# The Binding of Deoxycholate, Triton X-100, Sodium Dodecyl Sulfate, and Phosphatidylcholine Vesicles to Cytochrome $b_5$†

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ABSTRACT : Cytochrome $b_5$ is composed of two domains that can be isolated after tryptic cleavage as two polypeptide fragments. One fragment is globular and hydrophilic and contains the heme; the other fragment is rich in hydrophobic amino acids and is essential for recombination of cytochrome $b_5$ with microsomal membranes [Ito, A., and Sato, R. (1968), *J. Biol. Chem. 243,* 4922; Spatz, L., and Strittmatter, P. (1971), *Proc. Nat. Acad. Sci. U.S. 68,* 1042]. Equilibrium dialysis and sedimentation equilibrium measurements of the binding of deoxycholate, Triton X-100 and dodecyl sulfate show that neither intact cytochrome $b_5$ nor its proteolytic fragments possess high affinity binding sites for any of these amphiphiles. However, each detergent binds to the protein in a highly cooperative manner at concentrations near the critical micelle concentration. Binding measurements using the separated tryptic fragments show that deoxycholate and Triton X-100 (both nondenaturing detergents) bind to the hydrophobic fragment to the same extent as to intact cytochrome $b_5$, and not at all to the polar fragment. Sodium dodecyl sulfate (a denaturing detergent) is bound to both tryptic fragments, but 70% of the detergent is bound to the hydrophobic fragment although it comprises only 30% of the protein mass. Less detailed measurements were made with synthetic and natural phosphatidylcholines, and show that the intact protein is quantitatively incorporated into phosphatidylcholine vesicles, but that no interaction with the polar fragment occurs. These results are interpreted in terms of the hydrophobic domain of cytochrome $b_5$ having a diffuse hydrophobic surface that can act as a nonspecific nucleus for the formation of a micelle with a variety of amphiphilic substances. This domain of the molecule will insert into any available hydrophobic environment, whether it be detergent micelles, synthetic phospholipid vesicles, or the microsomal membrane. The incorporation of cytochrome $b_5$ into the microsomal membrane is only a specialized case of this general property.

Because biological membranes are a mixed system, containing substantial amounts of both protein and lipid, any description of membrane structure must include and describe the interactions that occur between the protein and lipid molecules of the membrane. A few proteins are known to be associated with the membrane primarily through ionic interactions, *e.g.* the ATPase of *Streptococcus fecalis* (Abrams and Baron, 1968), and the basic protein of myelin (Eng *et al.*, 1968; Eylar *et al.*, 1969). Each of these proteins can be removed from the membrane by conditions known to disrupt ionic interactions (removal of an essential cation; use of high salt concentrations or low pH). Many membrane proteins, however, are not removed from the membrane by such procedures and require conditions that are known to disrupt the entire membrane structure (*i.e.*, the use of detergents). These proteins are believed to penetrate at least part way into the phospholipid bilayer with direct contact between the nonpolar amino acids of the protein and the liquid hydrocarbon interior of the membrane bilayer. Although there is a great deal of evidence that such interactions do occur, the evidence is often indirect, and in no instance has an investigation of the mode of association between protein and lipid been made. Does some portion of a membrane protein have high affinity sites for single lipid molecules, or does interaction occur only with lipid in an aggregated state? Is the interaction specific for lipids or is there a general affinity for a variety of amphiphiles? Is the interaction purely hydrophobic or is there also a significant contribution from the polar head group of the lipid or other amphiphile molecule? What is the stoichiometry of association and what factors determine it? The answers to these questions are not known, and the purpose of the present paper is to provide such answers for one relatively well-characterized membrane protein, in terms of a conventional binding study between the protein and five amphiphilic substances: the bile salt deoxycholate, one ionic and one nonionic detergent, and two phosphatidylcholines.

The protein chosen for this work is cytochrome $b_5$ derived from liver microsomal membranes. This protein has been studied in considerable detail, especially by Strittmatter and coworkers (Spatz and Strittmatter, 1971; Strittmatter *et al.*, 1972). It consists of a single polypeptide chain of molecular weight about 16,000 which in its native membrane-bound state is folded into two distinct domains. One domain, comprising the $NH_2$-terminal 90 amino acids, lies wholly outside the membrane. The amino acid sequence of this fragment of cytochrome $b_5$ has been determined for several species (Ozols and Strittmatter, 1969; Tsugita *et al.*, 1970; Nóbrega and Ozols, 1971). In addition, the three-dimensional structure of this domain for the calf protein is known (Mathews *et al.*, 1971a,b). This domain contains the

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heme, is globular, and has a polar hydrophilic surface. The other domain is formed by the carboxy-terminal segment of the peptide chain. This segment, comprising about 40 amino acids, is relatively rich in hydrophobic amino acids and is essential for incorporation of the protein into the membrane (Spatz and Strittmatter, 1971). Neither its amino acid sequence nor its structure has been determined.

The two regions of cytochrome $b_5$ can be separated after mild tryptic proteolysis (Spatz and Strittmatter, 1971), which does not appear to alter the structure or properties of each part of the protein. Only a short polypeptide region of a few amino acids is extensively digested; this region may act as a link between the two domains permitting the polar heme containing portion to be more accessible to the solvent. This property of cytochrome $b_5$ is of considerable importance for this investigation, since it permits independent binding measurements for the whole molecule, and for the two domains, one of them presumed to be crucial for association with the membrane *in vivo*, while the other is not.

## Experimental Section

*Materials.* Sodium [$^{14}$C]deoxycholate (DOC)[1] (6.1 mCi/mmol) was obtained from Mallinckrodt Co. and found to be 99% pure by thin-layer chromatography. It was diluted with unlabeled DOC purchased from Fisher Scientific Co. No DOC solutions had any detectable absorbance above 255 nm. [R-$^3$H]Triton X-100 (TX) (0.28 mCi/g) was supplied through the courtesy of Dr. W. R. Lyman of Rohm and Haas Co. Its purification and molecular properties have been described previously (Makino *et al.*, 1973). Sodium dodecyl [$^{35}$S]sulfate (SDS) was purchased from Amersham–Searle (35 mCi/g) and was diluted with unlabeled SDS from Schwarz/Mann. Synthetic didodecylphosphatidylcholine was obtained from Supelco Inc.; egg phosphatidylcholine was obtained from Applied Science Laboratories, Inc.

*Methods.* All experiments other than the preparation of cytochrome $b_5$ and its tryptic fragments were done at room temperature, near 23°, in either 0.02 M Tris-acetate–0.2 mM EDTA (pH 8.10) ($I$ = 0.012) or in the same buffer to which 0.09 M NaCl had been added ($I$ = 0.102). Protein concentrations were measured using molar extinction coefficients: $\epsilon_{412.5} = 1.17 \times 10^5$ for both cytochrome $b_5$ and the polar fragment (Spatz and Strittmatter, 1971; Strittmatter and Velick, 1956), and $\epsilon_{280} = 1.93 \times 10^4$ for the hydrophobic fragment of cytochrome $b_5$. The value used for the hydrophobic fragment is based on the presence of two Tyr and three Trp residues per molecule, as determined for the corresponding fragment from rabbit cytochrome $b_5$ (Spatz and Strittmatter, 1971), and the molar extinction coefficients of 1340 and 5550, respectively, for these residues (Wetlaufer, 1962). We have measured Trp/Tyr ratios by the method of Edelhoch (1967) for the porcine protein and both proteolytic fragments: all three results are consistent with the Trp and Tyr contents of the rabbit protein. Our observed ratios are not consistent with the analytical data of Ozols (1974), who reported (by difference between the whole molecule and the proteolytic fragment) that the porcine hydrophobic fragment should contain two Trp and three Tyr residues.

All optical measurements were carried out using a Cary 15 spectrophotometer.

