Acid-Induced Unfolding of Cytochrome *c* at Different Methanol Concentrations: Electrospray Ionization Mass Spectrometry Specifically Monitors Changes in the Tertiary Structure†

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ABSTRACT: The acid-induced denaturation of ferricytochrome *c* (cyt *c*) was examined in aqueous solutions containing different concentrations of methanol by electrospray ionization mass spectrometry (ESI MS) and optical spectroscopy. Circular dichroism, fluorescence, and absorption spectroscopy show that at a low concentration of methanol (3%) a decrease in pH induces a cooperative unfolding transition at around pH 2.6 that is accompanied by a breakdown of the native secondary and tertiary structure of the protein. In 50% methanol the breakdown of the tertiary structure occurs at around pH 4.0, whereas the α-helical content remains largely intact over the whole pH range studied. In ESI MS different protein conformations in solution are monitored by the different charge state distributions they generate during ESI. The ESI mass spectra recorded at near-neutral pH for both methanol concentrations are very similar and show a maximum at (cyt $c + 8H^+)^{8+}$. Despite the different conformations of the protein in solution, the acid-denatured states for the two methanol concentrations also show very similar mass spectra with a maximum at (cyt $c + 17H^+)^{17+}$. This indicates that the charge state distribution generated during ESI is not sensitive to the differences in the secondary structure of the denatured protein. The observed transition from low to high charge states is due to the breakdown of the tertiary structure in both cases. These findings suggest that ESI MS might be a general method to selectively monitor changes in the tertiary structure of proteins.

Electrospray ionization mass spectrometry (ESI MS)[1] is a relatively new method to study conformational changes of proteins in solution. During the ESI process, positively charged ions are formed from proteins in solution by proton attachment (Smith et al., 1990; Loo, 1995; Przybylski & Glocker, 1996). Generally an unfolded protein shows higher charge states in its ESI mass spectrum than the same protein in the native state (Mirza et al., 1993; Wagner & Anderegg, 1994; Konermann et al., 1997a,c; Konermann & Douglas, 1997). The physical basis for this relationship is still not fully understood. Several studies ascribe the observed effects to changes in the steric accessibility of possible protonation sites and to changes in their specific pK_a values (Chowdhury et al., 1990; Katta et al., 1991; Loo et al., 1990). A different model proposes that the high charge states observed for denatured proteins are mainly due to the increased surface area of the unfolded polypeptide chain (Fenn, 1993).

Despite the widespread use of ESI MS to monitor conformational changes of proteins, it is still not established which alterations of the protein structure in solution give rise to the observed changes in the charge state distribution. For example, the distribution could be sensitive to changes in the secondary structure, the tertiary structure, or a combination of both. In this study we attempt to clarify whether the charge state distribution generated during ESI can be used to monitor specific structural aspects of proteins in solution. For this, the acid-induced denaturation of cyt *c* under different solvent conditions is studied by optical spectroscopy and the results obtained by these techniques are compared to the changes observed in the respective ESI mass spectra. In aqueous solutions of low ionic strength, cyt *c* is in the native state at neutral pH. Acidification of the solution induces a cooperative unfolding transition at around pH 2.5 (Theorell & Åkesson, 1941; Knapp & Pace, 1971; Babul & Stellwagen, 1972; Moore & Pettigrew, 1990). Under conditions of moderately low pH the transition to a molten globule-like state can be induced by the addition of methanol (Bychkova et al., 1996).

Cyt *c* was chosen for the experiments carried out in this study because it offers a large number of optical probes which allow a detailed characterization of the protein structure in solution. Optical absorption spectroscopy can be used to monitor changes in the ligation and spin state of the heme iron and hence in the tertiary structure around the heme iron. In the native state the heme iron is ligated by His18 and Met80 (Babul & Stellwagen, 1972; Stellwagen & Babul, 1975; Bushnell et al., 1990). Coordination of the iron with these strong-field protein ligands produces a low-spin complex having a Soret absorption maximum between 400 and 410 nm (Babul & Stellwagen, 1971, 1972; Drew & Dickerson, 1978). The coordination bond between iron and Met80 leads to the appearance of a weak charge transfer band in the absorption spectrum at 695 nm. (Kaminsky et al., 1973; Drew & Dickerson, 1978; Sosnick et al., 1996). Unfolding of the protein leads to the displacement of both

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[1] Abbreviations: 8^+, 9^+, etc., charge states of protonated cytochrome *c* ions in the gas phase [(cyt $c + 8H)^{8+}$, (cyt $c + 9H)^{9+}$, etc.]; CD, circular dichroism; cyt *c*, horse heart ferricytochrome *c*; ESI, electrospray ionization; MS, mass spectrometry; UV, ultraviolet.

