A Water–Lipid Interface Induces a Highly Dynamic Folded State in Apocytochrome *c* and Cytochrome *c*, Which May Represent a Common Folding Intermediate†

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ABSTRACT: In this study, we have used CD and NMR techniques to investigate the secondary structure of (apo-) cytochrome *c* both in solution and when associated with micelles. In aqueous solution, the holoprotein cytochrome *c* is tightly folded at secondary and tertiary levels and differs strongly from its random-coiled precursor. However, in the presence of 12-PN/12-Pglycol (9:1) micelles, we observed a remarkable resemblance between the CD spectra of these partially helical proteins. The water–lipid interface induces a secondary folding of apocytochrome *c*, whereas cytochrome *c* is suggested to partially lose its tertiary structure. The exchange of all amide protons and, using deuterium-labeled proteins, of all amide deuterons with the solvent was monitored by NMR. A rapid exchange rate was observed, indicating that these folding states are highly dynamic. Saturation-transfer NMR of micelle-associated apocytochrome *c* showed that the exchange takes place at the (sub-) second time scale. The holoprotein in the presence of micelles was found to have two distinct exchange rates: (1) a fast rate, comparable to that found for the micelle-associated precursor and 4.5 times slower than that of the random-coiled apocytochrome *c*, and (2) a slow rate which is 75 times slower than the precursor in solution. Urea denaturation studies showed the micelle-bound proteins to have a low helix stability, which explains the inability of the lipid-induced secondary structure to prevent its labile protons from rapid exchange. The uniqueness of this lipid-induced highly dynamic folding state of (apo-) cytochrome *c* is demonstrated by comparison with amphiphilic polypeptides like melittin, and its implications for membrane translocation and functioning are discussed.

Apocytochrome *c* is the cytosolically synthesized precursor of the mitochondrial protein cytochrome *c*, which functions in the intermembrane space as electron donor to the inner-membrane protein cytochrome *c* oxidase (Powell et al., 1990). In order to reach its functional location, apocytochrome *c* has to translocate across the outer mitochondrial membrane and a heme group has to be covalently attached by the enzyme heme lyase. The import route of apocytochrome *c* is unique, since the protein does not possess a cleavable amino terminus (Smith et al., 1979; Hennig & Neupert, 1981), unlike other mitochondrial precursors, nor is a membrane potential, ATP, or a cytosolic-exposed proteinaceous component (Nicholson et al., 1988; Stuart et al., 1990) involved in the translocation process. On the basis of biochemical and biophysical studies in model systems, it has been suggested that direct interaction between apocytochrome *c* and membrane lipids can result in its insertion and translocation across the lipid barrier (Jordi et al., 1989; Rietveld et al., 1985; Demel et al., 1989; Görrissen et al., 1986).

From previous circular dichroism (CD)[1] studies in model systems, it was shown that the basic apocytochrome *c* undergoes a conformational change from random coil to a partially α-helical structure upon binding to phospholipid vesicles (de Jongh & de Kruijff, 1990; Walter et al., 1987) or micelles (de Jongh & de Kruijff, 1990; Jordi et al., 1989). This effect is more pronounced for negatively charged lipids than for zwitterionic ones. The amino terminus has been shown to spontaneously reach the opposite site of model membranes, which contain negatively charged lipids (Jordi et al., 1989). It was suggested that during this process the N-terminus attains a helix extending from residues 1 to 22, implying that the 2 cysteines (positions 14 and 17) responsible for heme ligation are facing the same side, which could facilitate heme insertion in the translocation process (de Jongh & de Kruijff, 1990). Once the polypeptide contains the heme group it cannot cross a bilayer anymore (Geller & Wickner, 1985). These data point to an important role of lipid-induced folding for translocation and formation of the holoprotein.

The aim of the research presented here is to obtain greater insight into the nature of the folding states of apo- and holocytochrome *c* at a water–lipid interface, by studying the dynamics of the secondary structure induced in these proteins upon association with micelles, employing circular dichroism and ^{1}H-NMR techniques. In this study, we make use of micelles of phospholipid-based detergents as described in a previous paper (de Jongh & de Kruijff, 1990) containing 90% zwitterionic and 10% negatively charged lipids. We will focus on the exchange of the labile amide protons with the solvent, and we will present a new experimental procedure to detect this exchange by making use of amide-deuterated proteins. The observed data will be related to the stability of the helices as found by urea denaturation studies. It will be demonstrated that the interaction of apocytochrome *c* with micelles induces a folding intermediate which shows a strong resemblance to

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[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; CMC, critical micelle concentration; SDS, sodium dodecyl sulfate; 12-Pglycol, dodecylphosphoglycol; 12-PN, dodecylphosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.

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holocytochrome c, both in secondary structure and in the high dynamics of the secondary structure elements. However, the presence of a heme group within the holoprotein causes an additional folding of the protein around the heme, which might explain the differences in lipid–protein interactions between a translocation-competent precursor and a functional holoprotein.

Materials and Methods

Materials. Horse heart cytochrome c (type VI) and melittin were obtained from Sigma (St. Louis, MO), methyl iodide and ethanolamine were from Merck (Darmstadt, Germany), and urea was from Baker (Deventer, The Netherlands). Deuterium oxide and deuterium-depleted water were obtained from Isotec (Miamisburg, OH).

Preparation of Proteins. Cytochrome c was purified from various deamidated forms by chromatography over a (carboxymethyl)cellulose column, eluted with 85 mM sodium phosphate buffer (pH 7.0) (Brautigan et al., 1978). Apocytochrome c was chemically prepared by removal of the heme group of cytochrome c as described by Fisher et al. (1973). Before use, the proteins were checked for purity by SDS gel electrophoresis carried out under reducing and nonreducing conditions, where the precursor and the holoprotein migrated as single bands of 11.5 and 12 kDa, respectively. Cytochrome c was used in the oxidized form. Melittin was purified according to Batenburg et al. (1987).

