Tertiary Stability of Native and Methionine-80 Modified Cytochrome *c* Detected by Proton−Deuterium Exchange Using On-Line Fourier Transform Infrared Spectroscopy†

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ABSTRACT: The stability of the tertiary structure of cytochrome *c* and of a methionine-80 chemically modified form of this protein has been investigated by monitoring on-line the exchange of amide protons with deuterons using infrared spectroscopy. The modified protein has a structural stabilization energy of approximately 50% of that of native cytochrome *c*, whereas the secondary structure is not affected by the modification. In the modified protein the fraction of slow exchanging amides is smaller compared to that in the native protein, and the exchange rate constants are found to be 2−3 times larger for the slow (half-life of 5.5 h) and intermediate (half-life of 4.1 min) exchanging fraction of amides. The exchange rate of a fast exchanging fraction of amides (half-life smaller than 1 min), most likely surface exposed amides, is not influenced by tertiary destabilization of the protein. The results in aqueous solution agree well with data obtained by monitoring the amide-proton exchange using ^{1}H-nuclear magnetic resonance. In films, using attenuated total reflection infrared techniques, this difference in tertiary stability between modified and native cytochrome *c* could also be demonstrated. The various advantages and complications of this approach are discussed in detail.

The large abundance of literature on structural properties of proteins illustrates the need for detailed information on the secondary, tertiary, and quaternary structure and their dynamics to get a deeper insight into the function of proteins in biological systems. NMR[1] has been shown to be very useful in obtaining structural information at the atomic level of proteins [for review see Branden and Tooze (1991)]. An example of one of the first structural characterizations of a protein by amide-proton exchange using NMR is reported by Wagner and Wüthrich (1982) or recently by Kim and co-workers (1993), both for the basic pancreatic trypsin inhibitor. However, the application of NMR to membrane systems is limited, because the particle size has a positive correlation with the linewidth and therefore with the complexity of the spectra due to overlap of individual resonances.

IR spectroscopy is routinely used to obtain information at the level of secondary structure of both soluble and membrane-associated polypeptides. IR studies on the effect of exposure of proteins to ^2H_2O solvents (Haris et al., 1989; Azipiazu et al., 1993) and their exchange kinetics in ^2H_2O (Segawa & Kume, 1986; Wantyghem et al., 1990; Prestrelski & Arakawa, 1991; Rizzo et al., 1992) or analyses of films of aliquots of protein exposed for discrete periods of time to ^2H_2O (Heimburg & Marsh, 1993) have been reported.

In this work we present an application of IR spectroscopy to characterize tertiary folding states by monitoring on-line the amide-proton exchange. The results are compared to the corresponding NMR data. To illustrate the approach, a small (12 kDa) water-soluble protein, cytochrome *c*, was chosen, whose native structure (Bushnell et al., 1990; Feng et al., 1990) and tertiary destabilized conformation (Jeng & Englander, 1991) have been described extensively. The native protein was specifically chemically modified to obtain a protein with a comparable secondary structure but with a distinct tertiary folding state. We investigated the result of this modification on the exchange kinetics both in aqueous solution and in films. We will show that this application is suitable to characterize tertiary folding states and, as will be presented in the near future, can also be applied to investigate membrane-associated proteins.

MATERIALS AND METHODS

Horse heart cytochrome *c* (Type VI, Sigma, St. Louis, MO) was eluted on a Sephadex G-75 column using 10 mM phosphate buffer (pH 7.0), dialyzed extensively against demineralized water, and subsequently freeze-dried. The protein was used in the oxidized form. Cytochrome *c* was chemically modified by incubation of 4 mM cytochrome *c* with 24 mM iodoacetic acid (Sigma, St. Louis, MO) for 8 h at 37 °C in 50 mM sodium acetate (pH 1.5) in the presence of 0.5 M sodium chloride. Under these conditions: (1) Methionine-80 was dissociated from the heme group, as could be demonstrated by the absence of the absorbance at 695 nm, normally present in the native protein due to the ligation of this methionine to the heme (Sreentathan & Taylor, 1971; Folin et al., 1972). This folding intermediate resembles the native protein in terms of intrinsic viscosity and far-UV CD spectrum but shows reduced stability toward

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[1] Abbreviations: NMR, nuclear magnetic resonance; (ATR) FT-IR, (attenuated total reflection) Fourier transform infrared; CD, circular dichroism; Gdn·HCl, guanidine hydrochloride; TMS, 3-(trimethylsilyl)-[$^2H_4$]propionate; ppm, parts per million.