PREPARATION OF CYTOCHROME $b_5$. Porcine cytochrome $b_5$ was used, prepared at 4° following the general procedure of Spatz and Strittmatter (1971), but modified to permit a large scale preparation of protein. The starting material was a suspension of crude microsomes from porcine liver (Masters *et al.*, 1967), kindly provided for us by Drs. C. Hall and H. Kamin of this Department. The final product was homogeneous by electrophoresis in 10% SDS–polyacrylamide gels (Weber and Osborn, 1969) and by sedimentation velocity and sedimentation equilibrium in 10 mM DOC. The protein had a visible and ultraviolet (uv) absorption spectrum identical with that shown by Spatz and Strittmatter for the rabbit protein ($A_{412.5}/A_{275} = 2.65$).

PREPARATION OF THE POLAR AND HYDROPHOBIC FRAGMENTS OF CYTOCHROME $b_5$. The tryptic fragments of cytochrome $b_5$ were prepared by a method similar to that described by Spatz and Strittmatter (1971). Cytochrome $b_5$ ($4.5 \times 10^{-4}$ M) in pH 8.1 buffer (0.02 M Tris-acetate–20 mM $CaCl_2$) was incubated at room temperature for 4 hr with $1.5 \times 10^{-6}$ M trypsin (the trypsin had been purified by the method of Robinson *et al.* (1971) to remove contaminating chymotrypsin). Proteolysis was stopped by inactivating the trypsin with the active-site titrant *p*-nitrophenyl-*p'* guanidinobenzoic acid. The polar and hydrophobic fragments were separated on a Sephadex G-100 column (pH 8.10, 0.02 M Tris-acetate–0.09 M NaCl–0.2 mM EDTA). The polar fragment was homogeneous as judged by sedimentation equilibrium and the absorption spectrum was identical with the spectrum published by Spatz and Strittmatter ($A_{412.5}/A_{275} = 5.52$). The hydrophobic fragment was further purified by elution from the Sephadex G-100 column to which 10 mM DOC was added to the buffer and by a second elution on the Sephadex G-100 column without DOC.

BINDING BY EQUILIBRIUM DIALYSIS. Most binding experiments were made by the method of equilibrium dialysis in Lucite cells containing two 1-ml compartments separated by a Visking membrane (Steinhardt and Reynolds, 1969). Protein concentrations between $10^{-5}$ and $10^{-4}$ M were used except for data obtained with the hydrophobic fragment ($4 \times 10^{-6}$ M). Equilibrium was usually approached from both below and above the equilibrium concentration by adding an excess of detergent to either the protein chamber or the solvent chamber. Equilibrium was obtained in 48–72 hr at detergent concentrations less than the cmc.[1] At concentrations above the cmc, sometimes as long as 3 weeks was necessary for equilibrium to be reached. In the case of TX above the cmc, equilibrium across the membrane could not be obtained in a reasonable time even in the absence of protein. Therefore, TX equilibrium dialysis experiments above the cmc were done using a three-chambered equilibrium dialysis cell. The middle chamber contained excess TX, the other two chambers buffer and protein solutions, respectively. Using this method, both a buffer solution and a protein solution are simultaneously equilibrated with the same concentrated TX solution: $\bar{\nu}$ values can then be calculated from the difference in TX concentration between the two outer chambers. The value calculated for $\bar{\nu}$ was constant between 4 and 14 days although the concentration of TX increased very slowly in both compartments. By varying the excess TX in the center chamber, different concentrations of TX were obtained in the buffer chamber.

[1] Abbreviations used are: DOC, sodium deoxycholate; cmc, critical micelle concentration; $I$, ionic strength; TX, Triton X-100; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; HDL, high-density lipoprotein.

Concentrations of [$^{14}$C]DOC, [$^{35}$S]SDS, and [$^{3}$H]TX were determined by using a Beckman LS-100C scintillation counter. Conversion of observed counts to concentration units was done by comparison with standard radioactively labeled detergent solutions of the same specific activity. The quenching of $^{3}$H by the heme in cytochrome $b_5$ or polar domain was corrected for by appropriate controls at the same cytochrome $b_5$ concentrations. No corrections for quenching were necessary for either $^{14}$C or $^{35}$S. In some experiments with TX, unlabeled detergent was used. Concentrations of TX were calculated using an experimentally determined molar extinction coefficient for TX of 1465. Since TX did not perturb the spectrum of cytochrome $b_5$ at concentrations below 2 mM, its concentration in the presence of cytochrome $b_5$ could be calculated by measuring the absorbance at both 275 and 412 nm and using the $A_{412}/A_{275}$ ratios for the protein given above.

Binding data obtained for DOC at ionic strength 0.01 were corrected for the Donnan effect, with the assumption that counterion binding could be neglected. The required corrections to $\bar{\nu}$ for intact cytochrome $b_5$ under the experimental conditions used were: 0.2 at 0.1 mM DOC, 2.3 at 5 mM DOC, and 4.5 at 10 mM DOC. The data obtained by sedimentation equilibrium were corrected in the same way since Donnan equilibrium affects these measurements in the same way. No correction was required for DOC at ionic strength 0.10 and no correction was applied to the SDS data because it was assumed that counterion binding would largely neutralize the charge of the bound detergent, as it does in pure SDS micelles (Mysels and Princen, 1959).

BINDING BY GEL CHROMATOGRAPHY. Some binding measurements with TX were made using the gel chromatography method (Makino *et al.*, 1973). This method could only provide an estimate of the TX binding above the cmc due to the similar molecular size of the TX micelle and cytochrome $b_5$–TX complex. A minimum value for the saturation binding was obtained by loading the complex on the gel column in the absence of TX, and a maximum value for the saturation binding was obtained by loading the protein in an excess of TX. Using this method and evaluating the binding from the leading edge of the protein peak, the minimum and maximum binding values agreed within 20%.

BINDING BY SEDIMENTATION EQUILIBRIUM. Binding measurements at detergent concentrations far above the cmc were most easily measured using sedimentation equilibrium in a Spinco Model E ultracentrifuge that was equipped with a photoelectric scanner. Sedimentation equilibrium directly measures the thermodynamically defined term, $M_p(1 - \phi'\rho)$, where $M_p$ is the molecular weight of protein in the sedimenting particle (exclusive of hydration or bound detergent), $\phi'$ is an effective specific volume reflecting both the true partial specific volume of the protein ($\bar{v}_p$) and the effects of interaction with solvent and detergent, and $\rho$ is the solvent density. It is an excellent approximation to separate these contributions (Tanford *et al.*, 1974. Equation 1 is equivalent to eq 2 of that paper.)

$$M_p(1 - \phi'\rho) = M_p(1 - \bar{v}_p\rho) + \bar{\nu}M_D(1 - \bar{v}_D\rho) \quad (1)$$

where $\bar{\nu}$ is the number of moles of detergent bound per mole of protein, $M_D$ is the molecular weight of the detergent, and $\bar{v}_D$ is its partial specific volume. Equation 1 was used previously (Tanford *et al.*, 1974) to measure the molecular weight $M_p$ in a protein–detergent complex when the amount of bound detergent was known. It is used here to measure $\bar{\nu}$ when $M_p$ is known. Molecular weights and partial specific volumes of cytochrome $b_5$ and its fragments and of the detergents used in this study are given in Table I. The protein molecular weights are for monomeric species; it cannot be automatically assumed that the detergent–protein complexes contain single protein molecules, but the results themselves will be seen to demonstrate this for the experimental conditions used here.

