Interaction of Apocytochrome *c* and Derived Polypeptide Fragments with Sodium Dodecyl Sulfate Micelles Monitored by Photochemically Induced Dynamic Nuclear Polarization ^{1}H NMR and Fluorescence Spectroscopy

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ABSTRACT: The topology of apocytochrome *c*, the heme-free precursor of the mitochondrial protein cytochrome *c*, was investigated in a lipid-associated form. For this purpose photochemically induced dynamic nuclear polarization ^{1}H nuclear magnetic resonance (CIDNP ^{1}H NMR) spectroscopy and quenching of tryptophan and tyrosine fluorescence by acrylamide were applied to an apocytochrome *c*–sodium dodecyl sulfate (SDS) micellar system. A pH titration of the chemical shifts of the histidine C2 proton resonances of apocytochrome *c*, using conventional ^{1}H NMR, yielded pK_a's of 5.9 ± 0.1 and 6.2 ± 0.1, which were assigned to histidine-18 and -33 and histidine-26, respectively. In the presence of SDS micelles an average pK_a of 8.1 ± 0.1 was obtained for all histidine C2 protons. Photo-CIDNP enhancements of the histidine, tryptophan, and tyrosine residues, contained in the intact apocytochrome *c* and in chemically and enzymatically prepared fragments of the precursor, were reduced in the presence of SDS micelles. Similarly, the quenching of the tryptophan fluorescence of the polypeptides by acrylamide was diminished in the presence of SDS. These results indicate the aromatic residues studied are localized in the interface of the SDS micelle.

Apocytochrome *c*, the precursor of the mitochondrial cytochrome *c*, is synthesized in the cytoplasm and imported via a unique pathway into the mitochondrial intermembrane space, where it functions in the respiratory chain (Hartl et al., 1989). The translocation of apocytochrome *c* across the outer mitochondrial membrane is coupled to the covalent attachment of a heme group, a reaction catalyzed by cytochrome *c* heme lyase (Hennig & Neupert, 1981; Nicholson et al., 1987, 1988). Apocytochrome *c* is a unique precursor protein because it can readily be prepared in large quantities by chemical removal of the heme group from the mature protein (Fisher et al., 1973), yielding an import competent precursor that can be used for biophysical studies. Such studies of apocytochrome *c*–lipid interactions in model systems indicated an initial electrostatic interaction between the highly basic apocytochrome *c* and the negatively charged lipids (Rietveld et al., 1986; Demel et al., 1989), followed by penetration into (Görrissen et al., 1986) and partial translocation across the bilayer (Rietveld et al., 1986). The same behavior has been shown for derived amino-terminal fragments of apocytochrome *c*, in contrast to derived carboxy-terminal fragments, which only bind to and penetrate into but do not translocate across the bilayer (Jordi et al., 1989a). During the insertion process apocytochrome *c* undergoes a random-coil to α-helix transition as determined by circular dichroism (Rietveld et al., 1985; Walter et al., 1986). These structural changes are also induced in the amino- and carboxy-terminal fragments of apocytochrome *c* upon interaction with sodium dodecyl sulfate (SDS) micelles (Jordi et al., 1989a). Combination of the data on these model membrane studies and on mitochondrial import of apocytochrome *c* (Nicholson et al., 1987) leads to a working hypothesis in which the amino terminus of the protein dynamically interacts with the head groups of the negatively charged phospholipids, which become structurally highly perturbed, whereas the carboxy terminus is more rigidly inserted in the membrane. Exposure of the amino terminus to the cytochrome *c* heme lyase results in covalent attachment of the heme group to the two cysteines contained in this part of apocytochrome *c*. The now folded protein is in a translocation-incompetent state and is released from the outer mitochondrial membrane into the intermembrane space.

Little is known about the structure and topology of the precursor in a lipid environment. Therefore, we investigated the protein in a lipid-associated form using photo-CIDNP ^{1}H NMR[1] and fluorescence spectroscopy. To approximate an amphiphilic environment as found in biological membranes and to obtain sufficient quality in the ^{1}H NMR spectra, SDS micelles were used as a model for the negatively charged phospholipid component of the outer mitochondrial membrane, as in other recent studies [e.g., Olejniczak et al. (1988) and references cited therein and Mammi and Peggion (1990)].

The photo-CIDNP ^{1}H NMR technique gives information about the solvent accessibility of histidine, tryptophan, and tyrosine residues in proteins to photoexcited flavin dyes (Kaptein, 1982; Kaptein et al., 1978). The selective enhancement of the signals of only these three types of amino acid residues is particularly advantageous in ^{1}H NMR investigations on protein–micelle interactions [e.g., Zetta and Kaptein (1984) and Mayo et al. (1987)]. The strategic distribution of histidine, tryptophan, and tyrosine residues over

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[1] Abbreviations: photo-CIDNP ^{1}H NMR, photochemically induced dynamic nuclear polarization proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; CNBr, cyanogen bromide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ESR, electron spin resonance.

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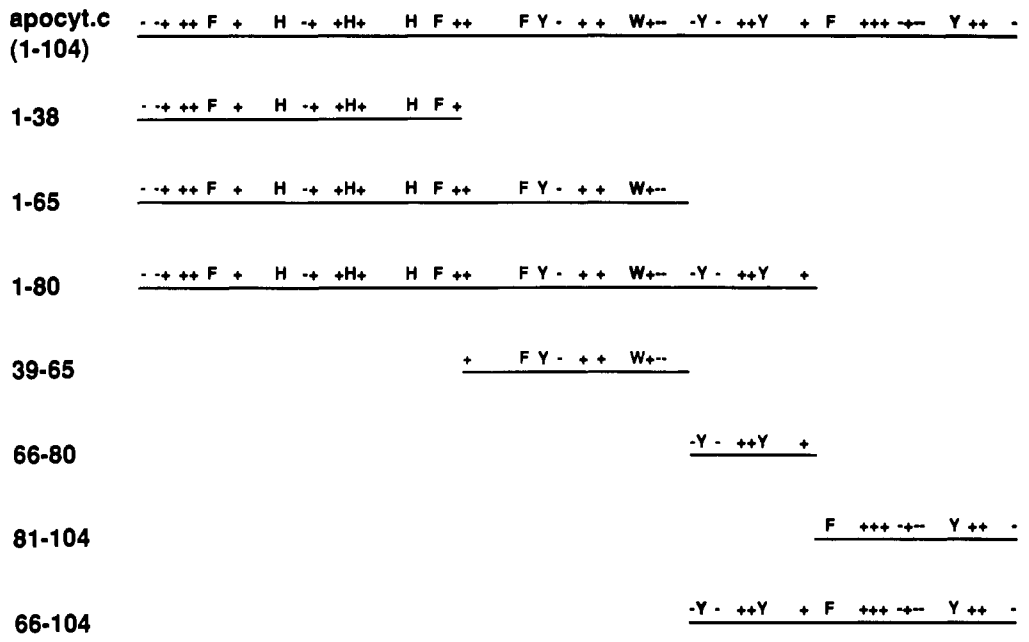