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thermal unfolding (Robinson et al., 1983; Potekhin & Pfeil, 1989; Jeng et al., 1990; Jeng & Englander, 1991). Also: (2) At this low pH the reaction of iodoacetic acid to methionines is specific over other amino acids (Gundlach et al., 1959). Next, the sample was dialyzed 4 times extensively against 250 times excess 10 mM phosphate buffer (pH 7.0) at 4 °C. The product, *S*-(carboxymethyl)methionine cytochrome *c*, was shown to contain 1.2 ± 0.1 mol of carboxymethyl groups/mol of protein, as detected by the sharp resonance at 1.87 ppm using difference ^{1}H-NMR spectroscopy between native and modified cytochrome *c* in 10 mM acetate-$^2H_5$ (p^{2}H 2.2) containing 0.5 M sodium chloride with 10 mM 3-(trimethylsilyl)[$^2H_4$]propionate (Merck, Darmstadt, Germany) as internal standard. Because (1) only one major NMR signal can be observed in the difference spectrum, which shifts from 2.06 to 1.32 ppm upon labeling, arising most probably from methionine ϵCH_3, and (2) under the conditions used during modification no significant enhanced level of fluorescence from the single tryptophan at position 59 could be detected, it is concluded that the second methionine present in cytochrome *c* at position 65 is not readily accessible for labeling due to tight folding of this part of the protein around the heme.

Fluorescence Measurements. The fluorescence of the single tryptophan of native/modified cytochrome *c* was recorded at room temperature on an SLM-Aminco 8000 using an excitation wavelength of 285 nm by detection of the emission at 345 nm. The protein concentration was 5 μM in 10 mM phosphate buffer containing guanidine hydrochloride (Gdn·HCl) (Sigma, St. Louis, MO) in concentrations ranging from 0 to 5 M. The pH of each sample was set to 7.0 individually.

Absorbance Measurements. The Soret band of 5 μM native/modified cytochrome *c* was recorded in samples containing 0−5 M Gdn·HCl in 10 mM phosphate buffer (pH 7.0). Spectra were recorded at room temperature on a Shimadzu UV-190 spectrophotometer in the wavelength region from 375 to 425 nm with the corresponding protein-free samples as reference.

Circular Dichroism Measurements. Samples of 100 μM native/modified cytochrome *c* in 10 mM phosphate buffer containing 0−5 M Gdn·HCl (pH 7.0) were measured at room temperature on a Jasco-600 spectropolarimeter in cells with a 0.02 cm path length. Spectra were recorded from 185 to 260 nm, using a scan speed of 50 nm/min, a time constant of 0.125 s, and a band width of 1 nm. Ten spectra were accumulated, averaged, and subsequently corrected for spectra of the corresponding protein-free samples. The analysis of the secondary structure was performed using a nonlinear least-squares fit procedure, where reference spectra of α-helical, β-stranded, and random coiled polylysine together with that of the β-turn, extracted from 24 proteins with known X-ray structure (Chang et al., 1978), were used to fit an experimentally obtained spectrum.

^{1}H-Nuclear Magnetic Resonance. Fifteen milligrams of native/modified cytochrome *c* was dissolved in 0.6 mL of 10 mM phosphate buffer in ^2H_2O (p^{2}H 7.0, corrected pH-meter reading) containing 10 mM TMS as internal standard. Addition of the solvent initiates the exchange of labile protons with the solvent, and this moment was defined as $t = 0$. The first spectrum was taken after 2.5 min, and during the next 27.5 min every 30 s a ^{1}H-NMR spectrum was recorded on a Bruker HX-360 with a spectral width of 5000 Hz as an average of 16 free induction decays using a single 4.2 μs 90° pulse and an interpulse time of 1 s, followed by Fourier transformation. Next, intervals of 2, 5, 10, 30, and 60 min were used between subsequent measurements up to $t = 24$ h. The temperature was controlled at 21 ± 0.1 °C during the experiment. The integral of the 9.2−5.6 ppm region of the spectrum was normalized to the integral of TMS, which frequency was set to 0 ppm. The maximal intensity of the integral of the 9.2−5.6 ppm region relative to that of TMS was determined by dissolving an identical sample in 0.6 mL of 10 mM phosphate buffer in H_2O (pH 7.0) containing 10 mM TMS and recording a spectrum using a selective 1−1 observation pulse sequence as described by Hore (1983) to reduce the H_2O signal without presaturation of the solvent. The reproducibility of these experiments was within 2%.

Infrared Spectroscopy in Aqueous Solutions. Typically 3 mg of protein was dissolved in 100 μL of 10 mM phosphate buffer in ^2H_2O (p^{2}H 7.0). The moment of addition of the solvent was defined as $t = 0$. Next, the sample was purged via a stop-flow mechanism into a cell of 6 μm thickness, and spectra were recorded at room temperature on a Bruker IFS-55 spectrometer, equipped with a liquid N_2 cooled mercury cadmium telluride detector in the range from 0 to 8000 cm^{-1} with a nominal resolution of 1 cm^{-1}. During the first 10 min of exchange every 30 s a spectrum was recorded as an average of 16 scans. Next, spectra were recorded with exponentially increasing intervals, with the final spectrum taken after 19 h of exchange. The effect of hydration of the dry material in ^2H_2O on the exchange of labile protons with the solvent was investigated by experiments where the sample was first dissolved in 5 or 10 μL of H_2O, prior to diluting it with 95 or 90 μL of ^2H_2O, respectively. No differences in the exchange rates due to prehydration were found (data not shown).