Table I: Molecular Weights and Partial Specific Volumes.

| | Mol Wt | Partial Sp Vol |
|---|---|---|
| Cytochrome $b_5$ | 16,200[a] | 0.738[b] |
| Apocytochrome $b_5$ | 15,600[a] | 0.736[b] |
| Polar fragment | 10,300[a] | 0.731[c] |
| Hydrophobic fragment | 4,600[a] | 0.758[b] |
| DOC[d] | 414 | 0.778 |
| TX[d] | 640 | 0.908 |
| SDS[d] | 288 | 0.870 |

[a] The molecular weights given here for the porcine protein were measured by sedimentation equilibrium, as will be reported in detail in a later paper. The values are very similar to those given by Spatz and Strittmatter (1971) for rabbit cytochrome $b_5$. The initial report on the extraction of cytochrome $b_5$ by detergents (Ito and Sato, 1968) suggested a molecular weight of 25,000 for the whole protein, but this was based on an empirical procedure that does not have general validity. [b] Calculated from the amino acid composition of Spatz and Strittmatter (1971) using the partial specific volumes of the amino acid residues given by Cohn and Edsall (1943). [c] Calculated from the amino acid composition of Ozols (1974). [d] Data from Tanford *et al.* (1974).

In all experiments where the sedimentation equilibrium method was applied, plots of log $c$ *vs.* $r^2$ were linear between the meniscus and the bottom of the cell. All protein concentrations were measured using the absorbance at 275 nm, and in the case of cytochrome $b_5$ and polar domain containing the heme molecule data were also collected at 412 nm. Each experiment was done using at least two different rotor speeds. In all experiments where binding was calculated, the same value of $M_p(1 - \phi'\rho)$ was obtained at all rotor speeds and at both wavelengths where two wavelengths were used.

MEASUREMENT OF CRITICAL MICELLE CONCENTRATION (CMC). The cmc of DOC was measured using the spectral shift of Methyl Orange accompanying its association with DOC micelles (Benzonana, 1969). Values of $6.0 \times 10^{-3}$ M ($I = 0.01$) and $3.2 \times 10^{-3}$ M ($I = 0.10$) were obtained which are close to those reported by Benzonana (1969) and by Makino *et al.* (1973). The cmc of TX was measured at $I = 0.10$ by its rate of dialysis and found to be $2.5 \times 10^{-4}$ M, which is similar to the result obtained by Makino *et al.* (1973) at pH 9.2. The cmc was also determined using gel chromatography. The column was first equilibrated with TX above the cmc. The eluent was then changed to buffer containing no TX. The plateau region that occurs after the elution of micelles, but before the internal volume, is a measure of the monomer concentration that was in equilibrium with micelles. Using this method, a value of $2.6 \times 10^{-4}$ M at $I = 0.01$ was determined.

The values used for SDS were determined from the data of Emerson and Holtzer (1965, 1967). These values were $1.3 \times 10^{-3}$ M ($I = 0.10$) and $4.4 \times 10^{-3}$ M ($I = 0.01$).

PREPARATION OF PHOSPHATIDYLCHOLINE VESICLES. Vesicles were prepared by ultrasonic irradiation of

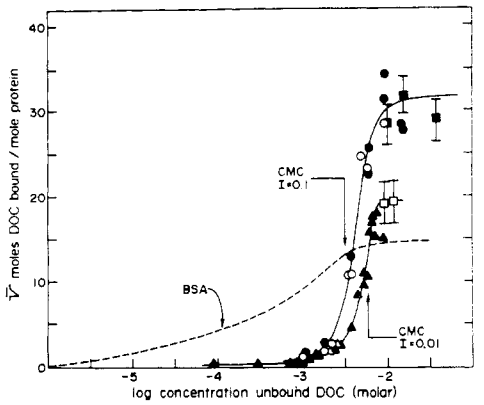


FIGURE 1: Binding of DOC to cytochrome $b_5$, pH 8.10, 23° obtained by: (●) equilibrium dialysis in forward direction (excess DOC added to dialysate side of cell), $I$ = 0.10; (○) equilibrium dialysis in reverse direction (excess DOC added to protein side of cell), $I$ = 0.10; (■) sedimentation equilibrium, $I$ = 0.10; (▲) equilibrium dialysis (forward direction), $I$ = 0.01; (△) equilibrium dialysis (reverse direction), $I$ = 0.01; (□) sedimentation equilibrium, $I$ = 0.01. The arrows indicate the cmc of DOC at $I$ = 0.10 and 0.01. The broken line indicates the binding of DOC to bovine serum albumin (Makino *et al.*, 1973).

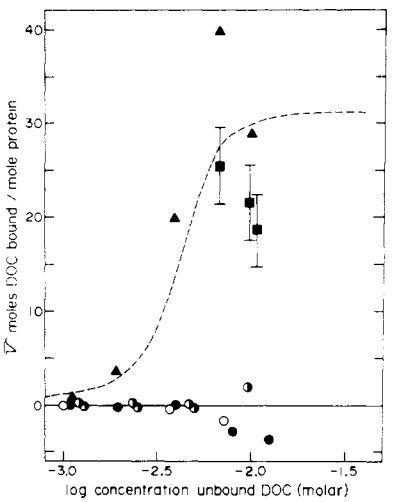


FIGURE 2: Binding of DOC to the polar and hydrophobic fragments of cytochrome $b_5$, pH 8.10, 23°: (▲) hydrophobic fragment, equilibrium dialysis (forward direction), $I$ = 0.10; (■) hydrophobic fragment, measured by sedimentation equilibrium, $I$ = 0.10; (●) polar fragment, equilibrium dialysis (forward direction), $I$ = 0.10; (○) polar fragment, equilibrium dialysis (reverse direction), $I$ = 0.10; (◑) polar fragment, equilibrium dialysis (forward direction), $I$ = 0.01. The broken line indicates the binding of DOC to the entire molecule of cytochrome $b_5$, $I$ = 0.10 (from Figure 1).

phosphatidylcholine suspensions in pH 8.10 buffer, ionic strength 0.10. A 20-kHz Branson Sonifier (Model W-85) was used at 30 W output for 3 ml of solution, or 75 W output for 150 ml of solution, under a nitrogen atmosphere, for 15 min. The temperature was maintained between 0 and 5° by use of an ice bath and sonicating for only 30-sec intervals at a time.

## Results

*Binding of DOC to Cytochrome $b_5$.* Experimental data for the binding of DOC to the entire molecule of cytochrome $b_5$ are shown in Figure 1. In this figure, the number of DOC ions bound per molecule of cytochrome $b_5$ ($\bar{\nu}$) is plotted as a function of the free DOC concentration in equilibrium with the protein. As can be seen, equilibrium was approached from both directions and the results obtained were the same within experimental error, demonstrating that the data represent true thermodynamic equilibrium. The results obtained by sedimentation equilibrium at the highest DOC concentrations show that no increase in binding occurs above the concentration level beyond which dialysis equilibrium measurements cannot be used. The monomeric molecular weight (16,200) was employed in obtaining $\bar{\nu}$ from the ultracentrifugal plots by eq 1. In fact, no other choice of $M_p$ was possible since the experimental parameter $M_p(1 - \phi'\rho)$ that is obtained directly from the sedimentation data was too small to be interpreted in terms of an aggregated protein species, *i.e.*, $M_p(1 - \phi'\rho)$ was greater than 16,200 $(1 - \bar{v}_p\rho)$ but less than 32,400 $(1 - \bar{v}_p\rho)$.

The results obtained previously for the binding of DOC to serum albumin (Makino *et al.*, 1973) are shown in Figure 1 for comparison; it is seen that the behavior observed for cytochrome $b_5$ is quite different. Cytochrome $b_5$ does not bind DOC until the concentration of unbound detergent is greater than 1 mM, indicating that this protein has no high affinity binding sites for DOC ions. However, between 1 and 10 mM DOC, a large increase in binding is observed, reaching a saturation value by 10 mM DOC. Such a large increase in binding over this narrow concentration range indicates that DOC binding to cytochrome $b_5$ is a highly cooperative process. It should be noted that cytochrome $b_5$ exhibits this cooperative binding near the same DOC concentrations at which micelles are first formed in solution (cmc). The point at which cytochrome $b_5$ binds DOC can be shifted to higher DOC concentrations by lowering the ionic strength, an effect analogous to the dependence of the cmc upon ionic strength. In fact, the cooperative binding was shifted by approximately the same amount by the change in ionic strength as was the cmc. In contrast, the binding of DOC to native serum albumin is not dependent upon the ionic strength. Makino *et al.* (1973) found the binding at ionic strength 0.01 to be indistinguishable from the binding observed at ionic strength 0.03.