FIGURE 1: Apocytochrome *c* and the derived polypeptide fragments used in this study. Aromatic amino acid residues are indicated by their one-letter code, the positively charged lysine and arginine residues by (+), and the negatively charged aspartic and glutamic acid residues by (−). Apocytochrome *c* contains the aromatic residues histidine-18, -26, and -33; phenylalanine-10, -36, -46, and -82; tryptophan-59; and tyrosine-48, -67, -74, and -97.

apocytochrome *c* (Figure 1) and the ability to chemically and enzymatically prepare polypeptide fragments of the precursor enable the identity of the regions of apocytochrome *c* that interact with the SDS micelles to be probed. Supplementary information about polypeptide–SDS interactions can be obtained from tryptophan and tyrosine fluorescence spectroscopy in combination with suitable quenchers of fluorescence. The results reported in this study indicate a localization of the aromatic residues contained in apocytochrome *c* in the interface of the SDS micelle.

Experimental Procedures

Materials. The tripeptides Ala-Tyr-Ala, Lys-Trp-Lys, and Lys-Tyr-Ser were purchased from Serva (Heidelberg, FRG), and Lys-His-Lys was purchased from Research Plus (Bayonne and Denville). DL-Tryptophan was purchased from Hoffmann-La Roche (Basel, Switzerland), and DL-tyrosine was from Fluka (Switzerland). CNBr was from Merck (Darmstadt, FRG).

Apocytochrome c. Apocytochrome *c* was prepared by removal of the heme group from horse heart cytochrome *c* (type VI; Sigma, St. Louis, MO; Fisher et al., 1973) and was stored at −20 °C after extensive dialysis at 4 °C against 10 mM ammonium acetate, pH 5.0, containing 0.01% (v/v) 2-mercaptoethanol and after lyophilization.

Fragments of Apocytochrome c. Fragments were prepared by cyanogen bromide treatment of cytochrome *c* on the basis of a method described by Corradin and Harbury (1970) and by digestion of fragment 1–65 with clostripain according to a procedure described by Parr et al. (1978). For both methods some modifications were introduced.

CNBr at a 150:1 molar ratio to protein and a reaction time of 20 h specifically cleaves the methionine peptide bonds at positions 65 and 80 in horse heart cytochrome *c* (type VI; Sigma, St. Louis, MO). The fragments were purified on a Bio-Gel P-10 (Bio-Rad, Richmond, VA) column, equilibrated with 10% formic acid, resulting in heme-containing fragments 1–80 and 1–65 and non-heme-containing fragments 66–80, 66–104, and 81–104. The heme group of fragments 1–80 and 1–65 was removed in the same way as described for cytochrome *c* (Fisher et al., 1973).

Clostripain (Boehringer, Mannheim, FRG) is a proteolytic enzyme with a high specificity for arginine residues (position 38 in fragment 1–65). A stock solution of clostripain (4 μL) in 2.5 mM dithiothreitol, 1 mM $CaCl_2$ and 1 mM Tris-HCl, pH 7.8, with an absorbance of 0.02 at 280 nm was added to a 1 mg/mL solution of fragment 1–65 in the above buffer, without dithiothreitol, and incubated for 1 h at 37 °C. The main resulting fragments, 1–38 and 39–65, were separated on a Bio-Gel P-10 column, eluted with 10% formic acid.

All purified fragments were lyophilized and stored at −20 °C after identification by migration as a single peak on the Bio-Gel P-10 column, amino acid analysis, ^{1}H NMR, and reversed-phase HPLC [Nucleosil 300-7 C_4 column, eluted with linear water–acetonitrile gradients containing 0.1% (v/v) trifluoroacetic acid].

NMR Sample Preparation. For NMR measurements weight amounts of the lyophilized polypeptides or tripeptides were dissolved as 0.6 mM solutions in ^2H_2O (ICN Biomedicals, Cambridge, U.K.) containing 10 mM sodium [$^2H_3$]acetate (Sigma, St. Louis, MO). Polypeptide samples were diluted to 0.3 mM either directly with the buffer or indirectly by addition of sodium [$^2H_{25}$]dodecyl sulfate (MSD Isotopes, Montreal, Canada) from a 1 M stock solution (final concentration 30 mM SDS), followed by dilution with the buffer. The pH was adjusted to the indicated values with 0.1 or 1.0 M ^{2}HCl or NaO^{2}H (both from Sigma, St. Louis, MO). All pH values reported in this paper are uncorrected readings of ^2H_2O solutions with an electrode standardized on H_2O buffers. The photo-CIDNP ^{1}H NMR measurements were carried out in the presence of 0.3 mM flavin dye, 3-*N*-(carboxymethyl)lumiflavin (a gift from Dr. F. Muller, Sandoz, Basel, Switzerland), added from a 4 mM stock solution. Just before each photo-CIDNP experiment, the pH was adjusted to the indicated value after addition of the flavin dye.

NMR Measurements. All NMR experiments were performed at 30 °C. Conventional ^{1}H NMR spectra were recorded either on a Bruker HX-360 spectrometer controlled by an Aspect 2000 computer or on a Bruker MSL-300 spectrometer controlled by an Aspect 3000 computer. Chemical

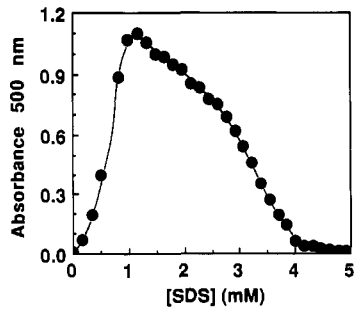

FIGURE 2: Influence of SDS on the absorbance at 500 nm of a 0.3 mM apocytochrome c solution.

shifts are given in parts per million from internal sodium 3-(trimethylsilyl)[$^2H_4$]propionate (Merck, Darmstadt, FRG). The H^2HO line was presaturated for 1–2 s prior to a 90° observation pulse and acquisition; 512–2048 free induction decays were accumulated. Photo-CIDNP ^1H NMR spectra were recorded on the Bruker HX-360 spectrometer, on the basis of a method developed by Kaptein (1982) and according to the modifications described by Mayo et al. (1987), by using a 0.5-s light pulse (5 W) and a 25-ms delay.