Infrared Spectroscopy on Films. A germanium crystal (50 × 20 × 2 mm, Harrick, Ossining, NY) was cleaned by washing with distilled water, methanol, and chloroform, and by subsequently placing it for 5 min in a plasma cleaner (PDC 23G, Harrick, Ossining, NY) in order to obtain a clean hydrophilic surface. For each experiment, 100 μg of protein was spread homogeneously on the crystal using the tip of a micropipet. On the crystal a 1 mm spacer was placed, and the crystal was covered by a second plate containing a gas inlet and outlet, allowing the film to be exposed to any experimental atmospheric condition. The crystal was placed under an aperture angle of 45°, yielding 25 internal reflections. For measurement of the proton to deuterium exchange, first 10 spectra were recorded with 30 s intervals to check the stability of the sample. Then the sample holder was connected ($t = 0$) to a setup where a constant flow of N_2 gas (30 mL/min) passes first a series of four chambers atmospherically equilibrated with ^2H_2O, resulting in a continuous flow of ^2H_2O-saturated N_2 gas over the sample on the crystal. A saturating hydration of the film of 0.60 ± 0.05 g of water/g of protein was reached within 30 s under these conditions, as detected from the intensity of the ^2H_2O band at 2100−2700 cm^{-1}. On the basis of the intensity at 3300 cm^{-1} before and after deuteration and the known extinction coefficient of H_2O at this frequency (Fringeli & Gunthard, 1980), we could calculate that after preparation of the film 0.25 ± 0.05 g of H_2O/g of protein was present. All experiments were carried out at room temperature.

Analysis of Infrared Spectra. The digitalization of the spectra was enhanced to 0.5 cm^{-1} by zero-filling of the data prior to Fourier transformation. From all spectra of an exchange experiment carried out as described above, spectra of protein-free samples corresponding to the same time point were subtracted, and they were corrected for contributions of atmospheric water and subsequently smoothed to a resolution of 2 cm^{-1}. A full automatic procedure for the integration of various bands of the IR spectra was used, revealing integrals for the amide I [(1704 ± 6)−(1600 ± 3) cm^{-1}], amide II [(1600 ± 3)−(1496 ± 12) cm^{-1}], and amide II′ [(1486 ± 8)−(1350 ± 10) cm^{-1}]. For all data presented, the amide II and amide II′ were normalized to the intensity of amide I to eliminate possible fluctuations in intensity. The obtained ratios amide II/I and II′/I were expressed as fractions of nonexchanged amides by determination of the 100% and 0% values (i.e., the intensities of the fully protonated and amide-deuterated proteins). The 100% value of the protonated protein in films was obtained from the spectra recorded prior to the start of the exchange. The analysis of the protonated form in aqueous solution was complicated by the absorbance of H_2O in the 1700−1600 cm^{-1} region, making a reliable integration of the amide I band impossible. To circumvent this difficulty, a sharp band of TMS at 830 cm^{-1} was used as internal standard. During the exchange experiment the TMS/amide I ratio remained the same, allowing us to use this ratio to estimate the intensity of amide I in an H_2O solvent. Notably, it was found that the amide II/I ratio was larger in films (0.65 ± 0.05) compared to aqueous solution (0.40 ± 0.05), in agreement with recent observations by Ishida and Griffiths (1993). Fully deuterated cytochrome *c* was obtained by incubation of the protein in ^2H_2O at a p^{2}H of 1.5 for 12 h at room temperature. After neutralizing the p^{2}H, spectra of the protein were recorded both in ^2H_2O solution and as a film while flushing the crystal with ^2H_2O-saturated N_2 gas. In both cases no intensity of amide II could be detected.

The proton−deuterium exchange experiments were repeated at least three times, and the reproducibility of the exchange curves and the fitted exchange rates was found to be within 5−10%.

Also, the intensity of amide II′ normalized to amide I can be used to determine the amide-proton exchange rates and size of the various populations. Analysis of these data revealed similar values as obtained from the analysis of amide II within 5−10% (data not shown).

The secondary structure of the proteins was quantified from the amide I band of proteins exposed for 60 min to ^2H_2O-saturated N_2 gas using a Fourier self-deconvolution procedure as described by Goormaghtigh et al. (1990). Although the absolute values of the secondary structure content so-determined are subject to uncertainties due to a number of theoretical limitations as has been described by Surewicz et al. (1993) and ourselves (Goormaghtigh et al., 1994), when differences in secondary structure are investigated, the accuracy is expected to be limited by the curve fitting procedure only. To avoid problems related to the lack of uniqueness in the solution of the curve fitting, an identical input parameter set was used for both native and modified protein. Mathematical subtraction of side chain contributions of cytochrome *c* [data from Venyaminov and Kalnin (1990)] had no influence on either the determination of the secondary structure of the proteins, in agreement with observations by

