomfield et al. (1967) which gives the Stokes radius of a particle consisting of linked spheres has been used. The two principal domains (including bound DOC for the hydrophobic domain) were considered to be spheres with a radius of 20 Å, i.e., close to the actual R_s values given in Table II. If the two spheres were touching, the predicted R_s value for the whole molecule would be 26 Å. To obtain the observed R_s of 31 Å, the spheres must be separated by a space of about 40 Å between their outer edges.

(2) We have taken the volume of the molecule to be that of two spheres with radius 20 Å, thereby making allowance for the solvation and/or asymmetry of the domains themselves, and have interpreted the observed R_s in terms of a prolate ellipsoid model (Tanford, 1961). With the minor semiaxis equal to 20 Å a major semiaxis of 60 Å is required to account for an R_s value of 31 Å. This again corresponds to a length exceeding the sum of the diameters of the spheres by about 40 Å.

Neither of these calculations can be taken seriously as a quantitative estimate. Both indicate, however, that the domains are quite far apart. If the peptide linking the domains is an α helix (as suggested below) it would account for a separation of 20 Å if the molecular weight of the link region given in Table I is used. The link region may be longer than that since the point at which proteolysis occurs between the link region and the hydrophobic fragment may not coincide with the point at which the functionally hydrophobic domain is joined to the rest of the polypeptide chain. The amino acid composition of the hydrophobic fragment (40 residues) given by Spatz and Strittmatter (1971) in fact contains ionic residues that presumably cannot be part of the functionally hydrophobic domain and thus might contribute to the interdomain spacing. Sequence information for assessment of this possibility is not available at present.

Circular Dichroism Associated with the Soret Absorption Band. Schnellbacher and Lumper (1971) and Huntley and Strittmatter (1972) have shown that the incorporation of heme into cytochrome b_5 induces optical activity in the form of several extrinsic Cotton effects in the heme absorption region. Their measurements were made using the polar peptide alone. We have confirmed their results for the Soret

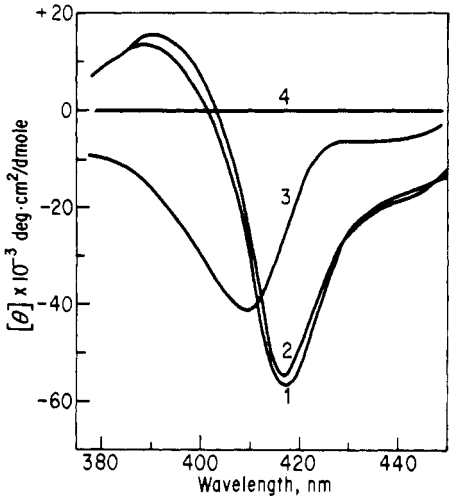

FIGURE 2: Circular dichroism in the Soret absorption region. Molar ellipticities are given for the polar fragment in aqueous buffer (curve 1) and for intact cytochrome b_5 in aqueous buffer (curve 2), in 1 mM SDS (curve 3) and in 20 mM SDS (curve 4).

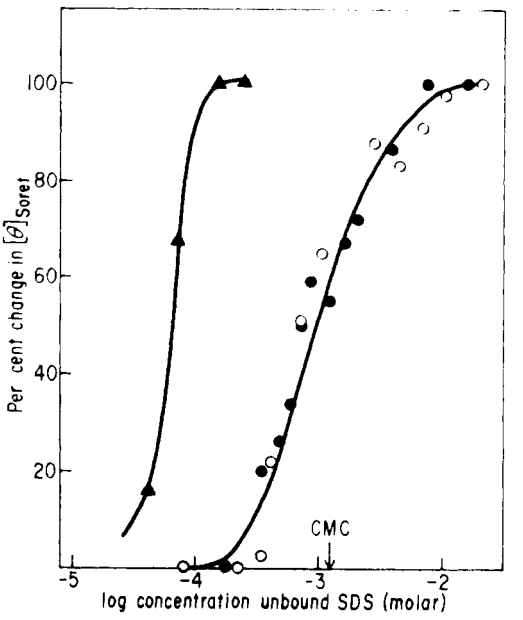

FIGURE 3: Progress in denaturation as a function of unbound SDS concentration for cytochrome b_5 (open circles) and its polar fragment (filled circles). A similar curve for myoglobin (triangles) is included for comparison.