The saturated DOC–protein complex formed by the cooperative binding (Figure 1) probably contains the protein in a conformation closely resembling the native state (evidence for this will be presented in later paper). Because the cooperative binding is dependent upon some of the same parameters as micelle formation (*i.e.*, a similar dependence upon ionic strength and detergent concentration) the process appears to resemble the cooperative formation of a micelle of detergent around at least part of the native protein molecule.

In order to determine the portion of cytochrome $b_5$ that binds DOC, both the polar, heme-containing fragment and the hydrophobic fragment were tested for DOC binding, with the results shown in Figure 2. The data show that DOC is not bound at all by the polar domain, while the hydrophobic domain binds essentially the same amount of DOC as the entire molecule of cytochrome $b_5$. (The larger amount of scatter in the data obtained with the hydrophobic fragment was due to the lower concentration of protein used in these experiments because of the limited amounts of purified peptide that were available.) Since the polar domain by all available criteria undergoes no conformational change upon proteolysis, the results clearly indicate that the binding of DOC to cytochrome $b_5$ is localized upon the small hydrophobic domain of the protein. The polar region neither has affinity for DOC ions, nor does it influence the binding of the detergent by the hydrophobic portion.

*Binding of Triton X-100.* The binding of the nonionic de-

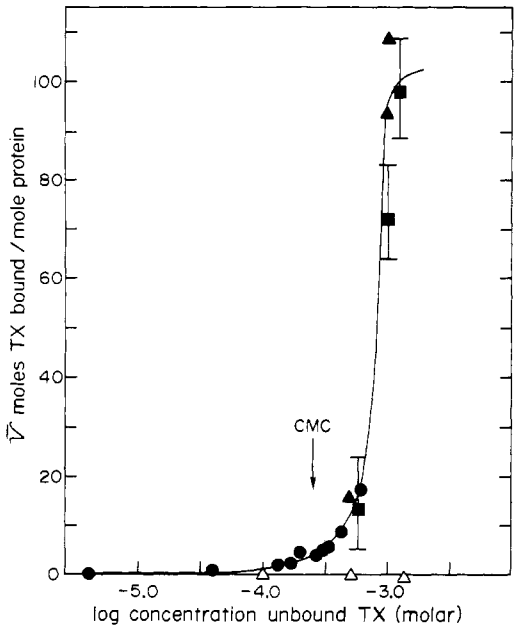


FIGURE 3: Binding of TX to cytochrome $b_5$ and polar fragment, $I$ = 0.10, pH 8.10, 23°: (●) cytochrome $b_5$, equilibrium dialysis; (▲) cytochrome $b_5$, column chromatography; (■) cytochrome $b_5$, sedimentation equilibrium; (△) polar fragment, column chromatography.

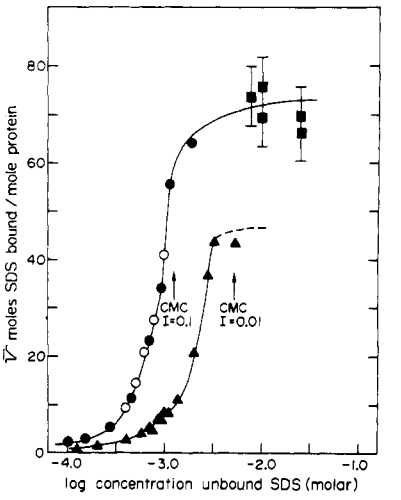


FIGURE 4: Binding of SDS to cytochrome $b_5$, pH 8.10, 23° obtained by: (●) equilibrium dialysis (forward direction), $I$ = 0.10; (○) equilibrium dialysis (reverse direction), $I$ = 0.10; (■) sedimentation equilibrium of apocytochrome $b_5$, $I$ = 0.10; (▲) equilibrium dialysis (forward direction), $I$ = 0.01. The arrows indicate the cmc for SDS at $I$ = 0.10 and 0.01.

tergent Triton X-100 (TX) to both cytochrome $b_5$ and the polar fragment is shown in Figure 3. Results obtained by dialysis equilibrium, column chromatography, and sedimentation equilibrium are all seen to be in good agreement. Once again, eq 1 was used to evaluate $\bar{\nu}$ from sedimentation equilibrium measurements of $M_p(1 - \phi'\rho)$. At the lowest concentration of TX used in such ultracentrifugal evaluations of $\bar{\nu}$, the experimental parameter, $M_p(1 - \phi'\rho)$, was once again too small to be interpreted in terms of an aggregated species, *i.e.*, $M_p(1 - \phi'\rho)$ was greater than 16,200 $(1 - \bar{\nu}_p\rho)$ but less than 32,400 $(1 - \bar{\nu}_p\rho)$. The value obtained for $\bar{\nu}$ assuming monomeric cytochrome $b_5$ agrees well with the binding obtained by either equilibrium dialysis or column chromatography. At the two higher TX concentrations, $M_p(1 - \phi'\rho)$ was sufficiently large to permit interpretation in terms of either monomeric protein and large $\bar{\nu}$ values or in terms of dimeric protein with a very small amount of bound TX. The value of $\bar{\nu}$ based on dimeric protein would have been zero at $1 \times 10^{-3}$ M unbound TX, a result clearly incompatible with the values of $\bar{\nu}$ that are firmly established at lower unbound TX concentrations, it being thermodynamically impossible for $\bar{\nu}$ to decrease with increasing detergent concentration when all other factors are held constant. The results obtained by sedimentation equilibrium as shown in the figure were thus all based on a monomeric state for the protein: the $\bar{\nu}$ values obtained are seen to agree with the values obtained by column chromatography. The latter are subject to a 20% error (see Experimental Section), but require no assumption about the state of aggregation of the protein.

The figure shows that the interaction of cytochrome $b_5$ with TX is analogous to its interaction with DOC. There are no high affinity binding sites on cytochrome $b_5$ for the TX molecules, for there is no binding of TX until concentrations of unbound TX are near the cmc of this detergent. At this point, cytochrome $b_5$ shows a very cooperative binding of TX while the polar domain does not bind the detergent. The saturation level of binding is much larger than for DOC, but TX micelles formed in the absence of protein are also much larger than DOC micelles (see later discussion of micelle sizes). Co-micellization of the hydrophobic domain of the protein with the detergent is again a reasonable interpretation of the data. The binding isotherm with TX is steeper than that shown in Figure 1 for DOC. This is consistent with the interpretation of micelle formation since the formation of a large micelle is a more cooperative process than the formation of a small micelle. The binding of TX was found to be unaffected by ionic strength: the same saturation level of 100 ± 15 mol of TX was observed at 0.01 and 0.10 ionic strength. This is again consistent with micelle formation. TX is a nonionic detergent and much less affected by ionic strength than DOC.

*Binding of SDS.* SDS is a detergent known to bind tightly and cooperatively to nearly all proteins. For a large number of water-soluble proteins and for some membrane proteins, a saturation level of about 1.4 g of SDS/g of protein is attained and a large conformational change accompanies the binding (Reynolds and Tanford, 1970a,b). The binding of SDS to intact cytochrome $b_5$ and to the polar fragment was measured, with the results shown in Figures 4 and 5. The binding to the intact protein at $I$ = 0.10 is superficially similar to the "typical" results cited above, reaching a maximum of 72 mol bound per mol of cytochrome $b_5$, which corresponds to 1.3 g of SDS/g of protein. However, in marked contrast to our previous observations for other proteins (Reynolds and Tanford, 1970a), the saturation level of binding depends strongly on ionic strength. In addition, the concentration at which the transition occurs (2.25 mM SDS at the lower ionic strength) is significantly higher than is usually observed.