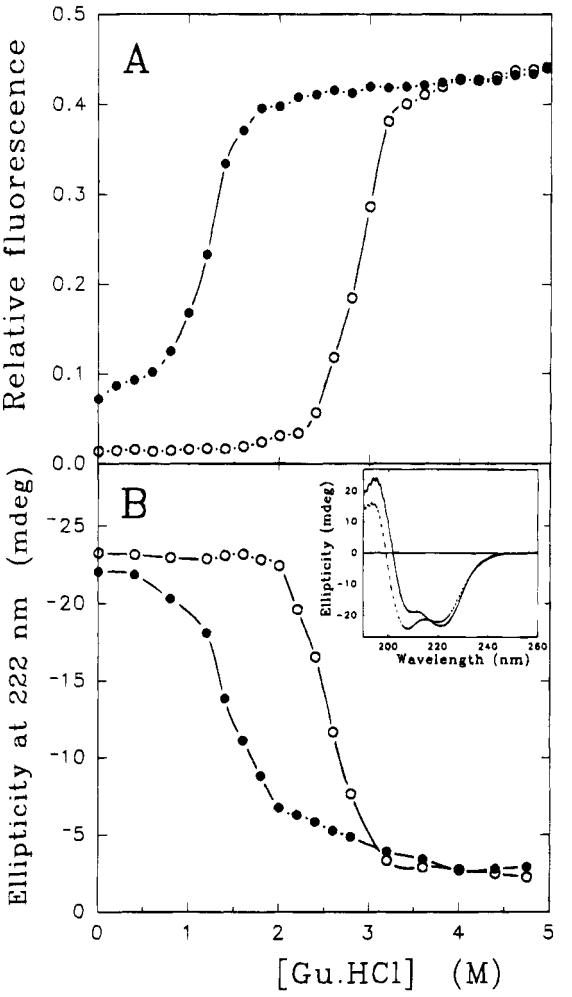

FIGURE 1: The structural stability of native (open circles) and methionine-80 modified (solid circles) cytochrome *c* by guanidine hydrochloride denaturing monitored by (A) the fluorescence of tryptophan-59, relative to that of D-tryptophan in aqueous solution, and (B) the circular dichroism ellipticity at 222 nm. The protein concentration in the fluorescence studies was 5 μM and in the CD studies 100 μM in 10 mM phosphate buffer (pH 7.0). The inset of panel B shows the CD spectra of native (solid) and modified (dashed) cytochrome *c* in the absence of denaturing agent.

Dong et al. (1992), or the integration of the various bands for monitoring the amide-proton exchange (data not shown).

RESULTS

In this work IR spectroscopy was used to monitor on-line the exchange of amide protons with the solvent in order to detect differences in tertiary folding states of proteins. The kinetics of exchange of both native and methionine-80 modified cytochrome *c* were measured and compared to NMR data.

Characterization of the Folding States of Native and Modified Cytochrome c. Differences in the tertiary folding of cytochrome *c* can be monitored by the fluorescence of the single tryptophan at position 59 in the protein. In the absence of Gdn·HCl (Figure 1A) no fluorescence can be detected for native cytochrome *c* due to quenching by the heme group (Tsong, 1975). However, upon unfolding of the protein using increasing concentrations of Gdn·HCl, the fluorescence increases (midpoint at 2.9 M Gdn·HCl) to approximately 40% of that of free tryptophan in the same solvent, a value which corresponds to a full exposure to the

Table 1: Free Energies of Structural Stabilization in kcal/mol of Native and Modified Cytochrome *c* As Determined by Various Techniques

	fluorescence	absorbance	circular dichroism
native	7.4 ± 0.1	8.5 ± 0.5	8.4 ± 0.2
modified	4.1 ± 0.1	4.0 ± 0.7	3.3 ± 0.5

solvent of this residue within a polypeptide (Tsong, 1975). Modified cytochrome *c* has a small but significant fluorescence in the absence of denaturing agent, indicating that the distance of the chromophore to the heme is larger than in native protein. The fluorescence increases, with a midpoint of 1.2 M Gdn·HCl, to a similar level at high concentrations of denaturing agent as found for the nonmodified protein. The lower midpoint value compared to that of the native protein indicates a lower stability of the tertiary structure for the modified protein.

An alternative way to monitor differences in tertiary stability is to detect the direct chemical environment of the heme characterized by the wavelength of maximal absorption of the Soret band of the heme (Pryse et al., 1992). In the absence of Gdn·HCl the Soret band of the modified protein absorbs at a wavelength 5 nm lower compared to that of the native protein (data not shown), indicating a more polar environment of the heme after methionine labeling. Upon increasing the Gdn·HCl concentration, the Soret band of native cytochrome *c* shifts to lower wavelength with a midpoint at 2.5 M Gdn·HCl, whereas for the modified protein a midpoint value of 1.4 M is found (data not shown), both comparable to the fluorescence data.

The inset of Figure 1B shows the CD spectra of native and modified cytochrome *c* in the absence of denaturing agents. Both spectra show three extremes (at 198, 207, and 222 nm), characteristic for (partial) helical proteins (Chang et al., 1978). Due to the presence of the heme group the spectrum of native cytochrome *c* is distorted, as demonstrated by the reduced intensity around 207 nm, as has been reported previously (Jeng et al., 1990; de Jongh et al., 1992). Remarkably, no spectral distortion is apparent from the spectrum of the modified protein, again demonstrating the different positions of the heme in the two proteins. At high concentrations of Gdn·HCl the ellipticity at 222 nm approaches zero for both the native and modified cytochrome *c* (Figure 1B), indicative of a complete loss of secondary structure. For the modified protein, the folding transition takes place at lower Gdn·HCl concentrations compared to the native protein (midpoints of 1.4 and 2.5 M, respectively) and indicates that the secondary structure of the first is less stabilized due to changed tertiary stability.