Absorbance Measurements. To determine the conditions that yield optically clear solutions of SDS and apocytochrome c, turbidity measurements at 500 nm were carried out on a Hitachi spectrophotometer, Model U 3200. To a solution of 0.3 mM apocytochrome c in 10 mM sodium acetate, pH 7.0, were added aliquots of a 100 mM SDS solution until the indicated SDS concentrations were attained (Figure 2). Up to a concentration of 1 mM SDS there is a strong interaction between apocytochrome c and SDS as evidenced by the large increase in turbidity. This is due to the ionic interaction between the highly basic apocytochrome c and the anionic detergent below its critical micelle concentration of 8.3 mM. At higher concentrations the turbidity decreases such that at a 5 mM SDS concentration the solution is virtually clear. Up to concentrations of 90 mM SDS the absorbance remained virtually constant. Under the experimental conditions for NMR and fluorescence, there is approximately one apocytochrome c molecule per SDS micelle present, i.e., 0.3 mM apocytochrome c and 30 mM SDS.

Fluorescence Measurements. Fluorescence experiments were carried out on a SLM-Aminco spectrofluorometer, Model SPF 500C. Tryptophan fluorescence was measured at its emission maximum (346–354 nm, 2.5-nm band-pass) upon excitation at 295 nm (2-nm band-pass). Tyrosine residues were excited at 275 nm (5-nm band-pass), and fluorescence intensities were measured at their emission maximum of 304 nm (5-nm band-pass). All experiments were performed at 30 °C.

Fluorescence intensities of 0.3 mM polypeptide or amino acid solutions in 10 mM sodium acetate, pH 7.0, were measured after sequential addition of small amounts of SDS micelles from a 0.5 M stock solution. Corrections were made for sample dilution, for inner filter effects caused by absorption of the polypeptide or amino acid/SDS solution at the excitation and emission wavelengths, and for scattering of the solution in the absence of fluorophore.

Fluorescence-quenching experiments were performed by adding aliquots of 3 M acrylamide to 0.3 mM polypeptide or amino acid solutions in 10 mM sodium acetate, pH 7.0, in the absence or presence of 30 mM SDS. Further experimental conditions and corrections were as described above, except for the additional correction for the absorption of acrylamide. The obtained fluorescence quenching data were fitted with a least-squares method to the Stern–Volmer equation $F_0/F = 1 + K_{SV}[Q]$, in which F_0 is the corrected fluorescence intensity in the absence of quencher and F that in the presence of quencher, [Q] is the concentration of quencher, and K_{SV} is the Stern–Volmer quenching constant that was determined.

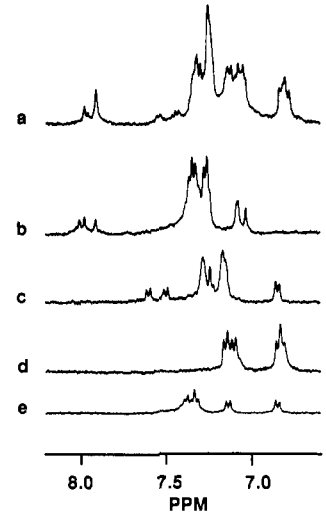

FIGURE 3: 360-MHz ^1H NMR spectra of the aromatic region of (a) apocytochrome c and some of its fragments used in this study: (b) 1–38, (c) 39–65, (d) 66–80, and (e) 81–104 at pH 6.5.

RESULTS

Conventional ^1H NMR. Figure 3a represents the aromatic region of a ^1H NMR spectrum of apocytochrome c in solution in the absence of SDS micelles. Resonances from individual amino acids cannot be assigned because of the strong overlap, which most likely is due to the unfolded nature of the protein (Walter et al., 1986). An exception is the unique tryptophan-59, which shows resonances at 7.43 and 7.53 ppm for the C7 and C4 protons, respectively. The protons are numbered and assigned according to Wüthrich (1976). Group assignments can be done from literature (Wüthrich, 1976) and by comparing the ^1H NMR spectra of the intact precursor protein with that of a selection of fragments (Figure 3b–e), which contain characteristic sets of aromatic amino acid residues (Figure 1). In this way the group of signals at about 6.8 ppm can be assigned to the tyrosine C3,5 protons and the cluster of strongly overlapping resonances at 7.0–7.4 ppm to the phenylalanine ring protons and the histidine C4, the tryptophan C2,5,6, and the tyrosine C2,6 protons. The signals around 7.9 ppm originate from the histidine C2 protons. At these polypeptide concentrations of 0.3 mM, the spectra show some line broadening, even those of the relatively small fragments. Because of the sharpening of the signals in the presence of 4 M perdeuterated urea (spectra not shown), the line broadening is probably due to some aggregation by hydrophobic interactions of the polypeptides in solution (Vincent et al., 1988).

It is well-known that the chemical shift values of the histidine C2 and C4 resonances are very sensitive to changes in pH in the vicinity of the imidazole ring. This property and their unique downfield position in the ^1H NMR spectra make the histidine C2 proton resonances very useful tools in probing the environment of the amino terminus of apocytochrome c in which they are located. As expected, the histidine C2 resonances show a large downfield shift from approximately 7.7 to 8.6 ppm when the pH is sequentially decreased (Figure 4). In Figure 4A, in the absence of SDS micelles, the signals from the three histidine C2 protons show mainly two peaks with a relative peak area of 1 (downfield) to 2 (upfield), indicating two kinds of histidine C2 protons in different

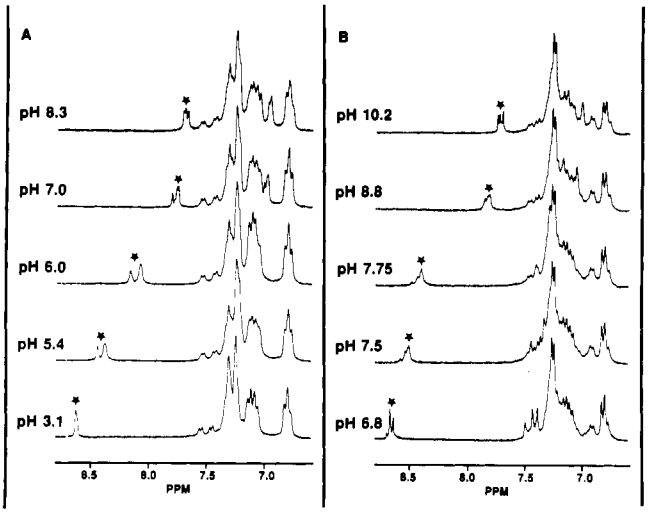

FIGURE 4: 300-MHz ^{1}H NMR spectra of apocytochrome *c* (A) in the absence and (B) in the presence of sodium [$^2H_{25}$]dodecyl sulfate micelles at the indicated pH values. The peaks marked (*) originate from the histidine C2 protons.