The abnormal behavior of cytochrome $b_5$ becomes more apparent when binding of SDS to both the polar and hydrophobic peptides is measured. Figure 5A shows a maximum of only 24 mol of SDS bound per mole of polar peptide (0.7 g of SDS/g of protein). This low value may be due to the resistance of the polar region to SDS denaturation because of stabilization of the protein structure by the heme moiety. The most significant finding, however, is the highly cooperative binding of relatively large amounts of SDS to the hy-

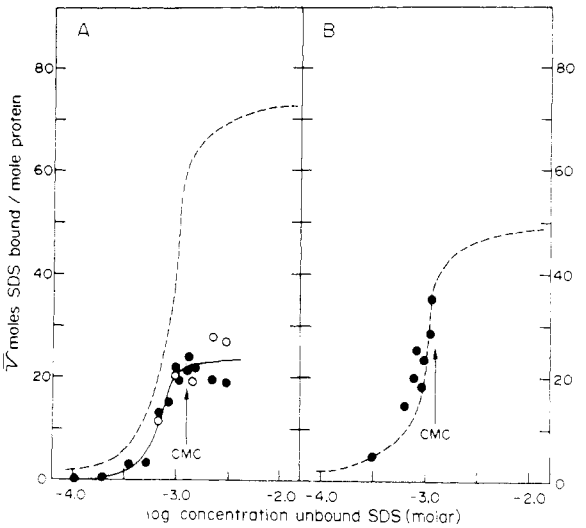


FIGURE 5: Binding of SDS to tryptic fragments of cytochrome $b_5$, $I$ = 0.10, pH 8.10, 23°. The arrows indicate the cmc of SDS: (A, polar fragment) (●) equilibrium dialysis (forward direction); (○) equilibrium dialysis (reverse direction); the broken line indicates the binding of SDS to the whole molecule of cytochrome $b_5$ (from Figure 4); (B, hydrophobic fragment) (●) equilibrium dialysis (forward direction); the broken line indicates the difference in binding of SDS between whole cytochrome $b_5$ and the polar fragment.

drophobic fragment at concentrations near the critical micelle concentration (Figure 5B). Saturation levels of binding could not be determined because of the limitation on protein concentration imposed by the limited amount of purified peptide we had available, but the data do fit a curve calculated from the difference in binding of SDS to cytochrome $b_5$ and the polar fragment (dashed line). Using this calculated curve, saturation occurs at 50 mol of SDS bound per mol of peptide. This small portion of cytochrome $b_5$ is, therefore, binding an unusually *large* amount of detergent (~3 g of SDS/g of protein). Even though the intact molecule binds a normal amount of detergent, the distribution of the detergent on the protein is unusual, the polar domain binding only 50% as much as a normal globular protein and the hydrophobic domain binding 2.5 times the normal amount. The large amount of SDS bound to the hydrophobic region, the fact that this binding occurs near the cmc of the detergent, and especially the ionic strength dependence seen in Figure 4 (which can now be interpreted as arising in large part from binding to the hydrophobic domain) suggest that the binding of SDS to the hydrophobic domain may be a process of micelle formation similar to that observed for DOC and TX.

*Comparison of Detergent Micelles with the Cytochrome $b_5$–Detergent Complexes.* Since both nondenaturing detergents (DOC and TX) as well as the denaturing detergent (SDS) appear to bind to the hydrophobic domain of cytochrome $b_5$ in a manner analogous with micelle formation, the Stokes radius of each detergent micelle and the Stokes radius of the corresponding cytochrome $b_5$–detergent complex were measured on calibrated Sephadex columns. The order of micelle size (TX > SDS > DOC) was the same as the order of the size of the corresponding cytochrome $b_5$–detergent complexes. In each case the complex was slightly larger than the pure micelle; for TX, for example, $R_s$ = 60 and 55 Å, respectively, for the complex and the pure micelle. This result is consistent with the proposal that the protein–detergent complexes resemble an approximately

Table II

| Detergent | $I$ | Micelle Aggregation No. | Max Binding per mol: Cyt $b_5$ | Max Binding per mol: Polar Peptide |
|---|---|---|---|---|
| DOC | 0.01 | 4[a] | 20 | 0 |
| | 0.10 | 13[a] | 31 | 0 |
| SDS | 0.01 | 65[a] | 45 | |
| | 0.10 | 85[b] | 70 | 19 |
| TX | | ~120[c] | 100 | 0 |

[a] Average values obtained by Small (1968). See text. [b] Emerson and Holtzer (1965, 1967). [c] Becher (1967); Lewis and Kirk (1974).

normal detergent micelle into which the hydrophobic domain of the protein has been inserted, leaving the polar domain exposed to solvent.

To further substantiate the similarity between detergent micelles and the detergent bound to cytochrome $b_5$, the maximum binding of each detergent was compared with the aggregation number of the pure detergent micelle (Table II). The results show that a good correlation exists between the number of monomers per micelle and the maximum binding for each detergent. In addition, perturbation of the micelle aggregation number by changes in the ionic strength is reflected in a similar perturbation of the maximum binding. Table II suggests that the binding of DOC may be anomalously high if the binding is to be interpreted as closely related to the formation of a normal micelle. This is actually not so because DOC micelles, unlike micelles formed by simple ionic detergents, do not have a preferred micelle size that is only mildly influenced by external conditions. For example, the average aggregation number is extremely sensitive to NaCl concentration and does not level off at a nearly constant value when a salt concentration sufficient for suppression of electrostatic repulsion between head groups is reached (Small, 1968). The results suggest a broad range of possible micelle sizes, the distribution among which will be sensitive to a variety of environmental factors. Work in this laboratory (J. A. Reynolds, unpublished results) has in fact shown that DOC micelles containing solubilized heme are hetergeneous (at ionic strength 0.125) and contain between 20 and 45 DOC monomers per micelle. These values are in the same range as we have observed here for complexes between DOC and cytochrome $b_5$.

*Binding of Cytochrome $b_5$ to Phospholipid Vesicles.* The binding of detergents to cytochrome $b_5$ has indicated nonspecific hydrophobic interactions between amphiphilic molecules and the hydrophobic domain of the protein; the binding of cytochrome $b_5$ to phosphatidylcholine vesicles was measured to test the general nature of this interaction. Two types of vesicles were used: synthetic didodecylphosphatidylcholine vesicles and egg-yolk phosphatidylcholine vesicles. To test the binding of cytochrome $b_5$ to vesicle preparations, the elution position of cytochrome $b_5$ from a Sepharose 4B column was measured after incubation of the protein with each type of vesicle for 1 hr at 37°, and was then compared with the elution position of cytochrome $b_5$ in the absence of phospholipid and with that of the corresponding vesicles alone (Figure 6). The protein in the ab-

sence of lipid or detergent is in aggregated form with $R_s$ = 60 Å. The pure lipid preparations elute as two peaks, similar to those observed for egg-yolk phosphatidylcholine preparations by Huang (1969), who showed that the peak eluting at the void volume contains multilayered vesicles and the peak included in the Sepharose 4B column contains uniform single walled vesicles.

Figures 6B and 6E demonstrate that the cytochrome $b_5$ aggregate disappears after incubation with phospholipid, and that the protein eluted instead at the positions characteristic of the lipid vesicles. In the case of egg-yolk phosphatidylcholine vesicles cytochrome $b_5$ eluted as two peaks, very similar to the elution pattern obtained with vesicles alone, indicating that the protein binds to both the single-walled and multi-walled vesicles. With didodecylphosphatidylcholine the result is less definite, since cytochrome $b_5$ eluted as a single peak at the void volume with a long trailing edge (Figure 6B). When the eluate from this column was monitored at 230 nm instead of 412 nm no peak was observed at the position corresponding to the included peak of Figure 6A. The most likely interpretation is, therefore, that incorporation of protein perturbs the vesicle size distribution in the case of didodecylphosphatidylcholine, causing all the vesicles to elute near the void volume.

