# Cytochrome *c* Folding Kinetics Studied by Time-Resolved Electrospray Ionization Mass Spectrometry†

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ABSTRACT: A new method for studying the folding kinetics of proteins is described. The method combines a continuous flow mixing technique with an electrospray mass spectrometer. Different protein conformations in solution are detected by the different charge states they produce during electrospray ionization. Unfolded proteins generally have more accessible protonation sites and give higher charge states than native proteins. The method is applied to study the refolding of acid-denatured cytochrome *c*. Global data analysis is used to obtain the exponential lifetimes which are associated with the refolding process. The kinetics can be described by two lifetimes of 0.17 ± 0.02 and 8.1 ± 0.9 s which are in accordance with the results of stopped flow experiments previously described in the literature. These lifetimes are associated with roughly 90 and 10% of the total intensity changes in the mass spectrum, respectively, and most likely reflect fast and slow refolding subpopulations of cytochrome *c* in solution.

Conformations of proteins have been studied by a variety of methods such as circular dichroism, fluorescence, NMR, or hydrogen−deuterium exchange. Folding kinetics can be studied by coupling these methods with a stopped flow apparatus or related techniques (Roder et al., 1988; Radford et al., 1992; Bycroft et al., 1990; Houry et al., 1996; Elöve et al., 1992, 1994). ESI MS,[1] which produces multiply charged intact gas phase ions from molecules in solution, is a relatively new method for probing conformational changes in proteins [for reviews, see Loo (1995), Przybylski and Glocker (1996), and Kebarle and Tang (1993)]. During ESI, positive protein ions are formed mainly by proton attachment to available basic sites on the polypeptide chain (Loo et al., 1990). The number of sites that can be protonated is related to the conformation of the protein in solution. An unfolded protein generally shows higher charge states in the ESI mass spectrum than the same protein in a folded state. This has been confirmed for several proteins such as myoglobin (Katta & Chait, 1991), lysozyme (Przybylski & Glocker, 1996; Loo et al., 1990), ubiquitin (Mirza et al., 1993; Hamdan & Curcuruto, 1994), and cyt *c* (Mirza et al., 1993; Chowdhury et al., 1990; Hamdan & Curcuruto, 1994; Wagner & Anderegg, 1994). However, the physical basis for the observed relationship between the protein conformation in solution and the charge state distribution generated during ESI is still a matter of debate. Several studies ascribed the observed effects to changes in the steric accessibility of possible protonation sites and to changes in their specific p*K* values (Chowdhury et al., 1990; Katta et al., 1991; Loo et al., 1990). A different model has been proposed by Fenn (1993) in which the highly charged states observed for denatured proteins are mainly due to the increased surface area of the unfolded polypeptide chain.

It has been demonstrated that time-resolved ESI MS in principle can be used to study mechanistic and kinetic aspects of reactions taking place in solution on the time scale of seconds to hours [see e.g. Lee et al. (1989), Chavez et al. (1996), and Sam et al. (1994, 1995)]. ESI MS has also been used in combination with hydrogen−deuterium exchange and stopped flow techniques to detect folding intermediates of proteins in solution with a time resolution of milliseconds (Miranker et al., 1993; Hooke et al., 1995). The information obtained from these measurements is complementary to that from two-dimensional NMR experiments. In the present study, we introduce a method for studying the folding kinetics of proteins which is comparatively simple in construction and use. This method combines ESI MS with a continuous flow mixing technique. Different conformations in solution are monitored by the different charge state distributions that they produce during ESI. The time resolution is in the range of ca. 0.1 s. The feasibility of this new technique is demonstrated by measuring the refolding kinetics of acid-denatured cyt *c*. The fact that at low ionic strength this protein has five pH-dependent conformational states labeled I−V is well-established (Theorell & Åkesson, 1941; Moore & Pettigrew, 1990). The reversible "acid transition" II → III has an apparent p*K* of roughly 2.5 and leads from a largely unfolded conformation (II) to the native state (III) (Theorell & Åkesson, 1941; Moore & Pettigrew, 1990; Robinson et al., 1983; Knapp & Pace, 1974; Babul & Stellwagen, 1972; Goto et al., 1990; Dyson & Beattie, 1982; Drew & Dickerson, 1978). In this work, time-resolved ESI MS is used to study the kinetics of the refolding from state II to state III. The lifetimes for refolding reported here compare favorably to those of previous studies of the folding of cyt *c*.

## EXPERIMENTAL PROCEDURES

*Chemicals.* Horse heart cyt *c*, purchased from Sigma (St. Louis, MO), was used without further purification. HPLC

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[1] Abbreviations: cyt *c*, cytochrome *c*; ESI, electrospray ionization; ESI MS, electrospray ionization mass spectrometry; i.d., inner diameter; NMR, nuclear magnetic resonance.