The free energy of structural stabilization under nondenaturing conditions can be obtained from the data presented in Figure 1 as described by Pace and Vanderburg (1979). For such an analysis we have to assume a two state model for the folding−unfolding process, which is suggested by the sigmoidal shape of the denaturing curves of Figure 1 and justified by the linear relation of the calculated free energy to the concentration denaturing agent (not shown). The free energies of the native and modified cytochrome *c* as determined by fluorescence, by monitoring the Soret band, and by CD are presented in Table 1. On the average, a stabilization energy of 8 kcal/mol is found for the native protein, whereas the structural stability of the modified protein is reduced by almost 50% to approximately 4 kcal/mol.

Table 2: Percentages of Secondary Structure of Native and Modified Cytochrome *c* As Determined by CD and ATR-IR

	infrared		circular dichroism	
	native	modified	native	modified
α-helix	41	45		42
β-strand	15	19		17
β-turn	24	17		23
random	20	19		18

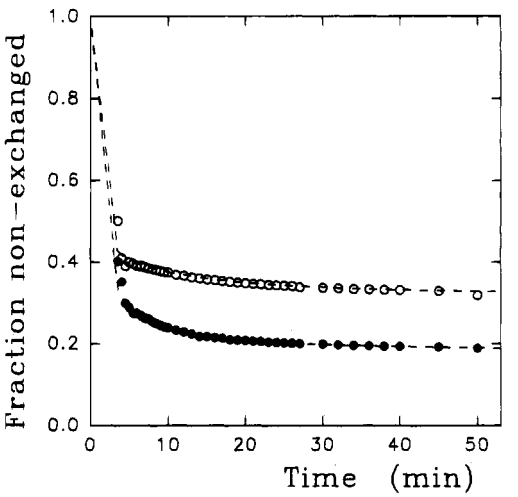

FIGURE 2: Monitoring the exchange of the amide protons of native (open circles) and methionine-80 modified (solid circles) cytochrome *c* (25 mg/mL) with a ^2H_2O solution containing 10 mM phosphate (p^{2}H 7.0) as detected by the integral in the 9.2−5.6 ppm region in ^{1}H-NMR spectra as a function of time. The dashed lines represent the three-exponential decay fits of the data.

To investigate the influence of the modification of cytochrome *c* on the secondary structure of the protein, we analyzed the CD spectrum of the modified protein and the amide I band in the IR spectra of films of both native and modified protein. The secondary structure content of the native protein (Table 2) is found to be comparable to that determined by X-ray (Bushnell et al., 1990) or NMR techniques (Feng et al., 1989, 1990). Due to the spectral distortion by the heme, the CD spectrum of the native protein could not be analyzed. The shape of amide I is not affected by the modification, as demonstrated by the comparable secondary structure content found for the two proteins (Table 2). The CD analysis confirms the similar secondary structure of the modified protein compared to that of native cytochrome *c*.

Amide-Proton Exchange Detected by ^{1}H-NMR. The exchange of the amide protons of both native and modified cytochrome *c* with a ^2H_2O solution as monitored by ^{1}H-NMR is shown in Figure 2, where the fraction of nonexchanged amides is plotted as a function of time of exchange. For the native protein roughly three classes of exchanging amides can be discriminated. Within the dead time of the measurement (2.5 min) 50% of the amides are exchanged. This fraction could arise from surface exposed amides of the protein. A slow exchanging fraction of approximately 35% dominates the decay curve from 20 min to 19 h, which most likely corresponds to amides deeply buried in the core of the folded protein. Also an intermediate exchanging component of approximately 15% can be discriminated, which

Table 3: Analysis of the Proton–Deuterium Exchange in Terms of Three Classes of Exchanging Components in Native and Modified Cytochrome *c* As Measured in ^2H_2O Solution by NMR and IR and in Films by ATR-IR Spectroscopy

		fraction	exchange rate ($\times 10^{-2}$ min^{-1})
NMR	native	0.56	
		0.10	9.6
		0.34	0.074
	modified	0.61	
		0.18	17
		0.21	0.21
IR			
solution	native	0.52	159
		0.07	7.8
		0.41	0.093
	modified	0.63	170
		0.20	12
		0.17	0.27
film	native	0.30	99
		0.19	4.4
		0.51	0.043
	modified	0.49	110
		0.20	11
		0.31	0.098

decays within 15 min. Although interpreting the exchange of 104 amide protons in only three classes is arbitrary, reasonable three-exponential fits of the exchange curves can be obtained using a nonlinear least-squares fit procedure as demonstrated by the dashed lines in Figure 2. The different fractions and exchange rates of these fits are presented in Table 3. For the fast exchanging component no rate constant has been denoted, because the half-life of the obtained rate was shorter than 1 min and therefore meaningless in an experiment with a dead time of 2.5 min. The half-life of the intermediate and slow exchanging component of cytochrome *c* are 7.2 min and 15.6 h, respectively. From the exchange curves in Figure 2 it can be observed that in modified cytochrome *c* the fraction of fast exchanging amide protons is larger and the slow component smaller compared to the native protein. This is also obvious from the analysis of the curves (Table 3). The exchange rate constants for the modified protein are 2–3 times larger than those of native cytochrome *c*, revealing half-life of the intermediate and slow exchanging components of 4.1 min and 5.5 h, respectively.