Preparation of Detergents. The detergent dodecylphosphoglycol (12-Pglycol) was synthesized as described previously (de Jongh & de Kruijff, 1990). Dodecylphosphoethanolamine was synthesized in a similar manner to 12-Pglycol, except that ethanolamine was used instead of ethylene glycol in the coupling reaction. Dodecylphosphocholine (12-PN) was synthesized via methylation of dodecylphosphoethanolamine by methyl iodide. Briefly, 0.15 mmol of lipid was dissolved in a 35-mL mixture of chloroform, dimethylformamide, 2-propanol, and a 1 M sodium hydrocarbonate/1 M disodium carbonate solution (pH 7.0, HCl), in a volume ratio of 2:2:1:2. The mixture was stirred overnight with 1 mmol of methyl iodide at 40 °C in a closed system, whereafter the product was purified according to established methods (de Jongh & de Kruijff, 1990). The purity of the detergents was checked by silica gel thin-layer chromatography, using a trichloroethane/methanol mixture with volume ratios of 7:2 and 1:2 as eluens. The products reacted positively with phosphorus reagents, and no contaminants could be detected by exposure to I_2 vapor or under UV light. Both ^{31}P- and ^{13}C-NMR identified the products positively.

Critical Micelle Concentrations. The CMC value of micelles consisting of 12-PN/12-Pglycol (9:1 mol/mol) in 10 mM phosphate buffer (pH 7.0) was determined by turbidity measurements on a Hitachi U-3200 spectrophotometer at various wavelengths (280, 300, and 336 nm) to exclude specific absorbance effects (de Jongh & de Kruijff, 1990). A CMC value of 1.0 mM was found between the previously described values for micelles of 12-PN and 12-Pglycol of 1.2 and 0.6 mM, respectively (de Jongh & de Kruijff, 1990).

Deuteration and Deuterium Depletion of Urea. Deuterated urea was prepared by incubation of 1 g of urea for 8 h at 70 °C in 22 mL of ^2H_2O (pH 12.5, NaO^2H). The material was then freeze-dried and stored at −20 °C. The degree of deuteration was >95% as determined by ^2H-NMR.

^2H-Depleted urea was obtained by incubation of 1 g of urea in 1.7 mL of ^2H-depleted water at 70 °C at pH 7.0. After the material was freeze-dried, it was stored at −20 °C. The content of natural-abundance deuterium was reduced by a factor 5.3 as determined by ^2H-NMR.

Deuteration of (Apo-) Cytochrome c. Amide-deuterated apocytochrome c was prepared by incubating the protein (10 mM) for 15 h at 37 °C in ^2H_2O (pH 7.2). After being freeze-dried for >12 h at <0.05 mbar, the protein was stored at −20 °C under nitrogen.

Amide-deuterated cytochrome c was prepared by dissolving the protein (10 mM) in 8 M deuterated urea in 10 mM phosphate/^2H_2O buffer (pH 8.0) and incubating for 15 h at 37 °C. Next, the urea was removed by three dialysis steps against 20 times excess 10 mM phosphate buffer (pH 7.0) in ^2H_2O at 4 °C. After dialysis, the CD spectrum of a control sample of cytochrome c in H_2O showed a partial helical structure, identical to the starting material, indicating refolding of the protein. Removal of all the solvent-accessible deuterons was obtained by subsequent dialysis of the deuterated protein against 250 times excess 10 mM phosphate H_2O buffer (pH 7.0) followed by elution over a Sephadex G-75 column with H_2O as eluens. After being freeze-dried, protein was stored under nitrogen at −20 °C.

This deuteration procedure did not result in a reduced electron-transfer activity of cytochrome c to cytochrome c oxidase (data not shown), assayed as described by Hovius et al. (1990). For this purpose, mitochondria were isolated as described by Wojtczak and Sottocasa (1972) and frozen to make the outer membrane semipermeable.

Circular Dichroism Measurements. For all experiments, a 9:1 molar ratio of the detergents 12-PN/12-Pglycol was used in the stock solutions, which were prepared by codissolving appropriate amounts of dry material in the desired buffer. The pH values mentioned are all direct pH-meter readings.

Stock solutions of 0.3 mM protein and of 75 mM detergents were prepared in 10 mM potassium phosphate (pH 7.0) or sodium acetate buffer (pH 4.75). Appropriate amounts of both stock solutions were mixed or diluted with buffer to obtain samples with a final protein concentration of 0.1 mM in the absence or presence of the detergents 12-PN/12-Pglycol (lipid to protein ratio of 120). For the denaturation studies, urea was added from a concentrated stock solution to obtain final concentrations ranging from 0 to 8.5 M. All samples were incubated for 20 min at room temperature prior to the CD measurements. The CD spectra were recorded at room temperature on a Jasco-600 spectropolarimeter from 185 to 260 nm, using 0.1-mm path-length cells with a scan speed of 20 nm/min, a time constant of 0.5 s, and a bandwidth of 1 nm. Ten spectra were accumulated and stored using a Laser 386 computer. After spectral subtraction of CD spectra from corresponding protein-free samples, spectral analysis was performed as described by de Jongh and de Kruijff (1990). For all data presented here, the root mean square of the fit was smaller than 10.

^1H-NMR Measurements. Concentrated stock solutions of protein (10 mM) and of the detergents 12-PN/12-Pglycol (1.2 M) in 10 mM phosphate buffer (pH 7.0) were mixed and incubated for 20 min at room temperature. The samples were then diluted 25 times in 10 mM phosphate buffer in ^2H_2O (pH 7.0) to a final concentration of 0.2 mM protein and 24 mM detergent. Spectra were recorded within 15 min after dilution at room temperature on a Bruker AM-600 spectrometer at a resonance frequency of 600.16 MHz, with a sweep width of 9 kHz and a single 90° pulse of 9.2 μs.