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protein ligands from the heme iron which are consequently replaced by two weak-field ligands from the solvent such as water or chloride. This produces a high-spin complex with a Soret absorption maximum between 390 and 395 nm and with a characteristic absorption band at 620 nm (Babul & Stellwagen, 1972; Drew & Dickerson, 1978; Robinson et al., 1983). Absorption spectroscopy can thus be used to monitor the accessibility of the heme group to the solvent.

The fluorescence of the single tryptophan residue (Trp59) is a sensitive probe for protein unfolding. In the native state the Trp fluorescence is largely quenched due to resonant energy transfer to the adjacent heme group. A breakdown of the tertiary structure leads to an increased distance between heme and Trp59, which causes a decrease in the quenching efficiency and thus an increase in Trp fluorescence (Tsong, 1975, 1976; Elöve et al., 1994; Bychkova et al., 1996; Sosnick et al., 1996). The near-UV CD spectrum monitors changes in the tertiary structure around Trp59 (Davies et al., 1993; Bychkova et al., 1996), whereas the far-UV CD spectrum monitors changes in the secondary structure of the protein (Greenfield & Fasman, 1969; Brahms & Brahms, 1980; Johnson, 1990).

In this work the acid-induced denaturation of cyt *c* is studied in aqueous solutions containing 3% and 50% methanol. A comparison of the results obtained by optical spectroscopy and those obtained by ESI MS indicates that the charge state distribution generated during ESI selectively monitors changes in the tertiary structure of cyt *c*.

EXPERIMENTAL PROCEDURES

Chemicals. Horse heart cyt *c* and Trp were purchased from Sigma (St. Louis, MO). HPLC-grade methanol, hydrochloric acid, and ammonium acetate were obtained from Fisher Scientific (Nepean, ON). Prior to measurements the protein solutions were extensively dialyzed against water containing 1 mM ammonium acetate. For ESI MS and optical spectroscopy the dialyzed solutions were diluted to a concentration of 10 mM in protein and 0.5 mM in ammonium acetate. The pH of the solutions was adjusted by addition of hydrochloric acid and measured with a calibrated accumet pH electrode (Model 15, Fisher Scientific).

ESI MS measurements were carried out as described previously on a quadrupole mass spectrometer constructed in house (Konermann et al., 1997a; Konermann & Douglas, 1997). The ESI source was operated at +4600 V and at a flow rate of 5 mL/min. All ESI MS experiments were carried out at room temperature (21 ± 2 °C). The signal stability and intensity for ESI MS were significantly increased by adding a small amount of methanol (3%) to the aqueous solution of cyt *c*. This effect has been observed previously (Przybylski & Glocker, 1996) and might be due to a decrease in the surface tension of the aqueous solution (Kebarle & Tang, 1993). The average charge state $\langle k \rangle$ of an ESI mass spectrum was calculated according to

$$\langle k \rangle = \frac{\sum_k I_k k}{\sum_k I_k} \qquad (1)$$

where the values of k are integer numbers denoting the charge states of protein ions corresponding to peaks in the mass spectrum and I_k are the intensities of the respective peaks (ions per second).

Optical Spectroscopy. Absorption spectra were recorded on a Cary 3E absorption spectrophotometer. The absorption values reported for the 695 nm band were corrected for contributions of lower wavelengths by the method of Kaminsky et al. (1973). Fluorescence measurements were made on a Perkin-Elmer LS-5E fluorometer using an excitation wavelength of 280 nm. The fluorescence intensity for cyt *c* under different solvent conditions was monitored at the maximum of the emission spectrum (around 340 nm) with a spectral bandwidth of 5 nm for excitation and emission. The fluorescence intensities for cyt *c* reported in this study are relative to the fluorescence intensity of free Trp under the same solvent conditions as in previous studies on the acid-induced denaturation of cyt *c* (Tsong, 1975, 1976; Myer et al., 1980). CD spectra were measured on a Jasco J-720 spectropolarimeter. The measured ellipticities were normalized as described by Bychkova et al. (1996).

RESULTS

HCl-Induced Denaturation in Water Containing 3% Methanol. ESI mass spectra of cyt *c* recorded between pH 6.4 and 2.3 are depicted in Figure 1. At pH 6.4 the protein shows a relatively narrow distribution of charge states with 8^+ being the most intense peak (Figure 1A). Lowering the pH to 4.2 slightly shifts the maximum of the charge state distribution to 9^+ (Figure 1B). At still lower pH the formation of cyt *c* ions in much higher charge states leads to the formation of a bimodal charge state distribution. At pH 2.6 the two parts of this distribution have about the same intensity; the maximum of the high charge states is at 17^+ whereas that of the low charge states remains at 9^+. Eventually, below pH 2.3 the high charge states dominate the ESI mass spectrum and the contribution of cyt *c* ions in low charge states becomes negligible (Figure 1D). These data are similar to previous measurements for the acetic acid-induced denaturation of cyt *c* and reflect the cooperative transition from the tightly folded, native state of the protein to the acid-unfolded conformation (Chowdhury et al., 1990; Li et al., 1993; Wagner & Anderegg, 1994; Hamdan & Curcuruto, 1994; Konermann et al., 1997a; Konermann & Douglas, 1997).