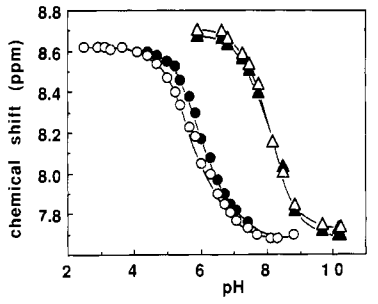

FIGURE 5: pH dependence of the chemical shifts of the histidine C2 protons in apocytochrome *c* [group 1 (●) and group 2 (○)] in the absence and [group 1 (▲) and group 2 (△)] in the presence of sodium [$^2H_{25}$]dodecyl sulfate micelles.

chemical environments, which could be caused by differences in primary or secondary structure. For operational purposes the downfield peak is defined to belong to histidine C2 protons of group 1 and the upfield peak to those of group 2. In Figure 4B, spectra of apocytochrome *c* in the presence of SDS micelles are shown. The addition of SDS micelles causes a large downfield shift of the resonances of all histidine C2 protons, demonstrating that all polypeptide molecules interact strongly with the SDS micelles. The chemical shift spread of the histidine C2 proton resonances is less in the presence of the SDS micelles. The histidine C2 signals remain relatively sharp, implying that the internal mobility of these residues, which are all located toward the amino terminus, is not affected by the interaction of the protein with SDS micelles.

The pH titration of the chemical shift of the histidine C2 resonances of apocytochrome *c* in the absence and presence of SDS micelles is presented in Figure 5. From this titration, pK_a values of 6.2 ± 0.1 and 5.9 ± 0.1 can be calculated for the histidine protons in the absence of SDS micelles. In their presence, the pH titration curves for the various histidine C2 proton resonances are not significantly different, and an average pK_a of 8.1 ± 0.1 is calculated. A similar pH titration experiment performed on the tripeptide Lys-His-Lys in the absence of SDS micelles yielded a pK_a value of 6.3 ± 0.1 (data not shown).

Photo-CIDNP ^{1}H NMR. The photo-CIDNP ^{1}H NMR method is based on the selective enhancement of ^{1}H NMR signals due to a reversible reaction between a photoexcitable flavin dye and specific solvent-exposed aromatic histidine,

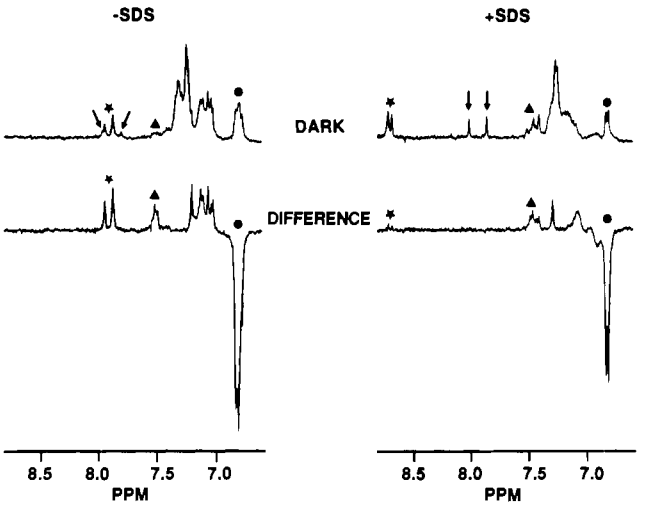

FIGURE 6: 360-MHz photo-CIDNP dark and difference ^{1}H NMR spectra of apocytochrome *c* without or with sodium [$^2H_{25}$]dodecyl sulfate micelles present at pH 6.5. In the spectra, signals are labeled originating from (*) the histidine C2 protons, (▲) the tryptophan C4 proton, (●) the tyrosine C3,5 protons, and (↓) the flavin protons. The difference spectra were obtained by subtraction of the scaled dark spectrum (128 free induction decays) from the light spectrum (16 free induction decays), which were alternately recorded in 16 experiments.

tryptophan, and tyrosine amino acids. The accessibility of the (partially) solvent-exposed aromatic residues to the flavin dye is reflected in the magnitude of photo-CIDNP enhancement of the ^{1}H NMR signals concerned.

Photo-CIDNP ^{1}H NMR dark and difference spectra of apocytochrome *c* in the absence and presence of SDS micelles are shown in Figure 6. The dark spectra are fully comparable to the earlier shown 300-MHz ^{1}H NMR spectra (Figure 4) with the exception of the signals originating from the flavin dye. The large downfield shift of the histidine C2 resonances upon addition of SDS micelles is clearly demonstrated. The difference spectrum of apocytochrome *c* in the absence of SDS micelles shows positively and negatively enhanced ^{1}H NMR signals. According to the photo-CIDNP ^{1}H NMR theory (Kaptein, 1982), the positively enhanced signals in this region arise from histidine and tryptophan protons and the negative enhancements from tyrosine C3,5 protons. The relatively small positive enhancements of the tyrosine C2,6 signals in this region are due to NOE's (nuclear Overhauser effects) from the tyrosine C3,5 resonances. The appearance of these enhancements indicates the accessibility of all aromatic residues to the flavin dye and is in agreement with the unfolded state of the precursor in solution. The photo-CIDNP ^{1}H NMR difference spectrum of apocytochrome *c* in the presence of SDS micelles shows the same qualitative characteristics as the one without SDS micelles. However, the magnitude of the photo-CIDNP enhancements is differently reduced and is almost zero for the histidine protons. In general, a decrease in intensity of the signal is explained by a decrease in accessibility of the flavin dye to the interacting residue.

To get further understanding of the way different parts of apocytochrome *c* interact with the SDS micelles, photo-CIDNP ^{1}H NMR experiments were performed on derived polypeptide fragments in the absence and presence of SDS micelles. The photo-CIDNP difference spectra of a selection of polypeptides are shown in Figure 7. The spectra in the absence of SDS micelles show photo-CIDNP enhancements similar to those of the intact polypeptide (Figure 6) and are as expected from the sequence shown in Figure 1, assuming accessibility of the histidine, tryptophan, and tyrosine residues

Table I: Interaction Parameters for Apocytochrome c, Derived and Synthetic Polypeptides, and Amino Acids As Obtained from Photo-CIDNP ^{1}H NMR and Fluorescence-Quenching Studies[a]

protein or peptide	photo-CIDNP ^{1}H NMR			fluor quenching	
	His[c]	Trp	Tyr	Trp	Tyr
apocytochrome c[b]	12.0 ± 1.9 (2.4)	2.0 ± 0.4	2.0 ± 0.3	1.8 ± 0.1	
1–38	16.7 (3.3)				
1–65	13.0 (2.6)	1.7	2.3	1.7	
1–80	13.3 (2.7)		2.4		
39–65		1.7	3.1	1.8	
66–80			1.8		0.6
81–104			1.1		1.0
66–104			2.5		0.8
Lys-His-Lys	13.5 (2.7)				
Lys-Trp-Lys		3.6		1.6	
Ala-Tyr-Ala			1.0		1.1
Lys-Tyr-Ser			2.6		1.0
Trp				1.0	
Tyr					1.0

[a] The interaction parameters were calculated for the aromatic residues as the ratio of photo-CIDNP enhancement (integrated peak areas) or quenching constant K_{sv} in the absence to that in the presence of SDS micelles. [b] The given errors represent the standard deviations; $n = 5$ in case of the ^{1}H NMR interaction prameters and $n = 3$ for the fluorescence interaction parameter. [c] Values in parentheses are corrected for the pH dependence of the photo-CIDNP intensity.