The exchange of amide protons with the solvent can also be detected by monitoring the amide II or amide II′ intensities from IR spectra, as illustrated by a typical experiment in Figure 3. In the time course of amide-proton to -deuterium exchange a decrease of the amide II (1600–1500 cm^{-1}) and an increase of the amide II′ intensity (1500–1350 cm^{-1}) can be observed.

Amide-Proton Exchange in Solution Detected by IR. In Figure 4 the intensity of amide II normalized to amide I is plotted as a function of time of exchange, initiated by dissolving the protein in ^2H_2O. The fraction of slow exchanging amides is smaller and the exchange rates are faster for the modified protein compared to native cytochrome *c*. This is confirmed by the analysis of the data presented in Table 3, where the exchange rate constants for the slow and intermediate component in the modified protein are 2–3 times larger than found for the native cytochrome *c*. Although the dead time of these experiments is 30 s and therefore the numbers for the fast exchanging components should be interpreted with some care, it is interesting to note

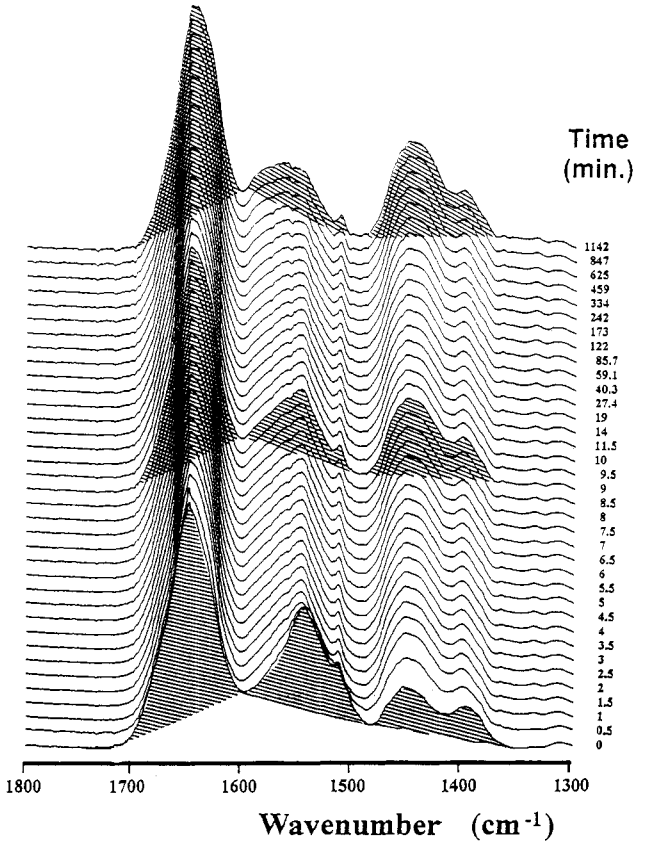

FIGURE 3: A typical experiment of a proton–deuterium exchange experiments of a film of 100 μg of native cytochrome *c* as detected by IR spectroscopy. The hatched areas correspond to the amide II (1600–1496 cm^{-1}) and amide II′ (1486–1350 cm^{-1}) regions, which are used to quantify the fraction of exchanged amide protons, after normalization to the integral of amide I (1704–1600 cm^{-1}).

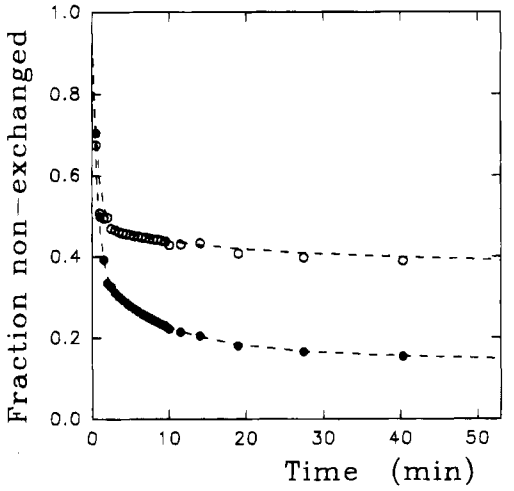

FIGURE 4: Monitoring the exchange of the amide protons of native (open circles) and methionine-80 modified (solid circles) cytochrome *c* (30 mg/mL) with a ^2H_2O solution containing 10 mM phosphate (p^{2}H 7.0) as detected by monitoring the intensity of amide II normalized to amide I from IR spectra as a function of time. The dashed lines represent the three-exponential decay fits of the data.

that these exchange rates are apparently not affected by the modification.