In Figure 2 the titration curves for cyt *c* obtained by ESI MS and several optical methods are compared. All the different probes show a pronounced transition at roughly pH 2.6. Figure 2A depicts the average charge state of the ESI mass spectrum as a function of pH. The native protein in the near-neutral range shows an average charge state of about 8.0. The shift in the maximum of the ESI mass spectrum from 8^+ to 9^+ leads to a slight increase in the average charge state at around pH 4.0. Unfolding of the protein at roughly pH 2.6 leads to a sharp increase to an average charge state of around 17.0. The transition observed for the CD signal at 289 nm (Figure 2B) indicates the breakdown of the well-ordered tertiary structure around Trp59. The sharp increase in Trp fluorescence (Figure 2C) shows that the efficient resonance energy transfer between Trp59 and the heme group becomes strongly perturbed. However, even at around pH 2.0 the fluorescence intensity reaches only about 70% of the fluorescence intensity measured for free Trp under the same

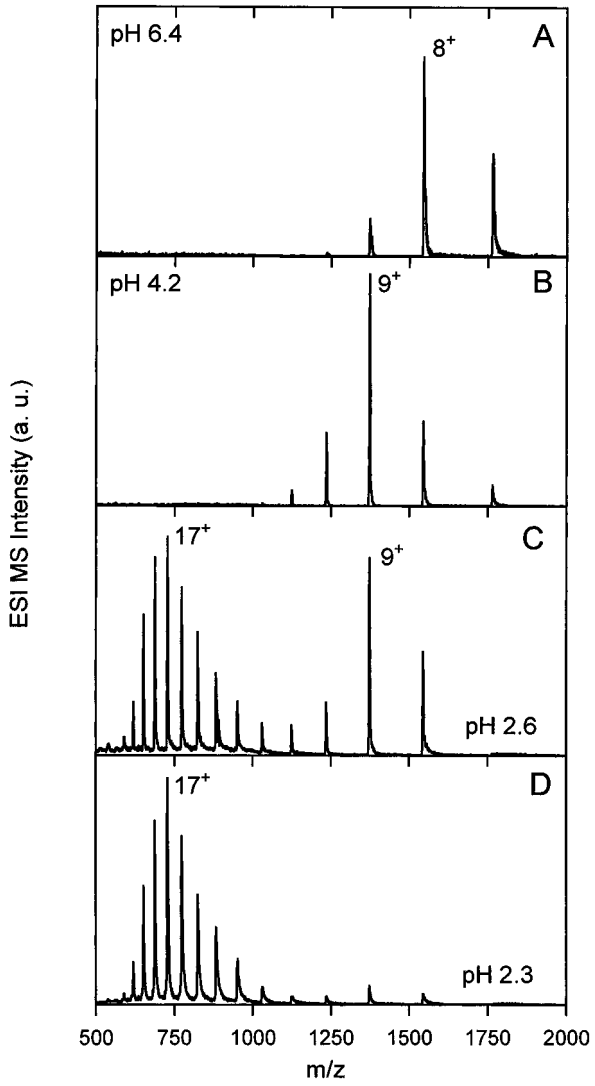

FIGURE 1: ESI mass spectra of cyt *c* in water containing 3% methanol and 0.5 mM ammonium acetate at (A) pH 6.4, (B) pH 4.2, (C) pH 2.6, and (D) pH 2.3.