For the ^1H to ^2H exchange experiments, samples of 0.2 mM protein in the absence or presence of detergents were prepared as above but using 10 mM [$^2H_5$]acetate buffers (pH 4.75). The spectra were recorded at room temperature on a Bruker

HX-360 spectrometer controlled by an Aspect 2000 computer, using a single 90° observation pulse of 8.1 μs. Integrals from 11.5 to 7.5 ppm were taken as a measure of the intensity of the amide region. The intensities obtained can be related to those of a similar sample diluted in H_2O instead of ^2H_2O in order to normalize the data to the maximal intensity of the amide region. For this purpose, at various time points, free induction decays were also accumulated using a selective 1-1 observation-pulse sequence, to reduce the water signal as described by Hore (1983) without presaturation of the solvent. Repeating the experiments yielded deviations in the final numbers of less than 1.5%.

The saturation-transfer measurements were performed on a sample of 0.2 mM apocytochrome *c* with 24 mM 12-PN/12-Pglycol, prepared as described for the CD samples, in 10 mM acetate buffer (pH 4.75) containing 10% ^2H_2O. Spectra were recorded on an AM-600 spectrometer with presaturation of water with 3 mW during 1.5 s, prior to a time-shared long observation pulse sequence (90° pulse of 22.5 μs) for solvent suppression (Redfield & Kunz, 1981). A T_1 relaxation time of 28 ms was found for H_2O.

All chemical shifts presented are relative to 3-(trimethylsilyl)[$^2H_4$]propionate at 0 ppm.

^{2}H-NMR Measurements. Fifteen milligrams of dry amide-deuterated protein was dissolved on ice in 0.4 mL of 10 mM acetate buffer in ^2H_2O (pH 4.75), containing, if desired, 12-PN/12-Pglycol (lipid to protein ratio of 120). The exchange was started by adding directly 3.6 mL of cold 10 mM acetate buffer (pH 4.75), and after given time intervals, aliquots of 0.4 mL were withdrawn from the sample. In order to quench the exchange, the samples were immediately frozen in liquid nitrogen and freeze-dried. Next, the samples were suspended in 0.4 mL of ^{2}H-depleted water at pH 9.0 and incubated for 60 min at 45 °C to obtain complete release of the residual deuterium from the protein. For the same reason, samples of cytochrome *c* were suspended in the presence of 8 M ^{2}H-depleted urea.

The amount of deuterium was quantified by ^{2}H-NMR at a resonance frequency of 46.06 MHz on a Bruker MSL-300 spectrometer. A quadrupolar-echo sequence (90° pulse of 11 μs, 35-μs echo delay, 2-s interpulse time, and 5-kHz sweep width) was used, and 1024 free induction decays were accumulated and stored using an Aspect 2000 computer. After Fourier transformation, the integral from 300 to −300 Hz was taken (zero-position at H^2HO) and compared to a calibration of standards of ^2H_2O in H_2O. The standard deviation in the experimental setup, both in deuteration and in determination of deuterium content, was less than 2%.

Corrections for amounts of deuterium arising from ^2H_2O that was not removed during freeze-drying were performed by determining the amount of water molecules present in the samples using ^{1}H-NMR on a Bruker HX-360 spectrometer (single 90° observation pulse of 8.0 μs). In all cases, approximately 0.95 molecule of water per detergent molecule was found to be present, resulting in corrections varying between 21.2 and 23.9 deuterons per protein. In the case of samples of cytochrome *c*, an additional correction needs to be made for the residual deuterons in the ^{2}H-depleted urea used, which was determined to be 2.9 deuterium atoms per protein. The data presented in this paper were corrected for these numbers.

Results

In a previous study, we showed that micelles of 12-Pglycol and of 12-PN mimic phospholipid bilayers very well in their ability to induce secondary structure in apocytochrome *c* and that up to high detergent concentrations optically clear solutions were obtained allowing accurate CD measurements (de Jongh & de Kruijff, 1990). In this study, we decided to make use of mixed micelles consisting of 90% of the zwitterionic lipid 12-PN and 10% of the negatively charged 12-Pglycol because (1) it mimics the content of surface charges of the outer mitochondrial membrane lipids and (2) this mixture not only allows accurate CD measurements but also permits high-resolution ^{1}H-NMR of apocytochrome *c* at a water–lipid interface. The ability of reduced cytochrome *c* to transfer its electron to cytochrome *c* oxidase in broken isolated mitochondria, assayed as described under Materials and Methods, was not reduced by its association to these micelles, indicating no irreversible denaturation of the protein (data not shown).

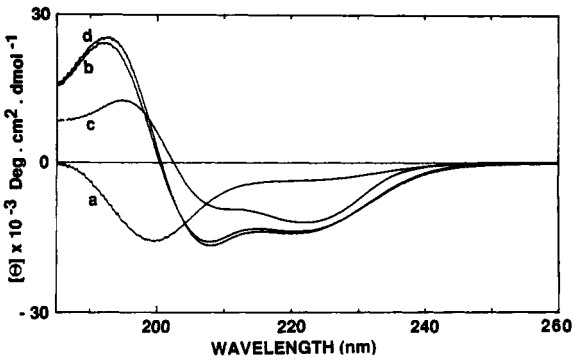

FIGURE 1: Circular dichroism spectra of 0.1 mM apocytochrome *c* in the absence (a) and presence (b) of 12-PN/12-Pglycol (9:1) micelles (lipid to protein ratio of 120) in 10 mM phosphate buffer (pH 7.0) and spectra of 0.1 mM cytochrome *c* in the absence (c) and presence (d) of these micelles.