absorption band and have obtained essentially identical data for intact cytochrome b_5, as shown by Figure 2. The figure also shows the effect of the addition of SDS. Similar results were obtained for intact protein and polar fragment: there is first a shift both in intensity and wavelength, followed by complete disappearance of the CD spectrum at sufficiently high SDS levels. The CD changes were accompanied by changes in the absorption spectrum (not shown).

A more detailed analysis of the effect of SDS is shown in Figure 3, where the change in ellipticity at 417 nm is plotted as a function of the unbound SDS concentration in equilibrium with protein. Identical denaturation profiles were obtained for intact cytochrome b_5 and for the polar fragment, which, coupled with the virtual identity of the CD spectra themselves (Figure 2), provides the strongest possible evidence that the conformation of the polar domain of cytochrome b_5 is the same in the intact protein and in the separated polar proteolytic fragment.

A denaturation profile obtained with another hemoprotein, myoglobin, is included in Figure 3 for comparative purposes. It is clear that the cytochrome b_5–heme association is exceptionally resistant to denaturation by SDS. The nature of this association has been discussed by Strittmatter and Ozols (1966a).

Circular Dichroism in the Far Ultraviolet. CD spectra in the presence of a saturating level of DOC and in the absence of DOC are shown in Figure 4. The intact molecule and the hydrophobic fragment are aggregated in the absence of DOC; all other measurements refer to monomeric protein. Negative dichroic bands were observed near 208 and 220 nm for all species and they suggest the presence of helical structure in the polypeptide backbone. However the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ ($\parallel$) transitions of the polar peptide are somewhat blue-shifted (as has also been observed by Schnellbacher and Lumper, 1971) and the $n \rightarrow \pi^*$ band of the hydrophobic fragment is somewhat red-shifted both in the aggregated state and in the monomeric DOC complex, i.e. the spectra deviate from the typical α-helix spectra given, for example, by Greenfield and Fasman (1969). The intensities of the CD bands of the hydrophobic fragment are different in the presence and absence of DOC. Removal of DOC has essentially no effect on the polar fragment, which is to be expected since DOC does not bind to it. Removal of DOC also has only a small effect on intact cytochrome b_5, but this could be accounted for within the rather large experimental error of these data simply by the fact that the polar domain makes the major contribution to the CD spectrum.

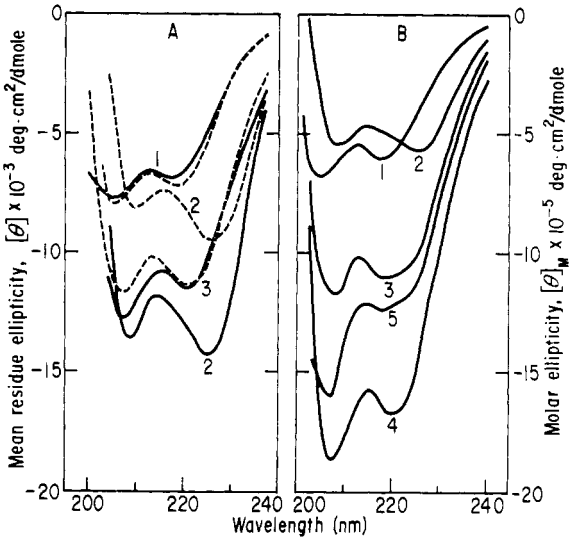

FIGURE 4: Circular dichroism data in the far ultraviolet. (A) Mean residue ellipticities in 10 mM DOC (solid lines) and in aqueous buffer in the absence of DOC (dashed lines) for the polar fragment (curve 1), the hydrophobic fragment (curve 2), and intact cytochrome b_5 (curve 3). (B) Molar ellipticities in 10 mM DOC for the polar fragment (curve 1), the hydrophobic fragment (curve 2), the sum of these (curve 3), intact cytochrome b_5 before proteolysis (curve 4), and the total product of proteolysis (curve 5).