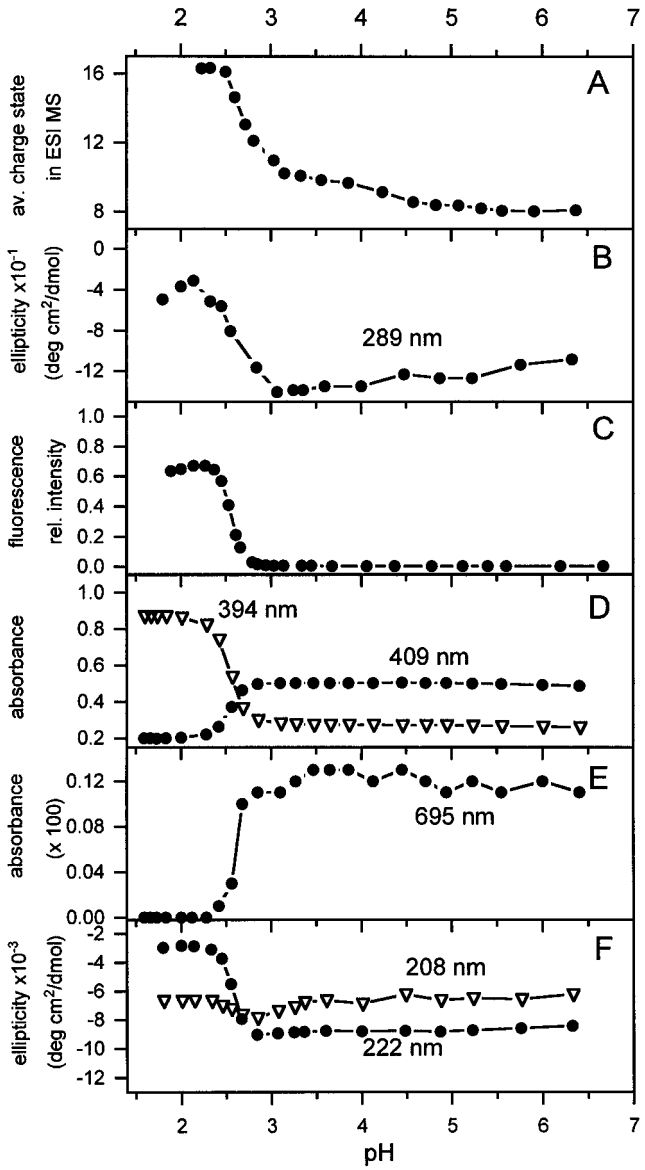

FIGURE 2: Acid-induced denaturation of cyt *c* in water containing 3% methanol and 0.5 mM ammonium acetate studied by various probes: (A) average charge state calculated from ESI mass spectra measured at different pH, (B) CD signal at 289 nm, (C) relative intensity of Trp fluorescence, (D) Soret absorption monitored at 394 and 409 nm, (E) absorption monitored at 695 nm, and (F) CD signal at 208 and 222 nm.

solvent conditions. Figure 2D shows the changes in the Soret region of the heme absorption as a function of pH. The acid-induced denaturation of the protein is accompanied by a change in the absorption maximum from 409 to 394 nm, indicating a transition from the low-spin state of the heme iron to the high-spin state. The change in the spin state is also accompanied by the appearance of an absorption band at 620 nm below pH 2.6 (data not shown). The disruption of the iron−Met80 coordination bond can be directly observed by the disappearance of the absorption band at 695 nm (Figure 2E). Changes in the far-UV CD are depicted in Figure 2F for two different wavelengths. The loss of ellipticity at 222 nm indicates the breakdown of the intact secondary structure. The CD signal measured at 208 nm stays fairly constant throughout the whole pH range, showing only a slight dip at around pH 2.9. The far-UV CD spectra for the native and for the acid-unfolded form of cyt *c* are depicted in Figure 3. The native state at pH 6.6 shows two pronounced minima at around 208 and 222 nm. The spectrum recorded at pH 2.0 shows a strong negative band at around 200 nm, whereas the ellipticity at 222 nm is greatly diminished.

HCl-Induced Denaturation in Water Containing 50% Methanol. The acid-induced unfolding of cyt *c* in an aqueous solution containing 50% methanol shows different behavior. ESI mass spectra measured at different pH are depicted in Figure 4. Note that the mass spectra measured in the near-neutral pH range for 3% and in 50% methanol are very similar (compare Figures 1A and 4A). The same holds for the spectra measured in the acidic range (Figures 1D and 4D). However, the transition from low to high charge states now occurs at about pH 3.8 The maximum of the low charge states undergoes a gradual shift from 8^+ at pH 6.4 to 10^+ at pH 3.8. The high charge states, attributed to a denatured conformation of the protein, also show a shift with decreasing pH: At pH 3.8 the most intense peak is around 14^+ and at pH 2.2 it is at 17^+. Even though the transition region is characterized by a bimodal charge state distribution, the observed gradual shifts in the maxima of the charge state distribution indicate that the unfolding of cyt *c* under these solvent conditions is not a simple two-state process (Konermann & Douglas, 1997). This conclusion is further

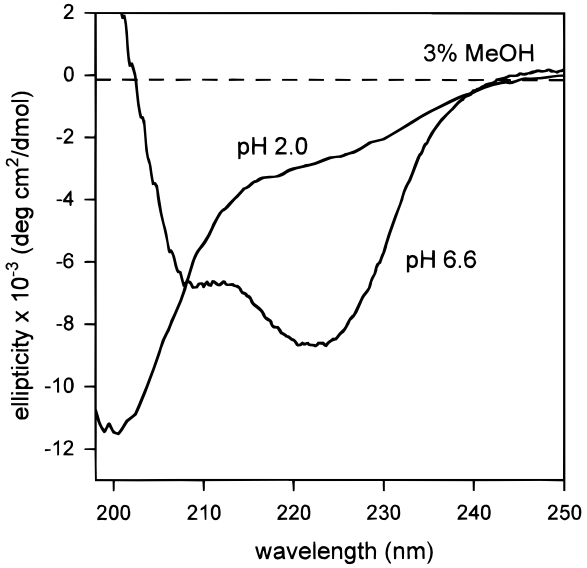

FIGURE 3: Far-UV region of the CD spectra of cyt *c* in water containing 3% methanol and 0.5 mM ammonium acetate at pH 2.0 and 6.6.