Amide-Proton Exchange on Films Detected by ATR-IR. Because of the relatively small quantities required and the additional advantage of the accessibility to information on the orientations of both lipids and protein secondary structure

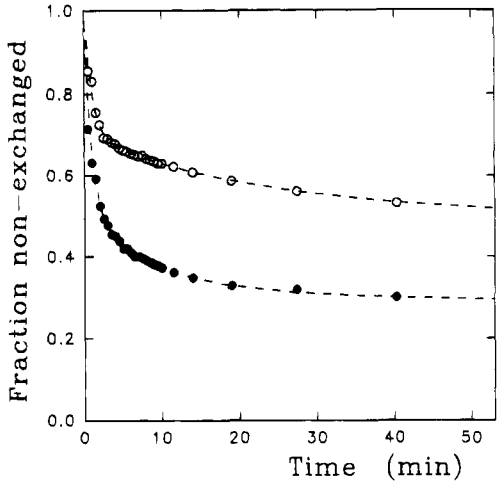

FIGURE 5: Monitoring the exchange of the amide protons of native (open circles) and methionine-80 modified (solid circles) cytochrome *c* (100 μg), spread on a germanium crystal from a 10 mM phosphate buffer (pH 7.0) and flushed with ^2H_2O-saturated N_2 gas, as detected by the intensity of amide II normalized to amide I from ATR-IR spectra as a function of time. The dashed lines represent the three-exponential decay fits of the data.

in membrane systems, ATR-IR measurements are more commonly performed to study films of the materials of interest (Goormaghtigh & Ruysschaert, 1990; Goormaghtigh et al., 1990). To investigate how amide-proton exchange in a hydrated film flushed with ^2H_2O-saturated N_2 gas relates to that observed in aqueous solution and whether also in films influences of tertiary stability can be detected by monitoring amide-proton exchange, we measured the exchange kinetics of both native and modified cytochrome *c* spread on a germanium crystal (Figure 5). From comparison of the exchange curves in films and in aqueous solution (Figures 5 and 4) it can be observed that in films the decay is slower than in aqueous solution. This is also demonstrated by the exchange rates determined by analysis of the curves (Table 3). With the exception of the intermediate rate constant of the modified protein, all rate constants are approximately 2 times smaller in films compared to those in aqueous solution. From Table 3 it is clear that also in films the exchange rate constants for the slow and intermediate component of the modified protein are 2−3 times larger than those of native cytochrome *c*. Again the rate constants of the fast component are not affected by tertiary (de)stabilization.

DISCUSSION

The chemical modification at methionine-80 of cytochrome *c* prohibits the ligation of this residue to the heme and results in a reduction of the tertiary stability of approximately 50% (Table 1), whereas the secondary structure is not affected (Table 2). Such a tertiary destabilized folding state is believed to be of biological relevance based on the observations that comparable folding states have been described in the presence of micelles (de Jongh et al., 1992) or negatively charged vesicles (unpublished results). A lipid-specific tertiary destabilization of cytochrome *c* at a membrane interface has also been reported previously (Muga et al., 1991; Spooner & Watts, 1991; Heimburg & Marsh, 1993). A partially tertiary unfolding could facilitate the transfer of electrons from and to its membrane-bound donor and acceptor proteins in the respiratory chain of mitochondria.

By monitoring the amide-proton exchange rates using ^{1}H-NMR or IR, it is shown that the modification of cytochrome *c* increases the exchange rates 2−3 times of those amides which are restricted in their exchange by protection of folding properties (Table 3). The comparable rate constants obtained for the fast component found for both the native and modified protein indicate that this population represents amides which are surface exposed or present in highly mobile regions of the protein, because the exchange rate is then expected to be insensitive to tertiary stabilization.

Comparison of the populations and corresponding exchange rate constants of native and modified cytochrome *c* in ^2H_2O solution, as determined by ^{1}H-NMR and IR spectroscopy (Table 3), shows that the populations correlate within 5%, whereas the rate constants vary within 20% for the two techniques. This good agreement justifies the use of amide II intensities to monitor amide-proton exchange with a solvent using IR spectroscopy.

However, in films the exchange rate constants are apparently 2 times smaller compared to those found in solution (Table 3). This result is somewhat surprising in view of the fact that, as in our case (see Materials and Methods section), above 0.15−0.4 g of water/g of protein the proton−deuterium exchange is not sensitive to the amount of water present (Schinkel et al., 1985) except for some amino acids involved in crystal lattice contacts (Gallagher et al., 1992). Since the use of films offers many advantages, we investigated in more detail the possible causes of this discrepancy between the solution and film IR data.

Increasing the ^2H_2O-saturated nitrogen flow rate up to 75 mL/min had no influence on the exchange rates of the protein films. However, decreasing the flow rate below 5 mL/min resulted in slower exchange rates (data not shown). Thus, under the conditions used the availability of ^2H_2O was not rate-limiting for the exchange. Moreover, varying the amount of material (from 10 to 150 μg) spread on the germanium crystal had no influence on the kinetics measured (data not shown). This indicates that in our measurements the thickness of the film does not influence the ^2H_2O diffusion in the film. From experiments where films were prepared from samples with different concentrations of buffer it appears that the ionic strength does not significantly affect the exchange rates (data not shown). Since the exchange rate is strongly pH-dependent, we investigated a possible pH shift upon preparation of a hydrated film. The IR bands at 1580 (COO^-) and 1715 (COOH) cm^{-1} of citric acid can be used to report the pH as shown in Figure 6A. Figure 6B shows the ratio of these two bands as a function of the pH for both citric acid in aqueous solution and in a film. Apparently, no shift of the pH can be observed upon preparation of a film, and therefore we conclude that the slower exchange observed in films of native and modified cytochrome *c* cannot be attributed to a different pH compared to the situation in aqueous solution.