On the right-hand side of Figure 4 the spectra in DOC are plotted as *molar* ellipticities instead of as mean residue ellipticities. Curve 3 is the sum of the molar ellipticities of the two major proteolytic fragments and it is evident that it falls far short of accounting for the molar ellipticity of intact cytochrome b_5, given by curve 4. Curve 5, which repre-

sents the CD spectrum after proteolysis,[2] but without removal of the proteolysis products, is, however, much closer to curve 3 than to curve 4. The major part of the difference between curves 3 and 4 thus arises from a conformational change accompanying proteolysis; only the relatively small difference between curves 3 and 5 reflects the contribution from the link region after proteolysis has taken place.

There is virtually conclusive evidence to show that the polar domain of cytochrome b_5 undergoes no conformational change upon proteolysis. The evidence for the hydrophobic domain is less firm, but the identical binding isotherms for DOC shown in our previous paper (Robinson and Tanford, 1975) strongly suggest that the hydrophobic domain of the intact protein in DOC has substantially the same conformation as the separated fragment in DOC. These results thus suggest that the conformational change accompanying proteolysis (curves 4 and 5 of Figure 4B) originates largely from the short peptide linking the two domains. If so, the data indicate that the link peptide is highly structured (largely helical) in the intact protein, losing that structure entirely when the peptide is excised. This conclusion comes from the following aspects of the data. (1) The difference between curves 3 and 5 of Figure 4B increases sharply below 210 nm, following roughly the CD curve for a randomly coiled polypeptide, so that it is reasonable to conclude that the link peptide *after* proteolysis is essentially structureless. (2) The difference between curves 4 and 5 of Figure 4B is greatest near 222 nm and has a crossover point near 205 nm. These are precisely the characteristics of the CD change accompanying conversion of α-helical polylysine to the randomly coiled polymer (Greenfield and Fasman, 1969).

Use of the numerical data of Greenfield and Fasman (1969) to quantitate the CD data indicates that 15 residues are involved in the helix–coil conversion, which, on the basis of the molecular weight data of Table I, suggests that the link region is entirely helical before proteolysis. The assumed equivalence of CD intensities in model polypeptides and in proteins is, however, of dubious validity and especially so here since the trough positions differ from those of polylysine. The quantitative unreliability of calculations of this kind is illustrated by the apparent helix content of only 18% that is obtained for the polar fragment from curve 1 of Figure 4A. This is much less than the actual helix content derived from the X-ray structure. This same observation has been made by Huntley and Strittmatter (1972).

Discussion

The two-domain model for cytochrome b_5 was proposed on the basis of investigations that showed that the single polypeptide chain could be split by proteolysis into a hydrophilic heme-containing fragment and a relatively hydrophobic fragment; the catalytic properties were recovered intact in the hydrophilic fragment, but the hydrophobic region was found to be essential for the binding of cytochrome b_5 to microsomal membranes (Spatz and Strittmatter, 1971; Strittmatter et al., 1972; Rogers and Strittmatter, 1973). We have studied the protein in DOC solutions and our measurements in this paper, together with the binding studies reported previously (Robinson and Tanford, 1975), support the two-domain model.