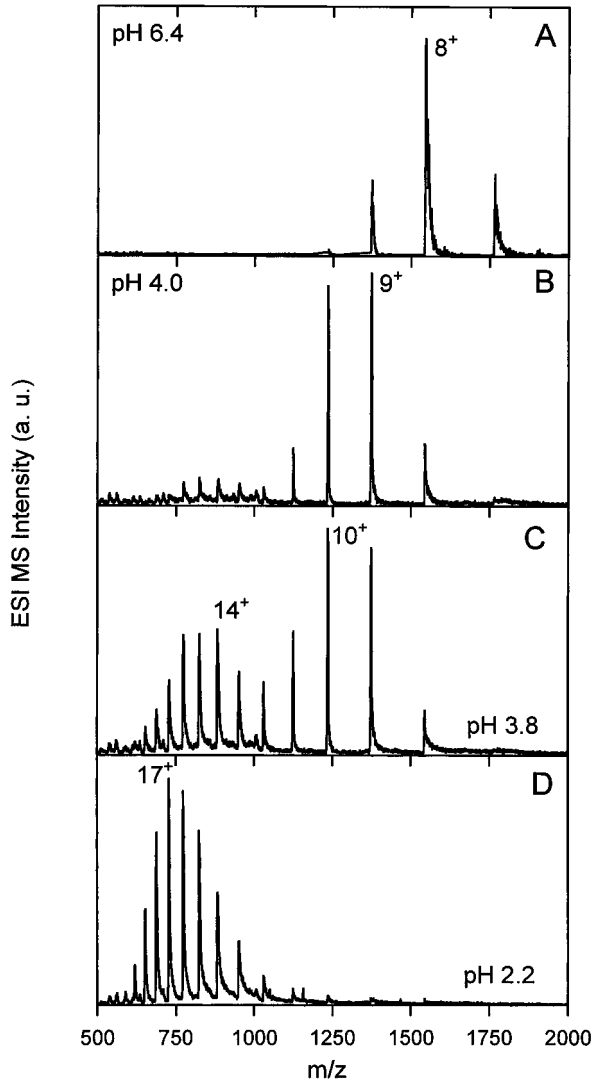

FIGURE 4: ESI mass spectra of cyt *c* in water containing 50% methanol and 0.5 mM ammonium acetate at (A) pH 6.4, (B) pH 4.0, (C) pH 3.8, and (D) pH 2.2.

supported by optical spectroscopy (see below). The average charge state of the ESI mass spectra as a function of pH is

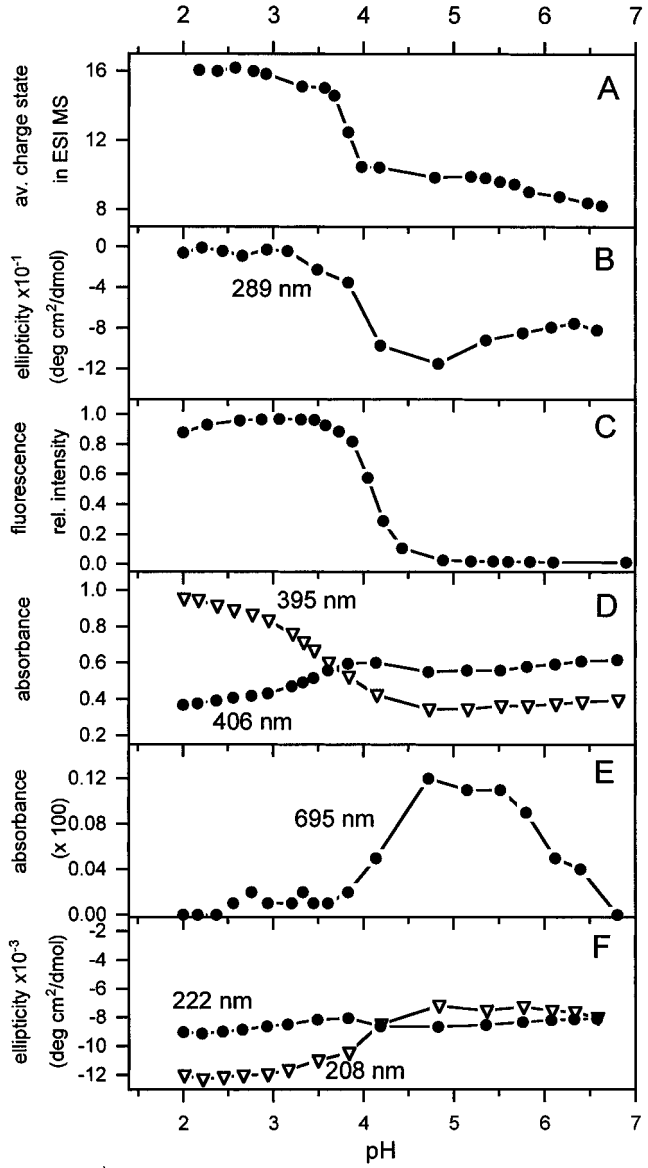

FIGURE 5: Acid-induced denaturation of cyt *c* in water containing 50% methanol and 0.5 mM ammonium acetate studied by various probes [see caption to Figure 2, except (D) Soret absorption monitored at 395 and 406 nm]. The curves depicted here are scaled equally to the ones shown in Figure 2.