Figures 6C and 6F show that the polar peptide of cytochrome $b_5$ is not incorporated into phospholipid vesicles, *i.e.*, as in the case of binding to detergent micelles or to microsomal membranes, the hydrophobic domain of the protein represents the site of interaction. (The small peak appearing at the void volume in Figure 6F arises from light scattering from the pure vesicles. Tubes from this peak were scanned and showed no Soret band, only a monotonically increasing absorbance as the wavelength decreased.)

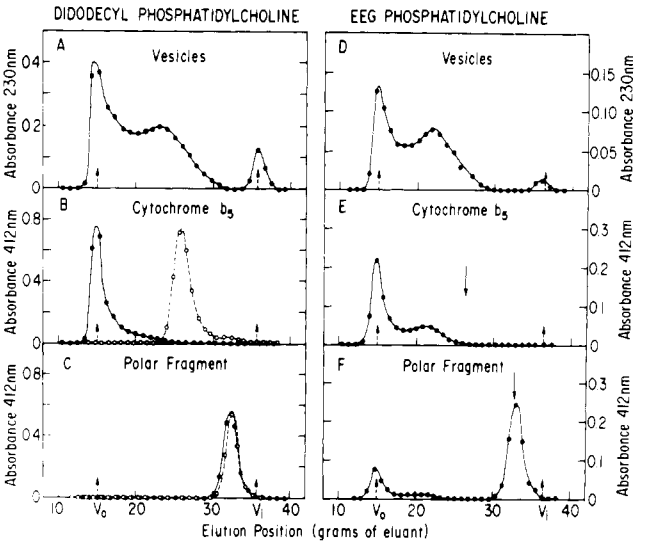


FIGURE 6: Determination of the binding of cytochrome $b_5$ to phosphatidylcholine vesicles by gel chromatography; 0.9 × 57 cm Sepharose 4B columns, pH 8.10, 23°, $I$ = 0.10 with a flow rate of ~2.5 g/hr were used. The dashed arrows at 15.0 and 36.5 g indicate the void volume ($V_0$) and the internal volume ($V_i$). (A) Elution of didodecylphosphatidylcholine vesicles (5 mM phospholipid). The column was equilibrated with 0.1 mM didodecylphosphatidylcholine prior to elution of the vesicles. (B) Elution of cytochrome $b_5$. Open circles illustrate the elution of cytochrome $b_5$ in the absence of didodecylphosphatidylcholine (*i.e.*, effluent contained only buffer). Filled circles illustrate the elution of cytochrome $b_5$ after incubation with 5 mM didodecylphosphatidylcholine. In this case, the column was equilibrated with 0.1 mM didodecylphosphatidylcholine. (C) Elution of the polar fragment of cytochrome $b_5$. Open circles represent the elution of the polar fragment in the absence of phosphatidylcholine. Filled circles represent the elution of the polar fragment after incubation with 5 mM didodecylcholine on a column equilibrated with 0.1 mM phosphatidylcholine. (D) Elution of egg phosphatidylcholine vesicles (5 mM phospholipid) after the column had been presaturated with phosphatidylcholine. (E) Elution of cytochrome $b_5$ after incubation with 5 mM egg phosphatidylcholine. The column had been presaturated with phospholipid. The arrow indicates the elution position of cytochrome $b_5$ in the absence of phosphatidylcholine (from Figure 6B). (F) Elution of the polar fragment of cytochrome $b_5$ after incubation with 5 mM egg phosphatidylcholine. The column had been presaturated with phospholipid. The arrow indicates the elution position of the polar fragment in the absence of phosphatidylcholine (from Figure 6C). The small peak appearing at the void volume was entirely due to light scattering of the phospholipid vesicles.

## Discussion

Cytochrome $b_5$ is a protein for which good indirect experimental evidence exists in favor of direct contact of the protein with the hydrophobic interior of the membrane. Recombination of cytochrome $b_5$ with microsomal membranes is possible and such recombination is dependent upon the presence of a 40 amino acid sequence with an unusually large content of nonpolar amino acids (Spatz and Strittmatter, 1971). We have shown that the protein is also readily incorporated into vesicles prepared from natural or synthetic phospholipids, and, while this paper was being written, interaction with a variety of phospholipids was also reported on the basis of immobilization of spin labels introduced into phospholipid molecules (Dehlinger *et al.*, 1974). All these data indicate that the native cytochrome $b_5$ molecule contains a hydrophobic binding region, localized on the hydrophobic domain of the molecule. We are concerned with the nature of this binding region and have approached this problem by studying the interaction of cytochrome $b_5$ and its proteolytic fragments with simple amphiphiles. Three amphiphiles have been used. Two of them, DOC and the nonionic detergent TX, do not combine with water-soluble proteins unless they contain specific hydrophobic binding sites, and when such sites are present they bind without causing denaturation (Helenius and Simons, 1972; Makino *et al.*, 1973). The third substance employed, SDS, binds to virtually all proteins with accompanying denaturation. The saturation level is usually very high, 1.4 g of detergent/g of protein (Reynolds and Tanford, 1970a,b).

We have shown that all three amphiphiles combine with intact cytochrome $b_5$ and with the hydrophobic fragment derived from it by proteolysis. As expected, only SDS binds to the heme-containing polar fragment, which has a highly polar surface. The maximal binding to this fragment was only 0.7 g/g of peptide, well below the amount usually observed. This aspect of our results has not been pursued, as our main interest is in the hydrophobic binding region.[2]

The mode of association of the three amphiphiles with the hydrophobic region was found to be different from any observed before, as may be seen from Table III, where a comparison is made between cytochrome $b_5$ and three proteins representing, respectively, a typical water-soluble protein with no affinity for amphiphiles (ovalbumin), a water-soluble protein with such affinity (BSA), and the apoprotein of a circulating lipoprotein. The unique aspects of the mode of association may be summarized as follows.

(1) Cytochrome $b_5$ has no high affinity binding sites for single amphiphile molecules or ions. The protein exists in

[2] The anomalous SDS binding to both fragments emphasizes again the danger of placing too much reliance on molecular weights empirically established on the basis of SDS gel electrophoresis.

Table III: Comparison between Cytochrome $b_5$ and Other Proteins.

| | DOC, TX | | SDS | |
|---|---|---|---|---|
| | High-Affin. Sites for Single Molecules | Coop. Binding | High-Affin. Sites for Single Molecules | Coop. Binding |
| Ovalbumin | No | Insignificant | No | With denaturation |
| BSA | Yes | No | Yes | With denaturation |
| HDL[a] apoproteins | Yes | With change in conformation[b] | Yes | With change in conformation[b] |
| Cytochrome $b_5$ | No | Yes, without denaturation | No | With denaturation of polar domain only |

[a] Reynolds and Simon (1974); Makino *et al.* (1975). [b] It was suggested that the conformation state induced in HDL apoproteins by the cooperative binding of SDS and of cationic detergents may differ from the denatured state adopted in these detergents by most water-soluble proteins.

aggregated form in aqueous solution (Spatz and Strittmatter, 1971) but there is good reason to believe that the forces involved are weak and that the aggregate is easily disrupted. With the substances we have tested there is no binding or disaggregation until the free amphiphile concentration is very close to the normal cmc of each amphiphile. The simplest interpretation is that hydrophobic amino acid side chains are not sufficiently concentrated in any portion of the surface of the hydrophobic domain to provide a *localized* site where the free energy of an isolated amphiphilic ligand would be comparable to or less than in a pure micelle. Instead the "hydrophobicity" of the surface may be viewed as being diffuse.