The secondary structure of (apo)cytochrome *c* in interaction with these micelles is investigated by CD (Figure 1). The spectrum of apocytochrome *c* in solution (spectrum a) shows a minimum at 198 nm, typical for a polypeptide in a random conformation (Fisher et al., 1973; Chou & Fasman, 1978). As expected, upon addition of 12-PN/12-Pglycol (9:1) micelles, a spectral change is observed, yielding three extrema at 196, 206, and 222 nm (spectrum b), indicating the presence of α-helical structures (de Jongh & de Kruijff, 1990; Chou & Fasman, 1978). The percentage of α-helices was estimated to be 42% by curve-fitting procedures. The CD spectrum of the holoprotein shows the presence of α-helices (spectrum c) which are known to be present within the protein (Bushnell et al., 1990; Wand et al., 1989). However, due to the presence of the heme group, the spectrum is distorted as has been reported previously (Jeng et al., 1990; Muga et al., 1991b), causing a red-shift and line broadening. Interestingly, the addition of micelles gives rise to a spectral change of cytochrome *c* (spectrum d), resulting in a spectrum which closely resembles that of the precursor in the presence of micelles, and no spectral distortions are apparent anymore. Under the same experimental conditions, identical spectra of the precursor and the holoprotein were also found in the presence of 12-Pglycol (58% α-helical) or SDS (38% helical), illustrating the generality of this observation.

In order to get insight into the dynamics of the secondary structure of apocytochrome *c* and its holoprotein at a lipid–water interface, we performed high-resolution ^{1}H-NMR measurements. Figure 2A shows the aromatic and amide regions of 600-MHz spectra of apocytochrome *c* in ^2H_2O buffer. In the region between 8.0 and 7.4 ppm, several well-resolved resonance lines are observed, and these are assigned to the C2-protons of the three histidines (residues 18, 26, and 33) at 7.93–7.85 ppm and the C4- and C7-protons of

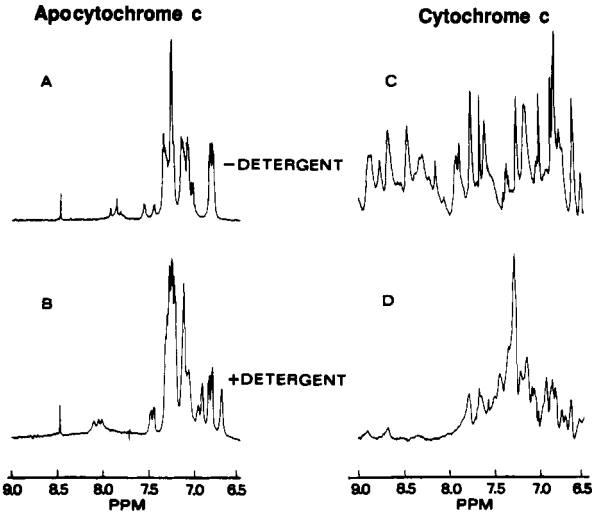

FIGURE 2: Amide and aromatic regions of 600-MHz ^{1}H-NMR spectra of apocytochrome *c* in the absence (A) and presence (B) of 12-PN/12-Pglycol (9:1) micelles (lipid to protein ratio of 120) in 10 mM acetate-d_5/^2H_2O buffer (pH 4.75) and spectra of 0.1 mM cytochrome *c* in the absence (C) and presence (D) of these micelles.

the single tryptophan (residue 59) at 7.55 and 7.45 ppm (Snel et al., 1991). The cluster of overlapping resonances from 7.4 to 7.0 ppm originates from the phenylalanine ring protons and the histidine C4-, the tryptophan C2,5,6-, and the tyrosine C2,6-protons; also, the resonances of the amino groups of the side chains of lysines, arginines, asparagines, and glutamines are expected in this region. The random-coil conformation of the protein is best illustrated by the resonances for the C3- and C5-protons of the four tyrosines present which are all overlapping around the resonance frequency of 6.85 ppm, typical for tyrosine in aqueous solution (Wüthrich, 1986). Most remarkable is the absence of resonances from amide protons, which are expected in the 9–7.5 ppm region. This absence is explained by exchange of these labile protons with ^2H upon exposure to ^2H_2O as is generally observed for a random-coiled polypeptide (Wüthrich, 1986). The sharp peak at 8.45 ppm is an experimental artifact.

The presence of 12-PN/12-Pglycol (9:1) micelles (Figure 2B) induces a slight line broadening of the resonance lines and changes in the chemical shift of several aromatic protons, demonstrating that all protein interacts with these micelles. Most notably, the histidine C2-protons, known to have a pH-dependent chemical shift of their resonances, are shifted 0.15 ppm downfield due to the local lower pH at negatively charged headgroups (Snel et al., 1991). A similar effect, but of larger magnitude (an almost 0.9 ppm shift), was observed previously for apocytochrome *c* bound to micelles which contain only the anionic detergent SDS (Snel et al., 1991). The tyrosine C3,5-protons do not overlap anymore at 6.85 ppm, indicating different chemical environments. Although the CD spectra (Figure 1) showed that the micelles induced large amounts of α-helical structures, which are expected to shield the amide protons of these residues from exchange with ^2H_2O, no amide protons are observed in the spectral region from 9 to 7.5 ppm 15 min after the addition of ^2H_2O. That this is due to exchange with the solvent is demonstrated by the observation that the amide protons of a similar sample in H_2O gave a large intensity in this region (see forthcoming Figure 5A, for example).