depicted in Figure 5A. It shows an increase from about 8.0 to 17.0 with a sharp transition around pH 3.8. The transitions observed in the CD signal measured at 289 nm (Figure 5B) and in the fluorescence intensity of Trp59 (Figure 5C) occur at roughly the same pH as the transition observed for the average charge state. At around pH 2.0 the single Trp in cyt *c* shows a fluorescence intensity almost as strong as the fluorescence intensity of free Trp in the same solvent. In 50% methanol the Soret region of the heme shows a maximum at 406 nm for pH 6.6 and a maximum at 395 nm for pH 2.0. Again, these changes indicate that the heme iron undergoes a transition from a low-spin to a high-spin complex. This is further confirmed by the appearance of an absorption band at 620 nm which occurs together with the band at 395 nm (data not shown). The changes in the absorption spectrum of the heme show a more gradual transition compared to the other probes for unfolding (Figure 5A−C) and compared to the absorption data of Figure 2D. The midpoint of these absorption changes is shifted to around

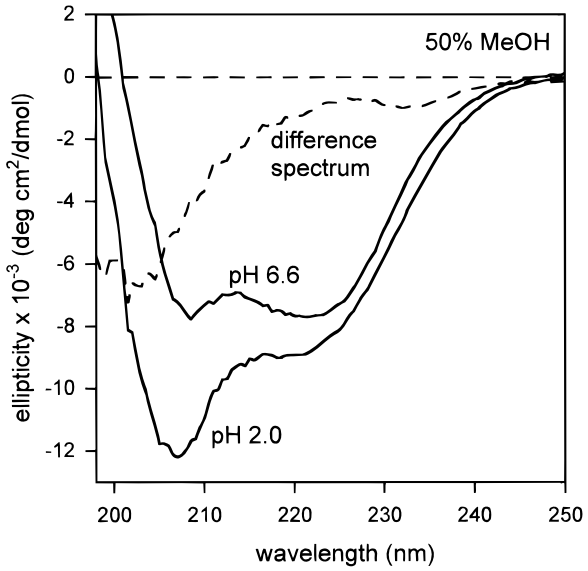

FIGURE 6: Far-UV region of the CD spectra of cyt *c* in water containing 50% methanol and 0.5 mM ammonium acetate at pH 2.0 and 6.6 (solid lines). Also shown is the difference spectrum between the two curves (dashed line).

pH 3.2. The pH dependence of the 695 nm absorption band (Figure 5E) shows that at pH 6.6 the heme iron is not ligated by Met80, as observed previously (Drew & Dickerson, 1978). Interestingly, the coordination bond between iron and methionine can be restored by slightly decreasing the pH to around 5.0 as can be seen by the increase in the absorption at 695 nm. Further acidification of the solution again leads to a disruption of the coordination bond. The accompanying absorption change shows a midpoint at around pH 4.3. Figure 5F shows the changes in the CD signal measured at 208 and 222 nm. The ellipticity at 222 nm shows only relatively small changes, from −8000 at pH 6.6 to −9100 deg cm^2 dmol^{-1} at pH 2.0. This indicates that acidification of the aqueous solution containing 50% methanol does not have a very pronounced effect on the overall α-helicity of the protein. In contrast the ellipticity measured at 208 nm shows a pronounced transition to more negative values at a pH which coincides approximately with that of the transitions shown in Figure 5A−C. The far-UV CD spectra of cyt *c* in 50% methanol at pH 6.6 and 2.0 and the difference between these two spectra are shown in Figure 6.

DISCUSSION

Unfolding in 3% Methanol Monitored by Optical Spectroscopy. For the acid-induced denaturation of cyt *c* in a solution containing 3% methanol the transition for all the different optical probes is observed at around pH 2.6. The simultaneous occurrence of these transitions indicates that the unfolding can be described as a cooperative, two-state process that leads to a breakdown of both the protein secondary and tertiary structure. This observation is in accordance with earlier studies on the acid-induced unfolding of cyt *c* in aqueous solutions at low ionic strength in the absence of methanol (Knapp & Pace, 1971; Babul & Stellwagen, 1972; Stellwagen & Babul, 1975; Robinson et al., 1983; Moore & Pettigrew, 1990).