(2) Cooperative binding of all three amphiphiles occurs near the cmc, and we suggest that it occurs to the native form of the protein. (Further evidence on this point will be presented in a subsequent paper.) The saturation levels of binding for the different amphiphiles and their dependence on ionic strength all suggest that the mode of association is similar to micelle formation as it occurs in the absence of protein. In other words, if P is native protein and Z a single amphiphilic ligand, the reaction

$$P + \nu Z \rightleftharpoons PZ_\nu \quad (2)$$

is slightly favored over the competing process of formation of a pure micelle

$$mZ \rightleftharpoons Z_m \quad (3)$$

with the saturation value of $\nu$ in reaction 2 being of the same order of magnitude as the optimal micelle size in reaction 3, with a similar dependence on ionic strength. This suggests that most of the free energy for the formation of $PZ_\nu$ arises from the same factors that lead to formation of pure micelles. The diffuse hydrophobicity of the protein surface represents only a small perturbation of the thermodynamics of the system, sufficient to cause co-micellization of the protein with the amphiphile, but not sufficient to greatly alter the characteristics of the micelle that is formed.

The statement that reaction 2 is slightly favored over reaction 3 of course implies that the protein will react with already existing micelles to form $PZ_\nu$, and equilibrium measurements cannot in fact distinguish between direct formation of $PZ_\nu$ as given by reaction 2 and the indirect process in which the micelle is formed first and the protein is then inserted into it. What can be eliminated is the possibility that the micelle is formed first and that the protein then binds *by polar forces* to the micelle surface. Considering the different kinds of micelle surface presented to the protein in these experiments, the latter process could not lead to the observed close correlation between the cmc and the critical concentration for formation of $PZ_\nu$.

(3) It is probable that even the binding of SDS to the hydrophobic domain of cytochrome $b_5$ is a process of this kind, occurring without denaturation of the protein, instead of the cooperative binding with denaturation commonly observed for water-soluble proteins. Evidence for this comes from the higher level of binding than the typical 1.4 g of SDS/g of protein, and from the effect of ionic strength on maximal binding. Preference for co-micellization is in fact predicted in terms of the same thermodynamic principle that has been used by Foster and Aoki (1958) and by Reynolds *et al.* (1967) to explain formation of the denatured SDS–protein complexes for typical water-soluble proteins. Let N and D be the native and altered states of a protein in the absence of detergent, and let $NZ_\nu$ and $DZ_\mu$ represent complexes that can be formed cooperatively in the presence of the detergent Z. The overall reaction scheme is then

$$\begin{array}{ccc} N & \rightleftharpoons & D \\ \downarrow\uparrow & & \downarrow\uparrow \\ NZ_\nu & \rightleftharpoons & DZ_\mu \end{array} \quad (4)$$

If $\mu > \nu$, as would be true for proteins that cannot bind SDS in the native state, or for BSA for which $\nu \simeq 10$ but $\mu$ (at the level of 1.4 g of SDS/g of protein) $\simeq 350$, the addition of SDS will favor denaturation even though the equilibrium ratio [D]/[N] in the absence of SDS may be extremely small. For the hydrophobic domain of cytochrome $b_5$, however, co-micellization of the native protein with SDS leads to relatively large values for $\nu$, *e.g.*, $\nu \simeq 50$ at ionic strength 0.10 (Figure 5B and Table II), whereas $\mu$ (corresponding to 1.4 g of SDS/g of hydrophobic domain) would be only $\simeq 20$. Thus the presence of SDS must stabilize the *native* state in this case. It may be noted that anomalously high SDS binding has also been observed for the major glycoprotein from erythrocyte membranes (Grefrath and Reynolds, 1975).

It is evident that the characteristics of the hydrophobic binding region of cytochrome $b_5$ deduced from these studies are uniquely designed for incorporation of the protein into an extended hydrophobic medium, such as the core of a bilayer membrane, and that they are quite different from the characteristics of the discrete hydrophobic binding sites of serum albumin and the one circulating lipoprotein that has been studied. The hydrophobic amino acid side chains that lead to the existence of a hydrophobic region are in cytochrome $b_5$ not concentrated in a narrow area where reaction with a single amphiphile would be possible, but spread diffusively over a wider area such that only envelopment in

an extended hydrophobic domain leads to a thermodynamically favored situation. There appears to be no discrimination between extended hydrophobic domains of different kinds: the core of a micelle (even the micelle of limited size formed by DOC) serves as well as the core of a lipid bilayer. The incorporation of cytochrome $b_5$ into a membrane is evidently nothing more than a special manifestation of a general phenomenon.[3,4]

Although the primary driving force for the incorporation of cytochrome $b_5$ into a membrane thus appears to be nonspecific, this does not rule out some selectivity at a secondary level. Once the protein has become incorporated into a membrane containing a mixture of lipids, weak forces may well lead to preferential migration of particular lipid molecules to the vicinity of the protein. This process would be analogous to preferential interactions of a soluble protein in a mixed solvent, and could arise from interactions between ionic groups on the protein and polar head groups of lipid molecules, as well as from preference for hydrocarbon chains of a particular length or degree of unsaturation. Similarly, the inability of cytochrome $b_5$ to associate with single amphiphile molecules as a *primary* process does not rule out interactions with discrete lipid molecules after incorporation in a membrane. Such a process would be analogous to the many different possible states of water molecules in the vicinity of a protein in aqueous solution. We suggest that the immobilization of lipid hydrocarbon chains by cytochrome $b_5$, as seen by spin label studies (Dehlinger *et al.*, 1974), is due to this kind of process. As is true for the analogous process in aqueous solution, this kind of selective interaction need not make a favorable contribution to the free energy of the primary incorporation process, *i.e.*, the hydrophobic free energy gained by incorporation can easily be sufficiently large to overcome some loss of free energy by vicinal lipid molecules.

In conclusion, it should be emphasized that we do not intend to suggest that the mode of association between cytochrome $b_5$ and membrane lipids will prove to be typical for all or even for a major fraction of membrane proteins in general. Membrane proteins can be expected to be as rich in diversity as soluble proteins have proved to be.