In general, tight folding of a protein, as in the case of cytochrome *c*, protects its labile protons from exchange, as is also demonstrated in the ^{1}H-NMR spectrum of this protein in

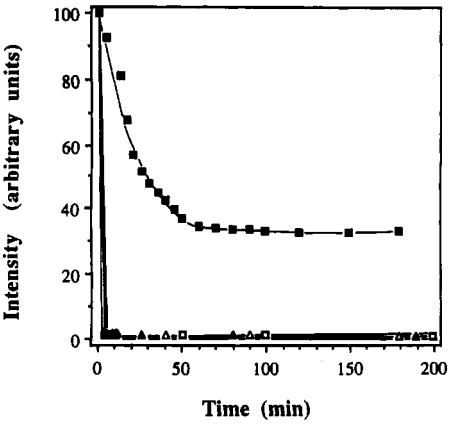

FIGURE 3: Amide proton exchange studies of apocytochrome *c* (open symbols) and cytochrome *c* (closed symbols) in 10 mM acetate-d_5 ^2H_2O buffer (pH 4.75) in the absence (□, ■) and presence (△, ▲) of 12-PN/12-Pglycol (9:1) micelles at a lipid to protein ratio of 120.

solution (Figure 2C). Next to the aromatic resonances, intensities of amide protons can be observed in the 9–7.5 ppm region (Wand et al., 1989). Remarkably, the holoprotein also appears to lose almost all intensity in the amide region upon addition of the 12-PN/12-Pglycol (9:1) micelles (Figure 2D). The residual resonances around 8.8 ppm are assigned to the histidine C2-protons, because their chemical shift was found to be pH-dependent (data not shown). The ~0.7 ppm downfield shift, with respect to the resonance position of these protons in the micelle-bound precursor, is attributed to the close proximity of the heme group.

The amide proton exchange is known to be slower at low pH values (Wüthrich, 1986). Below pH 4.5, aggregation of lipid–protein complexes was observed (not shown), and below pH 4, acidic denaturation of cytochrome *c* occurs (Jeng et al., 1990). We therefore selected a pH of 4.75 to further study the kinetics of this exchange. CD measurements revealed very similar spectra for all systems at pH 7.0 and 4.75 (data not shown).

The kinetics of the amide proton exchange with ^2H_2O were studied by recording ^{1}H-NMR spectra at given time intervals after the addition of ^2H_2O buffer at pH 4.75 (Figure 3). Only for cytochrome *c* in buffer could the amide protons be observed; after an initial decrease of the overall intensity in the amide proton region to about 30%, which was completed during the first hour, the intensity decreases very slowly, such that even after 3 weeks intensity of amide protons could be detected. In contrast, for apocytochrome *c* in solution as well as for both the precursor and the holoprotein in the presence of micelles, a complete loss of the intensity in the amide region was observed at the first time point (at 3.5 min), even at this low pH.

Since the experimental setup used in Figure 3 made it impossible to investigate the kinetics of the ^1H to ^2H exchange on a shorter time scale, another experimental approach was developed in which we made use of amide-deuterated proteins. These deuterated proteins were exposed for short time periods to added H_2O, whereafter the exchange was quenched by freezing the samples in liquid nitrogen and subsequent removal of the water by freeze-drying. The deuterium content of the protein was determined by ^{2}H-NMR as described under Materials and Methods. A sample of apocytochrome *c* which was not exposed to H_2O ($t = 0$) was found to contain 117 (±2) deuterons per protein (Figure 4), which is somewhat more than can be present as amide deuterons within the 104 residues which make up the protein. Due to the deuteration conditions at pH 7.2, it is not likely that the amino groups of lysines or

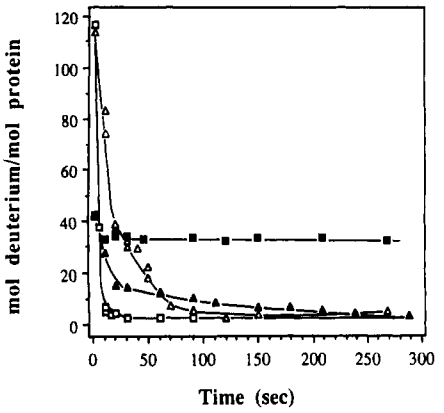

FIGURE 4: Amide deuterium exchange studies of apocytochrome *c* (open symbols) and cytochrome *c* (closed symbols) in 10 mM acetate buffer (pH 4.75) in the absence (□, ■) and presence (△, ▲) of 12-PN/12-Pglycol micelles at a lipid to protein ratio of 120. The deuterium content in the proteins, determined by ^{2}H-NMR, is plotted as function of the time after the addition of H_2O until quenching occurred.

arginines will be deuterated; however, the side chains of asparagines, glutamines, histidines, and the tryptophan contain labile protons which might contribute to a maximum of 20 additional deuterons per protein. After exposure of apocytochrome *c* to H_2O, virtually all the deuterons exchange rapidly with protons, as indicated by the fast decrease of deuterium content shown in Figure 4. The exchange takes place with a half-time of 4–5 s and is completed within 20 s. The presence of 12-PN/12-Pglycol (9:1) clearly decreases the rate of exchange of the amide deuterons, revealing a half-time of 18 s, while 100 s is needed for complete exchange. In cytochrome *c*, only 32 (±1) deuterons are present at the beginning of the experiment, less than found for apocytochrome *c* due to the selective back-exchange step in the preparation, which eliminates the fast-exchanging deuterons. That all deuterons present are shielded from the solvent is demonstrated by the observation that the deuterium content is independent of the time of exposure to H_2O (Figure 4). This number correlates very well with the amount of amide protons which was shown to be inaccessible to ^2H_2O (Figure 3). However, the presence of micelles induces a rapid decrease in the deuterium content and suggests the existence of two different exchange rates: a fast decrease for the first 30 s and a slower rate, revealing complete exchange within 5 min.