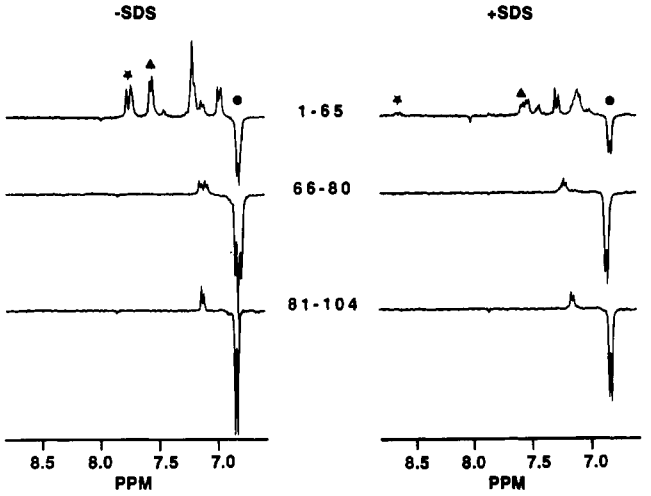

FIGURE 7: 360-MHz photo-CIDNP difference ^{1}H NMR spectra of the derived polypeptides of apocytochrome c in the absence and in the presence of sodium [$^2H_{25}$]dodecyl sulfate micelles. Indicated are (*) the histidine C2 protons, (▲) the tryptophan C4 proton, and (●) the tyrosine C3,5 protons.

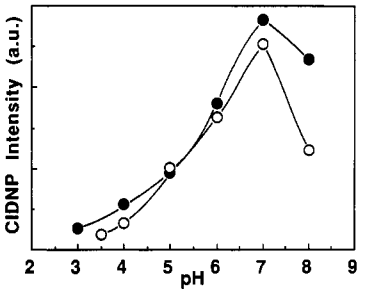

FIGURE 8: pH dependence of the photo-CIDNP intensity of the histidine C2 protons of (●) apocytochrome c and of (○) the synthetic tripeptide Lys-His-Lys.

to the flavin dye. In the presence of SDS micelles the signals of the histidine C2 protons of the amino-terminal fragment 1–65 also shift to a lower field, and their photo-CIDNP enhancements decrease remarkably, similar to those of the intact apocytochrome c (Figure 6). The same conclusion can be drawn for the effect of SDS on the photo-CIDNP enhancement of the tryptophan C4 and tyrosine C3,5 proton signals of fragment 1–65. In the difference spectrum of the small central fragment 66–80, the tyrosine signals have changed both in pattern and in intensity in the presence of SDS micelles, demonstrating a change in the accessibility of the residues to the flavin dye. The intensity of the tyrosine signal in the difference spectrum of the carboxy-terminal fragment 81–104 is slightly reduced. To treat these spectral results more quantitatively, a polypeptide–micelle interaction parameter is defined according to Mayo et al. (1987). This parameter is the ratio of the photo-CIDNP enhancement in the absence of micelles to that in their presence. The interaction parameters for apocytochrome c, its derived polypeptide fragments, and some synthetic water-soluble tripeptides are given in Table I. If the presence of SDS micelles does not influence the accessibility of the aromatic amino acid residue toward the flavin dye, an interaction parameter of 1.0 is obtained. This is observed for the tyrosine residue in the model peptide Ala-Tyr-Ala. Interaction parameters larger than 1.0 indicate a decreased accessibility of the residues to the dye. This is observed for most of the aromatics in almost all polypeptides studied. The magnitude of the interaction parameters varies between different residues and peptides. The extent to which the SDS micelles affect the accessibility of the residues is expressed in the magnitude of the interaction parameter: the higher the value of the interaction parameter, the less accessible the residue is. The accessibility of the tyrosine residue in fragment 81–104 is almost uninfluenced by the SDS micelles, whereas tyrosine-48 in fragment 39–65 is strongly shielded from the dye. The most conspicuous in Table I are the large magnitudes of the interaction parameters of the histidine residues; they suggest a very strong influence of the SDS micelles on these amino acids. However, this influence is 2-fold: the presence of SDS micelles affects the accessibility of the flavin dye, and it increases the local proton concentration in the environment of the imidazole ring as was shown in Figure 4 and 5. Kaptein and co-workers (Kaptein, 1982; Stob & Kaptein, 1989) showed that the photo-CIDNP intensity of histidine protons is dependent on pH. Therefore, the magnitude of the photo-CIDNP enhancement of the histidine C2 proton resonances for apocytochrome c and a model peptide Lys-His-Lys was determined as a function of the pH (Figure 8). As is shown in this figure, the photo-CIDNP intensity of the histidine protons increases as the pH is increased to 7, whereas it decreased both for apocytochrome c and for Lys-His-Lys. From the pH titration curves of Figure 5, it can be estimated that the histidine C2 protons in the presence of SDS micelles at pH 6.5 experience an environment comparable to

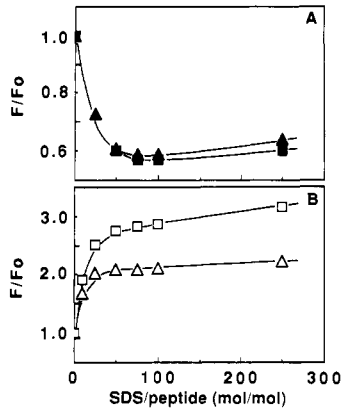

FIGURE 9: Change in fluorescence intensity of (A) tryptophan and (B) tyrosine residues upon addition of sodium dodecyl sulfate micelles to polypeptide solutions of (■) apocytochrome *c*, (▲) fragment 1–65, (□) fragment 66–80, and (△) fragment 81–104.