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## References

Abrams, A., and Baron, C. (1968), *Biochemistry 7,* 501.
Becher, P. (1967), in Nonionic Surfactants, Schick, M. J., Ed., New York, N.Y., Marcel Decker, p 495.
Benzonana, G. (1969), *Biochim. Biophys. Acta 176,* 836.
Cohn, E. J., and Edsall, J. T. (1943), Proteins Amino Acids, and Peptides, New York, N.Y., Reinhold, p 372.
Dehlinger, P. J., Jost, P. C., and Griffith, O. H. (1974), *Proc. Nat. Acad. Sci. U.S. 71,* 2280.
Edelhoch, H. (1967), *Biochemistry 6,* 1948.
Emerson, M. F., and Holtzer, A. (1965), *J. Phys. Chem. 69,* 3718.
Emerson, M. F., and Holtzer, A. (1967), *J. Phys. Chem. 71,* 1898.
Eng, L. F., Chao, F. C., Gerstl, B., Pratt, D., and Tavaststjerna, M. G. (1968), *Biochemistry 7,* 4455.
Eylar, E. H., Salk, J., Beveridge, G. C., Brown, L. V. (1969), *Arch. Biochem. Biophys. 132,* 34.
Foster, J. F., and Aoki, K. (1958), *J. Amer. Chem. Soc. 80,* 5215.
Grefrath, S., and Reynolds, J. A. (1975), *Proc. Nat. Acad. Sci. U.S. 71,* 3913.
Helenius, A., and Simons, K. (1972), *J. Biol. Chem. 247,* 3656.
Huang, C. (1969), *Biochemistry 8,* 344.
Ito, A., and Sato, R. (1968), *J. Biol. Chem. 243,* 4922.
Lewis, M. S., and Kirk, W. D. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1230.
Makino, S., Reynolds, J. A., and Tanford, C. (1973), *J. Biol. Chem. 248,* 4926.
Makino, S., Tanford, C., and Reynolds, J. A. (1975), *J. Biol. Chem.* (in press).
Masters, B. S. S., Williams, C. H., and Kamin, H. (1967), *Methods Enzymol. 10,* 565.
Mathews, F. S., Argos, P., and Levine, M. (1971b), *Cold Spring Harbor Symp. Quant. Biol. 36,* 387.
Mathews, F. S., Levine, M., and Argos, P. (1971a), *Nature (London), New Biol. 233,* 15.
Mysels, K. J., and Princen, L. H. (1959), *J. Phys. Chem. 63,* 1696.
Nóbrega, F. G., and Ozols, J. (1971), *J. Biol. Chem. 246,* 1706.
Ozols, J. (1974), *Biochemistry 13,* 426.
Ozols, J., and Strittmatter, P. (1969), *J. Biol. Chem. 244,* 6617.
Reynolds, J. A., Herbert, S., Polet, H., and Steinhardt, J. (1967), *Biochemistry 6,* 937.
Reynolds, J. A., and Simon, R. H. (1974), *J. Biol. Chem. 249,* 3937.
Reynolds, J. A., and Tanford, C. (1970a), *Proc. Nat. Acad. Sci. U.S. 66,* 1002.
Reynolds, J. A., and Tanford, C. (1970b), *J. Biol. Chem. 245,* 5161.
Robinson, N. C., Nozaki, Y., and Tanford, C. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1370.
Robinson, N. C., Tye, R. W., Neurath, H., and Walsh, K. A. (1971), *Biochemistry 10,* 2743.
Rogers, M. J., and Strittmatter, P. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1254.
Small, D. M. (1968), *Advan. Chem. Ser. No. 84.*
Spatz, L., and Strittmatter, P. (1971), *Proc. Nat. Acad. Sci. U.S. 68,* 1042.
Steinhardt, J., and Reynolds, J. A. (1969), Multiple Equilibria in Proteins, New York, N.Y., Academic Press.
Strittmatter, P., Rogers, M. J., and Spatz, L. (1972), *J. Biol. Chem. 247,* 7188.
Stittmatter, P., and Velick, S. F. (1956), *J. Biol. Chem. 221,* 277.

[3] The self-association of the protein or of the hydrophobic fragment in aqueous solution in the absence of detergent or lipid may be viewed as another special manifestation. It is evident, however, that self-association fulfills the requirements of the hydrophobic site only weakly, existence as a monomeric species in a continuous hydrocarbon medium being the more stable state.

[4] The observation of Strittmatter *et al.* (1972) that cytochrome $b_5$ cannot be as readily incorporated into mitochondrial or erythrocyte membranes as into liver microsomal membranes would seem to be at variance with our conclusion. These membranes were, however, not depleted of their natural protein content before incorporation was attempted and this could have been the reason for their results. Rogers and Strittmatter (1974) have shown subsequently that the presence of cytochrome $b_5$ reductase in microsomal membranes restricts the binding of cytochrome $b_5$ to membrane and *vice versa*.

Tanford, C., Nozaki, Y., Reynolds, J. A., and Makino, S. (1974), *Biochemistry 13,* 2369.
Tsugita, A., Kobayashi, M., Tani, S., Kyo, S., Rashid, M. A., Yoshida, Y., Kajihara, T., and Hagihara, B. (1970), *Proc. Nat. Acad. Sci. U.S. 67,* 442.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17,* 303.

# Comparison of the Resonance Raman Spectra of Carbon Monoxy and Oxy Hemoglobin and Myoglobin: Similarities and Differences in Heme Electron Distribution†

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ABSTRACT: With 441.6-nm excitation, which is near the Soret band, we observe that the resonance Raman spectra of hemoproteins contain not only the bands between 650 and 1700 $cm^{-1}$ which arise from vibrations of the conjugated macrocycle, but also bands below 650 $cm^{-1}$, some of which involve vibrations of the iron pyrrole–nitrogen bonds. The spectra of the oxygen and carbon monoxide complexes of both myoglobin and hemoglobin are sufficiently similar to those of low spin met derivatives, that the electronic distribution on the heme for both ligands can be interpreted as that of a low spin ferriheme. This agrees with an earlier interpretation, by others, of comparative optical absorption spectra and, as pointed out previously, would imply that in the complex the ligands are bound as $O_2^-$ and $CO^-$. However, band frequencies and relative intensities differ somewhat between the carbon monoxide and oxygen complexes of the same protein, which indicates differences between the details of the $\pi$-electron distributions in the corresponding complexes.

In a previous paper (Yamamoto *et al.,* 1973) we presented a comparative study of resonance Raman spectra, with excitation near the Soret band, of a number of hemoproteins including oxyhemoglobin ($HbO_2$)[1] and deoxyhemoglobin (Hb). These vibrational spectra showed that the electronic charge distribution on the iron in $HbO_2$ is essentially the same as that in low spin ferric hemoproteins. Although these spectra do not reflect directly the electron distribution on the ligand, the diamagnetism of $HbO_2$, as first proposed by Weiss (1964), implies that the oxygen is bound as $O_2^-$ with the unpaired electron ($S = \frac{1}{2}$) on the $O_2^-$ antiferromagnetically spin paired with the low spin ferric ($S = \frac{1}{2}$) ion. Similar correlations among the resonance Raman spectra of some of the same hemoglobin derivatives have also been noted more recently by Spiro and Strekas (1974) using excitation near the $\alpha$ and $\beta$ bands, at which excitation frequency the intensity distribution among the vibrational lines differs markedly from that observed with Soret excitation.

In this paper we show that, by the same Raman criteria, the electron distribution on the iron in HbCO also is similar to that of the low-spin ferrihemoproteins and, thus, from the diamagnetism of HbCO, the carbon monoxide should be bound formally as $CO^-$. Whereas our previous paper (Yamamoto *et al.,* 1973) was limited to the spectral range 1300–1700 $cm^{-1}$, this paper includes spectra in the frequency range 100–900 $cm^{-1}$ which contains bands with contributions from iron–pyrrole nitrogens. In both spectral ranges differences occur between the oxygen and carbon monoxide complexes. We also observe that the spectra of $MbO_2$ and MbCO are similar to those of the respective hemoglobin complexes, but spectral differences exist between corresponding myoglobin and hemoglobin derivatives which must reflect the influence of the different proteins on the heme. Spiro and Strekas (1974) have already suggested that the iron in HbCO is low spin ferric; however, their suggestion is based on the observation of only a single line in the high frequency region and they fail to report any differences between carbon monoxide and oxygen derivatives.

## Experimental Procedure

Raman spectra were recorded with the 441.6-nm line of a Spectra Physics He–Cd laser, Model 185, using a spectrometer described elsewhere (Rimai *et al.,* 1973). Samples were contained in standard 5 mm × 10 mm rectangular cuvets and temperatures were maintained at 4° by blowing cold gas through a jacketed cuvet holder.

In the 441.6-nm laser beam, both oxygen and carbon monoxide readily photodissociate from hemoglobin and myoglobin. In order to obtain spectra of the fully liganded proteins we utilized the highest possible sample concentrations consistent with preparative convenience and the need to monitor the $\alpha$–$\beta$ bands of the optical absorption spectra. In addition we controlled the gas phase over the sample utilizing a cuvet to which was attached a configuration of stopcocks whereby: (1) the sample could be isolated from the ambient atmosphere, (2) the gas over the sample could be exchanged, and (3) the gaseous ligands (*i.e.,* CO or $O_2$) could be maintained at 1 atm of pressure. Further the laser

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[1] Abbreviations used are: Hb, hemoglobin; Mb, myoglobin; $HbO_2$, $MbO_2$, HbCO, MbCO, corresponding oxygen and carbon monoxide liganded derivatives.