It should be noted that the exchange kinetics of amide protons and amide deuterons are not directly comparable. Therefore, it is possible that the exchange of amide protons of apocytochrome *c* bound at a lipid–water interface is within the (sub-) second time scale and that it occurs within the T_1 relaxation of water (here ~100 ms). By specific presaturation of H_2O, it is then expected that exchange will reduce the intensity of the amide proton resonances (Johnston & Redfield, 1977). This is indeed the case as demonstrated in Figure 5, where ^{1}H-NMR spectra are shown from the amide and aromatic region of apocytochrome *c* bound to 12-PN/12-Pglycol (9:1) micelles without presaturation (spectrum A) and with presaturation of water with 3 mW (spectrum B). The difference spectrum (spectrum C) shows clearly that the intensity of amide protons in the entire amide region is reduced due to presaturation of the solvent. The fact that the amide resonances can be observed in the 9–7.5 ppm region implies that the exchange is slow compared to the time scale dictated by the frequency difference between the amide protons and water (0.5 ms). This saturation-transfer effect showed a positive correlation to the power of presaturation. The specifity of the

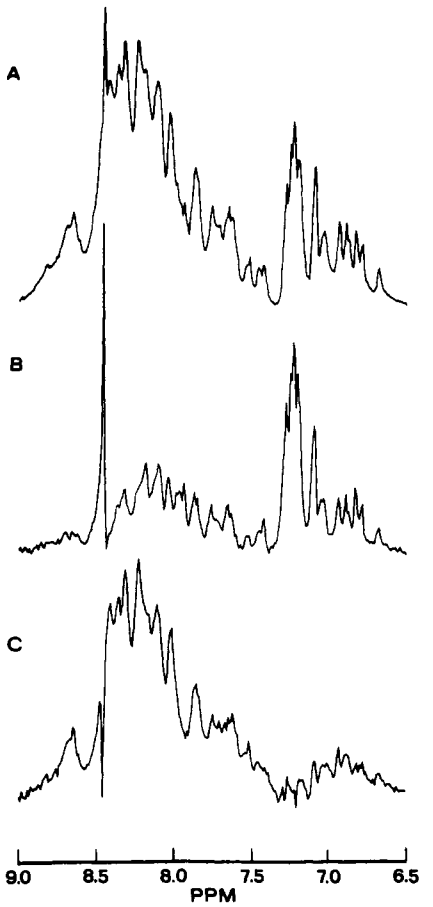

FIGURE 5: Amide and aromatic regions of 600-MHz NMR spectra of apocytochrome *c* in 10 mM acetate buffer (pH 4.75) in the presence of 12-PN/12-Pglycol (9:1) micelles (lipid to protein ratio of 120) without (A) and with presaturation during 1.5 s with 3 mW selectively at the water resonance (B). Spectrum C shows the difference spectrum.

presaturation is indicated by the unaffected intensity of the aromatic resonances at 7.5–6.5 ppm.

The fast exchange of all amide protons of the micelle-associated protein, including those in α-helices, strongly suggests that the helices are not very stable. In order to get insight into this possibility, we studied the susceptibility of the secondary structure to denaturation by urea as shown in Figure 6A. The percentage of α-helices found by analytical curve fitting of the recorded CD spectra is plotted as a function of the urea concentration for the different systems. Apocytochrome *c* in aqueous solution has a random conformation and therefore cannot be unfolded any further by urea. However, the ~40% α-helical precursor in the presence of 12-PN/12-Pglycol micelles gradually loses its helicity upon titration with urea, and is completely unfolded at 8 M. That cytochrome *c* in solution has a stable folded structure is demonstrated by the fact that even at 6 M urea the helices are still intact and it is only above this concentration that the α-helicity strongly decreases, which is in agreement with results from Myer (1968). However, in the presence of micelles, again the resemblance of the holoprotein to the precursor is striking in that the stability of the helices is gradually reduced above 1 M urea. Assuming that the proteins remain associated to the lipids throughout the urea titration, one can perform a quantitative analysis of these plots according to a two-state model as described by Pace and Vanderburg (1979) (Figure 6B). Extrapolation of the straight lines gives the free energy of unfolding under native conditions. A remarkable decrease of the

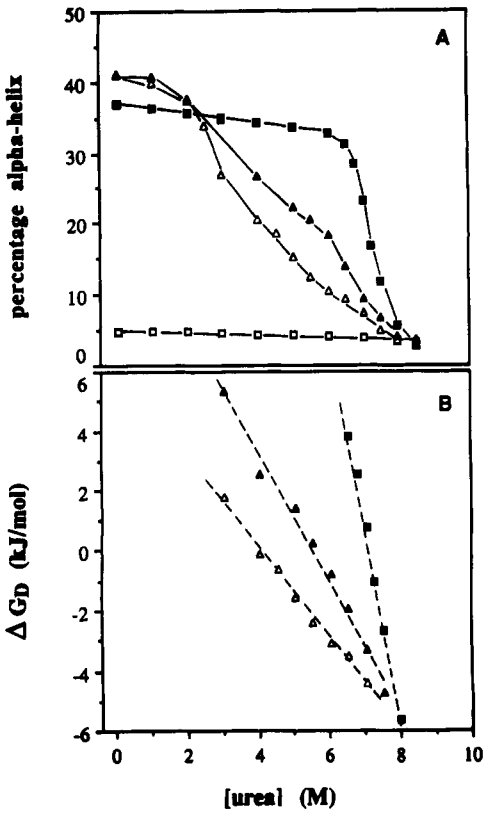

FIGURE 6: (A) Urea denaturation experiments of apocytochrome c (open symbols) and cytochrome c (closed symbols) in 10 mM acetate buffer (pH 4.75) in the absence (□, ■) and presence (△, ▲) of 12-PN/12-Pglycol (9:1) micelles at a lipid to protein ratio of 120. The percentage α-helix as found by analytical curve fitting of CD spectra is plotted as a function of the concentration of urea. (B) Analysis of the data of (A), based on the unfolding described by a two-state model. The free energy of unfolding is plotted against the urea concentration.

helix stability by a factor of 3.3 is observed upon addition of the micelles to the holoprotein. The helices of the holoprotein associated to the micelles are calculated to be 2 times more stable than those of the precursor under the same conditions.