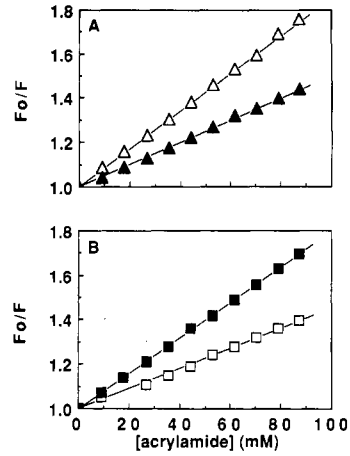

FIGURE 10: Stern–Volmer plots of the fluorescence quenching by acrylamide of (A) the tryptophan residue in fragment 1–65 and of (B) the tyrosine residues in fragment 66–80 in the absence (open symbols) and in the presence (closed symbols) of SDS micelles.

that of the free polypeptide at pH 4.0. This apparent change in pH leads to a decrease in photo-CIDNP intensity of about 80% both for apocytochrome *c* and for the tripeptide Lys-His-Lys (see Figure 8). Therefore, the interaction parameters in Table I of all histidine-containing polypeptides were corrected for this effect, and the pH-corrected values are given in parentheses in the table. These corrected values still indicate a lesser accessibility of the residues to the flavin dye and are now in the same range of magnitude as calculated for the tryptophan and tyrosine residues.

Fluorescence Spectroscopy. Fluorescence properties of tryptophan and tyrosine residues are dependent on their local environment (Lakowicz, 1983) and therefore are another useful probe to monitor the apocytochrome *c*–SDS micelle interaction. Interactions of the tryptophan and tyrosine residues in apocytochrome *c* and in its derived polypeptide fragments with SDS micelles can result in changes in fluorescence intensity for both types of residues with an additional blue shift of the emission maximum for tryptophan residues. To enable a direct comparison with the NMR results, identical polypeptide concentrations were used. The fluorescence intensity of both tryptophan and tyrosine residues depends linearly on the polypeptide concentration, up to 0.4 mM (data not shown). The changes in fluorescence intensity of tryptophan- or tyrosine-containing polypeptides are shown as a function of the SDS concentration in panels A and B of Figure 9, respectively. In Figure 9A, a decrease in tryptophan fluorescence intensity is observed for apocytochrome *c* and fragment 1–65. This is accompanied by a blue shift of the emission maximum from 354 to 346 nm in case of apocytochrome *c* and from 354 to 351 nm for fragment 1–65. The fluorescence emission upon excitation at 275 nm is dominated by the tryptophan fluorescence in spectra of polypeptides containing both tryptophan and tyrosine residues. Therefore, in our tyrosine fluorescence studies we have restricted ourselves to fragments that only contain tyrosine residues. In Figure 9B it is shown that when the SDS concentration is increased, the tyrosine fluorescence intensity of fragments 66–80 and 81–104 increases in a saturable manner. The emission maxima remained unchanged. Such behavior of tyrosine fluorescence in more hydrophobic regions was reported before (Lakowicz, 1983).

To determine the solvent accessibility of the tryptophan and tyrosine residues of the polypeptides in SDS micelle associated form, fluorescence-quenching experiments were carried out with acrylamide. Typical Stern–Volmer plots of tryptophan or tyrosine fluorescence-quenching experiments in the absence and presence of SDS micelles are given in panels A and B of Figure 10, respectively. From these plots the quenching constants, K_{SV}, were determined as described under Experimental Procedures.

In analogy with the interaction parameters calculated from the photo-CIDNP ^{1}H NMR data, a fluorescence interaction parameter is defined as the ratio of the quenching constants K_{SV} measured in the absence and presence of SDS micelles. These calculated interaction parameters are included in Table I. The interpretation of the fluorescence interaction parameters is parallel to that of the NMR data. An interaction parameter of 1.0 is calculated when the SDS micelles do not influence the accessibility of acrylamide to the fluorophores. If the fluorophores in the presence of SDS micelles are less accessible to the uncharged polar acrylamide, an interaction parameter larger than 1.0 is calculated, but if the fluorophores are more accessible, a value smaller than 1.0 is obtained.

As expected, the fluorescence interaction parameters for the free amino acids tryptophan and tyrosine are not influenced by the SDS micelles. This is also the case for the tyrosine residues of Lys-Tyr-Ser, Ala-Tyr-Ala, and fragment 81–104, whereas the other tyrosine-containing fragments, 66–80 and 66–104, show a better accessibility to the quencher in the presence of SDS micelles. All tryptophan-containing polypeptides show a decrease in accessibility of the residues toward the quencher in the presence of SDS micelles.

Discussion

The topology of apocytochrome *c* in a lipid-associated form has been investigated in the apocytochrome *c*–SDS micellar system. Our choice for SDS as a model for the interface presented by the lipids in a biological membrane is based on the findings that the conformational changes induced by phospholipid vesicles and SDS in the protein are very similar. Apocytochrome *c* undergoes a random-coil to partial α-helix transition upon addition of SDS micelles (Jordi et al., 1989a). The amount of α-helix induced by excess SDS (21%) is very close to the 22% of α-helix induced by the addition of dioleoylphosphatidylserine vesicles to the protein (de Jongh & de Kruijff, 1990), consistent with previous observations on α-helix induction in the protein by phosphatidylserine membranes (Walter et al., 1986). The analysis of the α-helix formation by SDS in apocytochrome *c* and derived fragments and secondary structure prediction programs (Jordi et al., 1989a) strongly suggest that these α-helices are induced in discrete regions of the protein that nearly coincide with the α-helices known to be present in the holoprotein. Therefore, it can be

excluded that SDS interacts in a nonspecific way with apocytochrome c. That all apocytochrome c molecules interact with the SDS micelles is proven by our observations that upon addition of SDS micelles (1) all resonances of the histidine C2 protons shift downfield (Figure 4B), (2) the fluorescence intensity decreases for all tryptophan-containing polypeptides (Figure 9A), which is accompanied by a blue shift of the emission maxima, and (3) the fluorescence intensity increases for all tyrosine-containing polypeptides (Figure 9B). It is highly unlikely that the polypeptides are present in an aggregated state in the micelles because of the high SDS-to-protein ratio used. Furthermore, in SDS–polyacrylamide gel electrophoresis apocytochrome c runs at its monomer weight (Rietveld & de Kruijff, 1984).