The far-UV CD spectrum recorded in 3% methanol at pH 6.6 is very similar to spectra of native cyt *c* recorded previously in aqueous solutions without methanol (Hennessey & Johnson, 1981; Bychkova et al., 1996). The two distinct bands at around 208 and 222 nm are indicative of the high degree of helicity in the secondary structure (Greenfield & Fasman, 1969; Brahms & Brahms, 1980; Hennessey & Johnson, 1981). A detailed analysis of this spectrum reveals an α-helical content of about 40%, consistent with the X-ray structure of cyt *c* (Hennessey & Johnson, 1981; Bushnell et al., 1990). At pH 2.0 the CD spectrum shows a pronounced negatively dichroic band at around 200 nm, which is typical for proteins in a random coil structure (Greenfield & Fasman, 1969; Brahms & Brahms, 1980; Hennessey & Johnson, 1981). The ellipticity at 208 nm stays fairly constant throughout the whole pH range, because the CD spectra of the native and of the acid-unfolded protein happen to show the same ellipticity at around 208 nm (see Figure 3) [note that this is not an isodichroic point throughout the whole pH range studied]. Thus the ellipticity monitored at 208 nm cannot be used to monitor changes in the helicity of the protein as a function of pH. It has been shown previously that the CD signal measured at 222 nm is a more selective probe for the helicity because interferences caused by other secondary structure elements are relatively weak at this wavelength (Chen et al., 1972). The observed transition of the CD signal at 222 nm indicates the breakdown of the helical structure in the protein at around pH 2.6.

Unfolding in 50% Methanol Monitored by Optical Spectroscopy. The unfolding of cyt c in 50% methanol can not be described as a simple two-state transition. At pH 6.6 the protein does not adopt a fully native conformation. The heme iron is not ligated by Met80, while the Soret absorption spectrum shows that the heme group is still in the low-spin state. This indicates that Met80 is replaced by another protein ligand (Babul & Stellwagen, 1972; Stellwagen & Babul, 1975; Drew & Dickerson, 1978; Dyson & Beattie, 1982; Elöve et al., 1994). A slight acidification of the solution to around pH 5.0 restores the native ligation of the heme iron with Met80. Thus under these conditions (pH 5.0, 50% methanol) the structure of cyt *c* appears to be similar to the native conformation at near-neutral pH in 3% methanol. The near-UV CD spectrum and the Trp fluorescence show that further acidification of the protein in 50% methanol leads to a breakdown of the tertiary structure. Also, the coordination bond between Met80 and the heme iron becomes disrupted. These conformational changes occur at around pH 4.0 and are thus shifted to higher pH compared to those of cyt *c* in 3% methanol. This shows that the tertiary structure of cyt *c* is significantly destabilized by the increased methanol concentration. The accessibility of the heme group for solvent molecules—as monitored by changes in the Soret absorption—increases gradually and this transition is clearly shifted to lower pH compared to the other changes in the tertiary structure.

These optical probes indicate that the tertiary structure of the protein is completely lost at around pH 2.0. However, the CD signal monitored at 222 nm stays roughly constant, indicating only minor changes in the helicity over the whole pH range. This is in line with previous observations made for cyt *c* (Bychkova et al., 1996) and other proteins (Harding et al., 1991; Buck et al., 1993; Dufour et al., 1993; Fan et al., 1993; Alexandrescu et al., 1994; Shiraki et al., 1995; Narhi et al., 1996), which show that alcohols can have a stabilizing effect on α-helices or can even induce a higher helicity than in the native state. The CD difference spectrum

(Figure 6) between the native and the acid state in 50% methanol exhibits typical features of a random coil structure (Greenfield & Fasman, 1969; Brahms & Brahms, 1980; Hennessey & Johnson, 1981). These data imply that a considerable portion of the polypeptide chain adopts a more disordered conformation than at near-neutral pH, while the α-helical content in the denatured protein is about the same as in the native state. The former leads to the transition in the 208 nm CD signal at around pH 4.0 that roughly coincides with the breakdown of the tertiary structure monitored by near-UV CD and Trp fluorescence. On the basis of the data presented here, however, it is not possible to decide whether the specific helices found in the native conformation are retained after denaturation.

These data show that cyt *c* in 50% methanol at around pH 2.0 has a pronounced secondary structure but a strongly reduced tertiary structure. Measurements carried out under similar solvent conditions showed that this form of the protein is relatively compact since it has a Stokes radius between that of the native and that of the acid-unfolded state in the absence of methanol (Bychkova et al., 1996). These three features (compactness, pronounced secondary structure, and strongly reduced tertiary structure) are characteristic for the "molten globule" state (Ptitsyn, 1992). Interestingly, the fluorescence intensity of cyt *c* in 50% methanol at pH 2.0 almost reaches the level of free Trp in the same solvent, whereas at the same pH in 3% methanol a relative fluorescence intensity of only about 70% was observed. The latter is consistent with data reported previously (Robinson et al., 1983). The fluorescence intensity is often regarded as a measure for the distance between heme and Trp59 and therefore this observation seems to be inconsistent with the Stokes radii measured by Bychkova et al. (1996). However, the rate for resonance energy transfer is also determined by the relative orientation of the donor and acceptor transition dipoles [Konermann et al. (1997b) and references therein]. Thus the higher fluorescence intensity observed for the acid-denatured state in 50% methanol may be due to an unfavorable orientation of the transition dipoles rather than a larger distance between heme and Trp59. The observed weaker fluorescence intensity of the acid-denatured state in 3% methanol might also be caused by an increased exposure of Trp59 to the polar solvent, which also acts as a quencher (Lakowicz, 1983).