In order to investigate whether the observed highly dynamic state is typical for apocytochrome c or if it is a more general feature of amphiphilic polypeptides associated to 12-PN/12-Pglycol (9:1) micelles, we performed similar experiments for melittin. Melittin adopts α-helices in the presence of these micelles, as was also found by Dawson et al. (1978) using SDS. However, in contrast to (apo-) cytochrome c, these helices were prevented from unfolding in up to 8 M urea (data not shown). The higher stabilization of the α-helices in micelle-associated melittin was also obvious from ^{1}H-NMR studies, which showed a protection of the amide protons from exchange with ^2H_2O over a period of hours (data not shown), which is in agreement with the literature for melittin bound to 12-PN micelles (Inagaki et al., 1989).

Discussion

In aqueous solution, apocytochrome c and the holoprotein exhibit a completely different secondary and tertiary structure as revealed by CD (Fisher et al., 1973; Jordi et al., 1989). However, the resemblance of the CD spectra of the two proteins bound to 12-PN/12-Pglycol (9:1) micelles is remarkable (Figure 1). For cytochrome c, the absence of spectral distortions, generally caused by the presence of a heme, upon association to micelles might point to loosening of the protein tertiary structure. Partial unfolding of cytochrome c has recently been reported for the protein bound to cardiolipin bilayers (Spooner & Watts, 1991a,b). Roder and co-workers showed that cytochrome c could be unfolded at acidic pH and high ionic strength (Jeng et al., 1990; Roder et al., 1988), accomplished by replacement of methionine-80 by histidine-26 or -33 for the axial ligation to the heme group (Roder & Elöve, 1991). Folding around the heme group prohibits the propagation of the amino-terminal helix at position 14 (Bushnell et al., 1990). However, unfolding from the heme group would allow this helix to extend to residue 22, which could explain the relatively high helix content as found for example in the presence of 12-Pglycol micelles.

Investigation of the dynamics of the folded states of both the precursor and the holoprotein bound to micelles was performed by monitoring the solvent accessibility of their labile protons. The amide proton exchange of apocytochrome c, both in solution and when bound to 12-PN/12-Pglycol (9:1) micelles, appeared to be too fast, even at the low pH of 4.75, to detect by on-line ^{1}H-NMR measurements of the amide intensity. However, with the newly developed experimental procedure using deuterated proteins, it was possible to detect the exchange kinetics taking place on the seconds time scale. Analysis of the amide deuterium exchange data (Figure 4) shows that in the presence of micelles there is a retardation of the exchange rate by a factor of 2.9 compared to apocytochrome c in solution. The cause of this retardation can be either due to shielding by the direct interaction with the micelles or by the micelle-induced α-helical conformation. The exchange at the (sub-) second time scale was demonstrated by a strong reduction of the amide intensity of apocytochrome c bound to these micelles due to their exchange with selectively presaturated water. The intruiging observation of a fast exchange of all the amide protons of apocytochrome c at a water–lipid interface, including regions adopting α-helices, has also recently been observed using infrared spectroscopy (Muga et al., 1991a). These authors observed an exchange of all amide protons to deuterium within 2 h of exposure of the protein bound to DMPC/DMPG bilayers.

In contrast to apocytochrome c, the fast exchange of deuterium in cytochrome c occurs only in the presence of 12-PN/12-Pglycol micelles. By analyzing the kinetics of the exchange of amide protons with ^2H_2O, the amide protons are found to be at least 10^4 times more protected in the protein in solution compared to the micelle-associated form. This protection factor is in the same order as Jeng et al. (1990) observed comparing native and acid-denaturated cytochrome c. The deuterium proton exchange data show only in the presence of 12-PN/12-Pglycol micelles a loss of deuterium from the protein, whereas in the absence of micelles these deuterons did not rapidly exchange in time. Since the protein was labeled specifically at those positions enclosed within the folded protein, this rapid exchange can only occur when a partial unfolding of the protein occurs. Analysis of the kinetic data shows that for cytochrome c bound to micelles there are two different exchange rates: (1) a fast rate, comparable with that found for the micelle-associated precursor and 4.5 times slower than the random-coiled apocytochrome c, and (2) a slow rate which is 75 times slower than the precursor in solution. This slow rate might represent the dynamics of unfolding from the heme as described by Roder and Elöve (1991). The faster rate may be related to the observation by Spooner and Watts (1991a) that the α-helices present in cytochrome c bound to a cardiolipin bilayer exhibit a lifetime no longer than 10^{-6} s.

The suggestion that the high dynamics of the secondary structured protein associated to micelles cause the fast amide

proton exchange is supported by the observation that the stabilization energy of the holoprotein helices is reduced upon addition of micelles (Figure 6). That the α-helices of the micelle-bound holoprotein are more stable than the precursor can be ascribed to its ability to fold around the heme group. The uniqueness of the highly dynamic secondary structure of (apo-) cytochrome *c* is illustrated by comparison with melittin, another micelle-associated amphiphilic polypeptide, which in contrast showed increased stabilization of the helices upon binding to these micelles. Thus far, only one case of fast exchange of all amide protons (<30 min) of a secondary structured polypeptide bound to micelles has been described in the literature: the mainly micelle surface associated myelin basic protein which has a mean hydrophobicity comparable to apocytochrome *c* (Mendz et al., 1990). However, several micelle-associated amphiphilic helical presequences, both surface-associated and also deeply buried in the lipid phase, showed much slower exchange of their amide protons with the solvent (up to 54 h) (Karslake et al., 1990; Rizo et al., 1991).