The conventional ^{1}H NMR experiments give insight mainly into the localization of histidine residues, which represent the amino-terminal part of apocytochrome c. The pK_a values of the histidine C2 protons have been determined in both the absence and presence of SDS micelles. In the absence of SDS, pK_a values of 6.2 and 5.9 are obtained. This appearance of different pK_a values in the essentially random-coil conformation of apocytochrome c in aqueous solution (Rietveld et al., 1985; Walter et al., 1986) may arise from different neighboring amino acids in the primary sequence. Histidine-26 is flanked by two positively charged lysine residues, histidine-18 is flanked by cysteine-17 and threonine-19, and histidine-33 is flanked by leucine-32 and glycine-34. On account of the primary sequence the histidines could be divided into two groups: histidine-26 with charged neighbors and histidine-18 and histidine-33 with uncharged neighbors. According to the relative peak areas and the pK_a value of 6.3 ± 0.1 determined for the synthetic tripeptide Lys-His-Lys, the pK_a of 6.2 ± 0.1 is assigned to the histidine C2 proton of residue 26 and the pK_a of 5.9 ± 0.1 to those of residues 18 and 33. These pK_a values are in agreement with the average pK_a of 6.2 reported for the three histidine residues in apocytochrome c by Cohen et al. (1974). At intermediate pH values these authors observed a splitting of the histidine C2 peak and relatively little fine structure in the downfield region, which they explained by a broadening of the resonances due to viscosity and aggregation.

In the presence of SDS micelles all the histidine C2 protons are still very sensitive to pH changes in the vicinity of the imidazole ring, and a pK_a of 8.1 has been determined. This increase in pK_a is in good agreement with the rise of 2 pK_a units reported for the N-terminal groups of substance P bound to SDS micelles (Woolley & Deber, 1987) and the model studies on L-histidine and charged derivatives performed by Sachs et al. (1971) in which it was shown that the presence of negatively charged neighboring groups causes an increase in the pK_a of the imidazole ring.

The photo-CIDNP ^{1}H NMR interaction parameters for the histidine residues of apocytochrome c and the derived fragments are comparable with that observed for the synthetic tripeptide Lys-His-Lys. On account of the positive charges on the lysines and the hydrophilicity of the histidine residue, we assume that this tripeptide is localized in the vicinity of the SDS head groups, i.e., in the interface of the SDS micelle. From the pK_a shifts and photo-CIDNP ^{1}H NMR interaction parameter of the histidines in apocytochrome c, we conclude that these residues are localized in the head-group region of the micelle in which they have a decreased accessibility toward the flavin dye. Extrapolating to the mitochondrial situation, this implicates that the cysteine residues 14 and 17 also are localized in the lipid head-group area prior to attachment of the heme group by cytochrome c heme lyase, which has its active site in the intermembrane space. To reach the active site of the heme lyase, the cysteines and thus the amino terminus of apocytochrome c will have to move across the outer mitochondrial membrane. Passage of the amino terminus across the lipid bilayer could be demonstrated by using a translocation assay (Jordi et al., 1989a). This might be accomplished by local changes in lipid organization. Recent NMR and ESR studies demonstrated the pronounced lipid structure perturbing ability of the amino terminus of the protein (Jordi et al., 1989b, 1990).

Insight into the topology of the other aromatic residues in apocytochrome c in the presence of SDS is provided by the analysis of the observed interaction parameters. An interaction parameter larger than 1.0 is observed when the aromatic residues concerned become less accessible to the flavin dye or quencher in the presence of SDS micelles. The decreased accessibility could be explained theoretically in two ways: as the result of the SDS-induced conformational transition, which could bury residues in the protein matrix, or as the result of a protein–micelle interaction, which could bury residues in the micelles. Rietveld et al. (1986) have shown the (partial) translocation of apocytochrome c across negatively charged model membranes. This requires penetration of the precursor into the membrane as has been demonstrated unequivocally in monolayer studies (Pilon et al., 1987) and in ESR and NMR studies on bilayer model membranes (Görrissen et al., 1986; Jordi et al., 1990). Therefore, we are inclined to interpret the decreased accessibilities of the residues as penetration of the protein into the SDS micelle. The interaction parameters of tryptophan in apocytochrome c and the derived fragments vary from 1.7 to 2.0, and both the NMR and the fluorescence interaction parameters are in good agreement. The localization of the tryptophan residues will be analyzed in the light of the data obtained for the tripeptide Lys-Trp-Lys. On the basis of the analysis of Jacobs and White (1989) concerning the interface-seeking properties of tryptophan and lysine residues, the interaction parameters of this tripeptide are considered to be representative of an interfacial localization of the tryptophan residue. That the NMR interaction parameter of Lys-Trp-Lys (3.6) is higher than that of the similarly localized Lys-His-Lys (2.7) could be due to the hydrophobicity of the tryptophan residue, which localizes it deeper in the micellar interface, where it is more shielded from the negatively charged flavin dye. The relatively large value of the NMR interaction parameter of Lys-Trp-Lys as compared to that obtained by fluorescence could be the result of the electrostatic repulsion of the negatively charged dye by the SDS head groups. In case of apocytochrome c (fragments) at a similar tryptophan concentration, more SDS head groups are shielded by the protein (polypeptides), enabling the flavin dye to reach the tryptophan residue more readily. The acrylamide used to determine the fluorescence interaction parameters is uncharged and assumed not to be repelled from the micellar surface, being consistent with the similar fluorescence interaction parameters observed for tryptophan in the polypeptides and the tripeptide. The NMR interaction parameters of tryptophan in apocytochrome c and the derived fragments are exceeded by that observed for tryptophan in the tripeptides. However, as stated above, the very similar fluorescence interaction parameters of apocytochrome c (fragments) and the tripeptide lead to the conclusion that the tryptophans contained in the polypeptides have an interfacial localization. It is of interest to compare these findings to previous tryptophan fluorescence studies on apocytochrome

c. From quenching experiments with iodide in cholate or phosphatidylserine systems, a surface location of part of the tryptophan-containing part of the protein was suggested (Rietveld et al., 1985), agreeing with the present study. Fluorescence-quenching studies with brominated lipids in vesicular systems (Berkhout et al., 1987) showed that part of the tryptophan residues can penetrate into the hydrophobic core of the bilayer to a depth of about 7–8 Å from the polar head groups. At the same time the absolute quenching efficiency was found to be relatively low, suggesting that apocytochrome *c* can adopt different orientations in the vesicle-associated form.

To get insight into the localization of the four tyrosine residues mainly contained in the carboxy-terminal part of apocytochrome *c*, we use the results of the tyrosine-containing fragments, assuming that the localization of these residues may be extrapolated to the situation in apocytochrome *c*. This is considered to be a reasonable assumption because the results obtained for the other aromatic residues (histidine and tryptophan) in apocytochrome *c* and the derived fragments agree very well. For tyrosine residues 48, 67, and 74, we conclude that they are localized in the micellar interface in such a way that in the presence of SDS their accessibility to the flavin dye is decreased. This conclusion follows from the interpretation of the NMR interaction parameters obtained for the tyrosine residues in the polypeptides in the light of the data observed for the tripeptide Lys-Tyr-Ser. This tripeptide is also predicted (Jacobs & White, 1989) to be localized in the interface of the SDS micelle. The interaction parameter of 2.6 found for Lys-Tyr-Ser agrees with this localization and with that of Lys-His-Lys and Lys-Trp-Lys. For all the tyrosine-containing fragments, except fragment 81–104, NMR interaction parameters in the range of 1.8–3.1 were obtained, all around the value observed for Lys-Tyr-Ser.