Measurements of the Stokes radius provide the most direct physical evidence for the existence of distinct domains in the intact molecule. The results resemble those obtained for immunoglobulin G, which was first characterized as consisting of globular domains linked by intervening extended portions of polypeptide chain by similar measurements in this laboratory (Noelken et al., 1965). In both proteins $R_s/R_{min} \simeq 1.5$ for the whole molecule, whereas the fragments obtained after proteolysis have smaller R_s/R_{min} ratios typical of compact globular particles. In both proteins the biological function is unaffected by proteolysis, indicating that the globular fragment carrying the active site has not undergone a large change in conformation, and in cytochrome b_5 additional evidence for this is provided by the CD spectrum associated with the heme prosthetic group and by the course of disappearance of this spectrum as a function of SDS concentration (Figure 3). The evidence that the hydrophobic domain retains its conformation after proteolysis is less secure, but the identical binding isotherms for DOC binding before and after proteolysis are certainly consistent with it. There is one difference between the effects of proteolysis on immunoglobulin and on cytochrome b_5. No loss of optical activity occurs for the former, whereas a substantial loss of structure accompanies proteolysis of cytochrome b_5. This loss has been ascribed to the interdomain peptide, i.e., we suggest that part or all of the link between the two domains may be highly ordered (and perhaps a rigid helix) in cytochrome b_5.

Since these results were obtained in DOC solution and since the hydrophobic fragment undergoes a substantial conformational change when DOC is removed (Figure 4), the question arises as to whether the results in DOC in fact reflect the state of the protein in its native state in a microsomal membrane. We believe that they do. A purely aqueous medium is not representative of the native environment of a membrane protein, whereas the binding of an amphiphilic ligand (in this case solely to the hydrophobic domain) simulates the association with phospholipid that occurs in the membrane. The data of the previous paper (Robinson and Tanford, 1975) are important in relation to this point, for they indicate that the mode of association between cytochrome b_5 and DOC is similar to its mode of association with several detergents and with phospholipid vesicles. In each case the process appears to represent a co-micellization of the hydrophobic domain with the amphiphilic ligand.

Although the complete amino acid sequence of the hydrophobic fragment has not been determined for any species, it appears probable that the hydrophobic combining region is smaller than the whole fragment. Ozols (1974) has reported the sequence of a few residues at the COOH terminus for three species: the terminal sequence is -Glu-Asp-COOH for all three. The sequence contains three negative charges, which presumably cannot be within the hydrocarbon core of the microsomal membrane or of solubilizing micelles. The fragment contains at least two other ionic residues (Spatz and Strittmatter, 1971). Since the point of trypsin cleavage that separates the fragment from the rest of the molecule does not necessarily coincide exactly with the beginning of the functionally hydrophobic segment, these residues might be part of the interdomain link and might contribute to its length. In any case, it is clear that the hydrophobic segment is joined at *both* ends to residues that project into the aqueous medium, which could mean that the polypeptide chain traverses the bilayer or micelle

[2] Proteolysis by trypsin after solubilization of monomeric protein in DOC leads to the same products as are obtained in the absence of DOC.

from one side to the opposite surface. Alternatively, there could be a 180° turn (Crawford et al., 1973) within the hydrocarbon core, with both ends of the chain emerging from the same side. The CD spectrum in DOC (Figure 4A) suggests that the hydrophobic fragment has a partially helical backbone, but there is no experimental basis for assigning the helical region or regions to the hydrocarbon-embedded portion rather than to the presumed external residues.

It may be noted in conclusion that both this paper and the previous binding study indicate that the denaturation of the polar region of cytochrome b_5 by SDS is anomalous. Figure 3 shows that the polar fragment (or the corresponding domain in the intact protein) is much more resistant to SDS than myoglobin, possibly indicating that the heme-protein interaction is stronger, as has been inferred from the crystal structure by Mathews et al. (1971a). However, the polar fragment also binds an abnormally low amount of SDS after the heme has been extracted (Robinson and Tanford, 1975), and this could also contribute to making the free energy of denaturation less favorable than for myoglobin.

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