The loose folding of apocytochrome *c* might facilitate its spontaneous translocation over a lipid barrier. However, the ability of the holoprotein to fold around the heme group causes important differences between the translocated precursor and cytochrome *c*, which might be reflected in their nature and strength of interaction with lipids. In many studies, the differences between apocytochrome *c* and the holoprotein with respect to lipid interaction have been illustrated, showing in general a reduced interaction for cytochrome *c* (Jordi et al., 1989; Li-Xin et al., 1988). However, unfolding of cytochrome *c* by heat treatment or urea resulted in a more pronounced interaction, suggesting the importance of folding for the protein–lipid interaction (Demel et al., 1989). Monolayer studies with various lipids showed a considerable effect of cytochrome *c* on the surface pressure with cardiolipin only (Demel et al., 1989), whereas Spooner and Watts (1991a,b) showed that cytochrome *c* partially unfolds when associated to cardiolipin bilayers. Because cardiolipin is only a minor component of the outer mitochondrial membrane, we speculate that after heme linkage to the precursor the protein tertiary folds and detaches from the membrane, illustrating the importance of folding for the translocation process. Although no tight association of cytochrome *c* with the mitochondrial inner membrane has been observed (Gupte & Hackenbrock, 1988), the inner membrane is enriched in cardiolipin, which is associated with cytochrome *c* oxidase (Powell et al., 1990). Therefore, we consider it possible that partial unfolding, as observed specifically in the presence of cardiolipin and upon its association to the micelles used in this study, is of importance for the functional activity of cytochrome *c* upon interaction with its redox partner.

In summary, it appears that in spite of the completely different properties of apocytochrome *c* and its holoprotein in solution, the presence of 12-PN/12-Pglycol (9:1) micelles induces α-helical structures in both the precursor and the holoprotein with a striking resemblance. However, the stability of the helices induced in (apo-) cytochrome *c* by the micelles is low compared to that of the holoprotein in solution. It is this helix instability which explains the fast exchange of all the amide protons of (apo-) cytochrome *c* at a micellar surface. The differences between the precursor and the holoprotein in the exchange and denaturation studies suggest an additional folding around the heme in the holoprotein, due to a reversible ligation of methionine-80 to the heme group, which is absent for the precursor protein. This folding event is likely to occur upon conversion of the translocated precursor to the holoprotein and may therefore be of importance for the translocation process.

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Differential Scanning Calorimetric Study of the Thermal Unfolding of Mutant Forms of Phage T4 Lysozyme†

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ABSTRACT: In two recent papers, we reported the effects of several point mutations on the thermodynamics of the thermal unfolding of the lysozyme of phage T4 as determined by differential scanning calorimetry. The mutants studied were R96H [Kitamura, S., & Sturtevant, J. M. (1989) *Biochemistry 28*, 3788–3792] and T157 replaced by A, E, I, L, N, R, and V [Connelly, P., Ghosaini, L., Hu, C.-Q., Kitamura, S., Tanaka, A., & Sturtevant, J. M. (1991) *Biochemistry 30*, 1887–1891]. Here we report the results of a similar study of the single mutations A82P, A93P, and G113A and the double mutation C54T:C97A. The three single mutants all show small apparent stabilization at pH 2.5 and 46.2 °C (the denaturational temperature of the wild-type protein), amounting to -0.5 ± 0.4 kcal mol^{-1} in free energy, whereas the double mutant shows a weak apparent destabilization, $+0.8 \pm 0.4$ kcal mol^{-1}. As in all our previous studies of mutant proteins, the enthalpy changes produced by these mutations are in general of much larger magnitude than the corresponding free energy changes and frequently of opposite sign.

It has often been remarked that globular proteins in solution, despite their highly organized and nearly perfectly cooperative structures, nevertheless are only marginally stable, their stabilization free energies of 5–20 kcal mol^{-1} being the results of cancellations of many positive and negative contributions. Consequently, as pointed out by Hawkes et al. (1984), thermodynamic parameters for the reversible thermal denaturation of proteins afford very little basis for understanding the wide range of forces involved in protein structures. Nevertheless, such data have proven to be useful in assessing the effects resulting from known amino acid replacements and in attempting to correlate these effects with observed changes in structure as determined by X-ray crystallography or 3-D NMR, and in supplying numbers with which the results of theoretical calculations can be compared.

A recent paper by Tidor and Karplus (1991) illustrates very clearly the formidable difficulties involved in reaching a detailed understanding of the energetic results of a single amino acid replacement in a protein. These authors used free energy simulation methods to analyze the effects of the R96H mutation in T4 lysozyme, one of the mutations on which we have run DSC experiments (Kitamura & Sturtevant, 1989). Limiting their treatment to interactions involving 7 residues, 3 on each side of residue 96, they tabulated some 35 free energy contributions involving the change of the folded WT to the folded mutant and 21 involving the unfolded forms, some positive and some negative with magnitudes up to 7 kcal mol^{-1}. These contributions added up to a total destabilization of 1.9 kcal mol^{-1} at 300 K compared with the extrapolated DSC result of 3.2 ± 1.2 kcal mol^{-1}.

The lysozyme of bacteriophage T4 is a single-chain polypeptide containing 164 residues and having a molecular weight of 18 700. Its crystal structure has been accurately determined (Weaver & Matthews, 1987), and numerous known amino acid replacements have been produced by appropriate mutagenesis and cloning. Although the apparent stabilities of many of these mutants have been estimated by means of optical melting curves, mainly employing circular dichroism and fluorescence emission, to date only eight have been studied by DSC. We believe it is important to subject as many as possible

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