Tyrosine-48 is a unique tyrosine residue in fragments 1–65 and 39–65. The interaction parameters of tyrosine observed for these fragments, which are 2.3 and 3.1, respectively, lead to the conclusion that they are localized in the vicinity of the SDS head group, like the histidines. Fragment 81–104 contains another unique tyrosine residue, namely tyrosine-97. According to the definition of the interaction parameters, the observed values of about 1 for both the NMR and fluorescence interaction parameters either are the result of a lack of interaction between this polypeptide and the micelle or are due to a localization of tyrosine-97 in the interface, where it is fully exposed to the dye and the quencher. We favor the latter interpretation because the increase in fluorescence intensity of tyrosine-97 upon addition of SDS (Figure 9B) strongly suggests an interaction of the polypeptide with the micelle. In this respect the tyrosine-97 residue differs from the other residues studied, which reside in the interface, whereas tyrosine-97 is more localized at the periphery of the interface.

Tyrosine residues 67 and 74 are contained in fragments 66–80 and 66–104; the latter also contains tyrosine-97. The NMR interaction parameters observed for these fragments result in an interfacial localization for tyrosine-67 and tyrosine-74, assuming that tyrosine-97 does not contribute to the magnitude of the value. Tyrosine residues 67 and 74 are located close together and are in the vicinity of tryptophan-59, so that a localization in the interface for both residues is plausible.

The fluorescence interaction parameter observed for Lys-Tyr-Ser is 1.0, in agreement with an interfacial localization in which the tyrosine residue is fully accessible to the acrylamide in the presence of SDS. The fluorescence interaction parameters obtained for fragments 66–80 and 66–104 are below 1, indicating an increased exposure of the tyrosine residues to the quencher in the presence of SDS micelles. In principle, according to the definition of the interaction parameter, these values were not expected. Nevertheless, they also indicate an interaction between the polypeptides and the SDS micelles, in agreement with the NMR data and the increase in fluorescence intensity of the tyrosines upon addition of SDS. The increase in exposure of the tyrosine residues contained in the fragments can be explained by an SDS-induced dissociation of aggregated polypeptides; however, the interpretation may be more complicated due to the presence of more than one fluorophore per fragment (Eftink & Ghiron, 1976).

Interestingly, in this study we observe a fully exposed tyrosine-97 in fragment 81–104, which is unexpected in view of protease experiments performed by Dumont and Richards (1984) in a vesicular system in which they observed a carboxy-terminal fragment of apocytochrome *c* that became partially protected against digestion and in view of ESR experiments in which fragment 81–104 was able to restrict strongly the motion of lipids, spin labeled near the methyl end of the *sn*-2 acyl chain, indicating a deep penetration of the polypeptide into the bilayer (Jordi et al., 1989b). As reported by Jordi et al. (1989a), the α-helical content of fragment 81–104 in aqueous solution is increased by SDS, in agreement with the prediction of an α-helix in this fragment (residue numbers 88–101). The exposed tyrosine-97 residue is located in this α-helix. Apparently, if the α-helix is inserted into the micelle, then at least tyrosine-97 is localized at the periphery of the interface of the SDS micelle. The remaining aromatic residues studied are not located within, nor are they situated at one end of the predicted α-helices [residue numbers 4–17 and 58–69 (Rietveld et al., 1985)]. Taking into account the distribution of the aromatic residues over the apocytochrome *c* molecule, the data indicate a position of the precursor at the SDS micellar surface, which does not exclude a possible insertion of the α-helices into the micelle.

Registry No. SDS, 151-21-3; L-His, 71-00-1; Lys-His-Lys, 106326-02-7; Lys-Trp-Lys, 38579-27-0; Ala-Tyr-Ala, 81075-03-8; Lys-Tyr-Ser, 72829-55-1; cytochrome *c*, 9007-43-6.

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Effects of Oxygen on the Relative Photodissociability of Cytochrome P-450·CO Complex in Rat Liver Microsomes†

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ABSTRACT: Flash photolysis of cytochrome P-450 in phenobarbital-induced rat liver microsomes was examined by observing the decay of absorbance change, $A(t)$, at 450 nm after photolysis of the heme·CO complex by a depolarized laser flash. The relative photodissociability, $A(0)/Ab$, is significantly decreased upon an increase in oxygen concentration. $A(0)/Ab$ is 96%, 86%, and 51% at oxygen concentrations of 90, 115, and 155 μM when compared with $A(0)/Ab$ at 0 μM O_2. In the presence of aminopyrine, the oxygen-dependent decrease in the relative photodissociability was enhanced about 2 times. The rate of CO recombination with cytochrome P-450 was decreased by increasing O_2 concentration. By going from 0 to 155 μM O_2, about 17% decrease and 58% decrease in the recombination rate were observed in the absence and presence of aminopyrine, respectively. In the absence of CO, no absorbance change $A(t)$ at 450 nm was observed even at 155 μM O_2, excluding a possible contribution of P-450·O_2. The present observation leads to the speculation that oxygen molecules around the heme·CO complex affect the electronic state of the heme, resulting in an increase in Fe–CO bond strength.

The liver microsomal monooxygenase system containing cytochrome P-450 catalyzes the metabolism of a wide variety of endogenous and xenobiotic compounds. Cytochrome P-450 receives two electrons from NADPH–cytochrome P-450 reductase and cytochrome b_5 and activates molecular oxygen by donating these electrons, resulting in N-demethylation or aromatic hydroxylation of substrates (Lu & Coon, 1968; Hildebrandt & Estabrook, 1971).

Oxygen concentration is essentially very important for the drug oxidation activity of cytochrome P-450. In vitro biochemical studies have usually been performed under an air-saturated high oxygen concentration of about 220 μM, although the practical oxygen concentration in liver is very low, about 35 μM (Erickson et al., 1982). It is therefore very important to investigate a possible difference in the catalytic mechanism of cytochrome P-450 depending on the oxygen concentration between in vivo and in vitro conditions (Jones, 1981).

So far, little is known about the oxygen concentration dependence for drug oxidation activity of cytochrome P-450 (Fujii et al., 1981; Tsuru et al., 1982; Holtzman et al., 1983; Pohl et al., 1984; Stevens et al., 1984; Webster et al., 1985). We have reported that the amount of metabolites from a

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