A potential explanation for the discrepancy between the solution and film IR data lies in the evaluation of the area of both amide I and amide II in films before deuteration. In particular, overestimation of amide I before deuteration would result in apparent slower exchange kinetics. Nonremoved H_2O associated to the protein and buffer materials upon preparation of a film gives rise to an IR band [δ(O-H)] in the amide I region. This H_2O will exchange rapidly upon deuteration as can be seen from the reduction of the

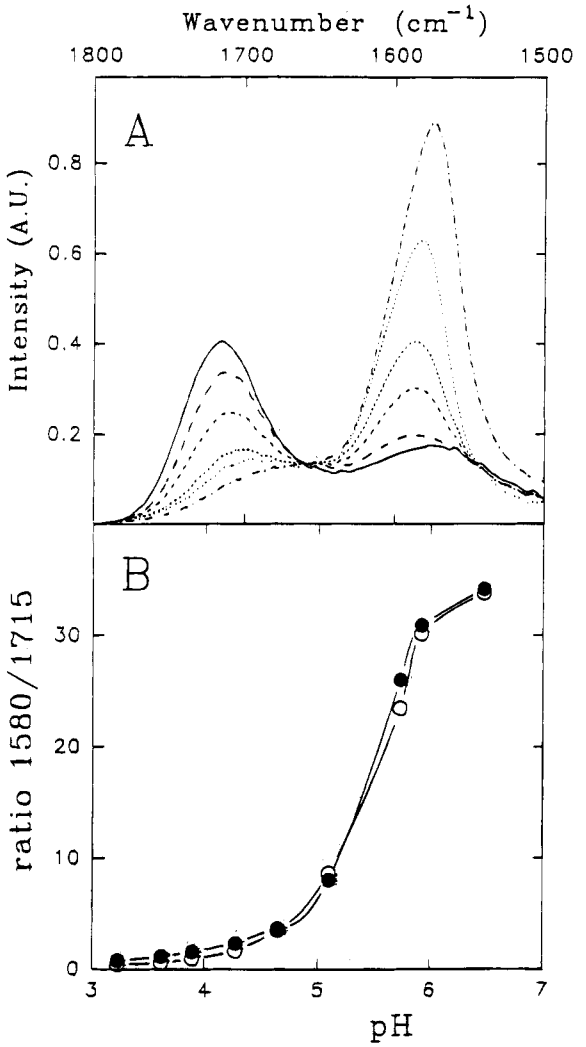

FIGURE 6: pH titration of citric acid in solution and in films. (A) ATR spectra of the COOH (1715 cm^{-1}) and COO$^-$ (1580 cm^{-1}) IR vibrations of 100 μg of citric acid spread on a germanium crystal: (—) = pH 3.22; (— —) = pH 3.61; (— –) = pH 4.27; (- - -) = pH 4.68; (•••) = pH 5.09; and (— • —) = pH 5.73. (B) The ratio of COO$^-$/COOH intensities of 50 mg/mL citric acid in solution using a 6 μm cell (closed circles) and of 100 μg of citric acid spread on a germanium crystal (open circles) is plotted as a function of the pH of the aqueous solution.

water ν(O-H) intensity in the 3500−3000 cm^{-1} region. Using the extinction coefficients of ν(O-H) and δ(O-H) of H_2O, the nonremoved H_2O contribution to the amide I might give rise to an overestimation of maximally 5−10% for the fractions and exchange rates in films. Also, the optical properties of amide I (i.e., extinction coefficient, reflectivity of the film, etc.) might change when going from a poorly to a fully hydrated state, as suggested by Gerothanassis and Vakka (1994) and by the 1.5 times higher amide II/I ratio found in films compared to aqueous solution (see Materials and Methods section). When monitoring the intensity of amide I upon hydration of a film with H_2O, and correcting this intensity for the H_2O contribution using the known extinction coefficients (Fringeli & Gunthard, 1980), we could not detect any significant change in the amide I intensity due to the hydration. The intensity of amide II, however, was found to be reduced by approximately 15% in the fully hydrated film. This would result in an underestimation of the film data as presented in Table 3 by 10−15%. Finally, we cannot rule out the potential influence of protein−protein interactions in films and the concomitant reduction of the freely accessible protein surface or that the tertiary stability of cytochrome *c* is increased upon preparation of films.

Conclusively, we can state that, by monitoring on-line the exchange of amide protons with a ^2H_2O solvent using IR spectroscopy, differences in tertiary structure can be accurately detected and that the results can directly be compared to those obtained using ^{1}H-NMR. Also in films tertiary stabilization determines the exchange rate in a comparable way as in aqueous solution. The applicability of (ATR-)IR to study membrane systems, where additional information on the orientation of lipids and polypeptides is available, makes monitoring on-line amide-proton exchange a promising approach for the conformational characterization of membrane proteins.

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