Comparison of the Results from Optical Spectroscopy and ESI MS. Electrolytic oxidation of water can lead to changes in the pH during ESI. This might complicate the relationship between the charge state distribution observed in ESI MS and the protein conformation in solution (Chowdhury et al., 1990; van Berkel & Zhou, 1995; van Berkel et al., 1997). However, this work and previous studies (Konermann et al., 1997a; Konermann & Douglas, 1997) indicate that, for the experimental conditions here, such electrolytic processes are negligible since the major folding transitions observed by ESI MS are accompanied by almost simultaneous transitions in other probes for the protein structure.

It remains unclear whether some of the observed minor changes in the charge state distribution outside the transition region are also due to conformational changes of the protein or whether they are caused by "secondary" solvent effects. For example, a higher acidity of the solvent might induce a slight increase in the observed charge states even when the protein conformation does not change. The shift in the maximum from 8^+ to 9^+ in 3% methanol (Figure 1A,B) could be due to such a secondary effect. On the other hand, the shift from 8^+ to 10^+ in the maximum of the charge state distribution observed in 50% methanol (Figure 4A−C) might be related to the conformational transition that causes the changes of the iron ligation in this pH range. Similarly, the shift from 14^+ to 17^+ at lower pH (Figure 4C,D) is most likely related to the gradual increase in solvent accessibility to the heme iron, which is monitored by the changes in the Soret absorption. It could be partly caused by an increased availability of His18 for protonation after disruption of the coordination bond to the heme iron.

It would be most interesting to correlate the pH-induced changes in the charge state distribution for different solvents with the number of solvent-accesible basic amino acid residues (Chowdhury et al., 1990; Loo et al., 1990; Katta & Chait, 1991). Unfortunately, at the present state, any attempts to make such correlations would be highly speculative due to the limited information on the detailed structure of the acid-denatured states in 3% and in 50% methanol, respectively.

The acid-induced unfolding of cyt *c* at different concentrations of methanol results in the formation of two different protein conformations. In 3% methanol the denaturation leads to a substantial breakdown of both the native secondary and tertiary structure. In contrast, denaturation in 50% methanol results in the breakdown of only the tertiary structure while the protein retains a high degree of secondary structure. Despite their pronounced differences in secondary structure, the acid-denatured states for the two methanol concentrations show a very similar charge state distribution. In both cases the transition observed between the low and the high charge states is observed at the pH where the tertiary structure breaks down. These observations indicate that the charge state distribution generated during ESI is not sensitive to changes in the secondary structure of the protein. The transition from low to high charge states is apparently brought about by changes in the *tertiary* structure. A similar observation was made by Pan et al. (1997), who showed that ESI MS can monitor an acid-induced conformational change in RNase A that is accompanied by the loss of enzymatic activity. During this transition the secondary structure of the protein as measured by the ellipticity at 222 nm remains intact. These findings suggest that ESI MS might be a general method to selectively monitor changes in the tertiary structure of proteins in solution.

Using ESI MS as a selective probe for changes in the tertiary structure of proteins would have advantages over other techniques. For example, the optical probes for tertiary structure used in this study all rely on the presence of specific chromophores in the protein. For ESI MS chromophores are not required. One-dimensional proton NMR is another relatively simple method to monitor changes in the tertiary structure (Griko et al., 1988; Buck et al., 1993; Fan et al., 1993; Alexandrescu et al., 1994). It is a disadvantage of NMR that the interpretation of the spectra is complicated by the large number of overlapping peaks. This is in contrast to ESI MS, where native and unfolded states of the protein usually show distinct, well-separated charge state distributions that allow an assessment of the folding cooperativity and the detection of coexisting conformations (Konermann & Douglas, 1997). Moreover, ESI MS requires only a relatively low protein concentration (millimolar or less)

compared to NMR. However, it is a limitation that ESI MS cannot be used for protein solutions containing high concentrations of salt, urea, or guanidine hydrochloride. It might also be considered a limitation that different proteins show different charge state distributions even when they are all in their native conformation. This is because the ESI mass spectrum depends on specific parameters like the number of basic amino acid residues exposed to the solvent (Loo, 1995).

The results presented here show that the charge state distribution generated during ESI specifically monitors changes in the tertiary structure of cyt *c*. Future experiments will be required to show whether this selectivity is also observed for other proteins and under different solvent conditions.

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