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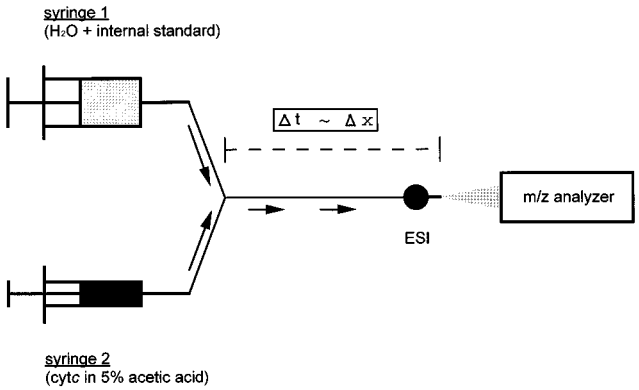


FIGURE 1: Experimental apparatus for the time-resolved ESI mass spectrometry experiments carried out in this work. Arrows indicate the flow directions of the solutions from the two syringes. ESI = electrospray ionization. The flow time $\Delta t$ in the reaction capillary is proportional to the capillary length $\Delta x$.

grade glacial acetic acid and HCl were obtained from Fisher Scientific (Nepean, Canada), and $N(CH_3)_4Cl$ was from Aldrich (Milwaukee, WI).

*Time-Resolved ESI MS.* The method is based on the continuous flow setup, depicted in Figure 1. Two syringes are advanced simultaneously by a syringe pump (model 22, Harvard Apparatus, South Natick, MA) at a total flow rate of 33 $\mu$L/min. Syringe 1 (volume of 1 mL, flow of 30 $\mu$L/min) contains $5 \times 10^{-4}$ M $N(CH_3)_4Cl$ in water; syringe 2 (volume of 0.1 mL, flow of 3 $\mu$L/min) contains cyt *c* ($1 \times 10^{-5}$ M) at pH 2.4 in a 5% solution (v/v) of acetic acid in water. The pH of each solution was measured with a calibrated accumet pH electrode (model 15, Fisher Scientific). Each syringe is connected to a fused silica capillary (TSP075150, i.d. of 75 $\pm$ 3 $\mu$m, Polymicro Technologies, Phoenix, AZ) by a connector (P742, Upchurch Scientific, Oak Harbor, WA). These two capillaries are connected to a third "reaction" capillary of the same i.d. by a homemade tee which had a dead volume of approximately 3 nL corresponding to a calculated mixing time (dead time) of roughly 5 ms for the flow rates given above. Refolding of the protein is initiated upon mixing the liquids from the two syringes in the tee. The reaction time is controlled by the length of the reaction capillary between the mixing point and the electrospray source. A distance of 1 cm in the capillary corresponds to a time of 81 $\pm$ 6 ms. For this study, reaction capillaries between 1.2 and 186 cm long were used, corresponding to times between 0.1 $\pm$ 0.007 and 15.5 $\pm$ 1.1 s, respectively. The uncertainties in the times are due to the manufacturers' stated uncertainties in the capillary diameters. In order to accommodate the shortest reaction capillaries, an ESI sprayer was designed which had a stainless steel spray capillary (i.d. = 200 $\mu$m) with an overall length of only 7 mm. The high voltage for the sprayer was applied to this capillary. Protonated gas phase protein ions were formed at the exit of the reaction capillary by pneumatically assisted electrospray and mass-analyzed in a spectrometer constructed in house. The ions were passed through a dry nitrogen "curtain" gas, a 0.25 mm diameter sampling orifice directly into an RF only quadrupole, and a short RF prefilter and finally to the mass analyzing quadrupole. The ion optics are similar to those described previously (Collings & Douglas, 1996). Fluctuations in the sensitivity of the instrument were compensated for by using $N(CH_3)_4^+$ ions as an internal standard. The presence of this internal standard at $5 \times 10^{-4}$ M did not have any noticeable effects on the mass spectra of cyt *c* at pH 2.4 or 3.0. All measurements were carried out at room temperature (21 $\pm$ 1 °C).

*Global Data Analysis.* The intensity for each charge state as a function of time was found to be well described by a sum of two exponentials plus a constant term. Intensities were fit to

$$I(m/z, t) = a_1(m/z)\exp(-t/T_1) + a_2(m/z)\exp(-t/T_2) + a_3(m/z) \quad (1)$$

where *m/z* is the mass to charge ratio for a given peak in the ESI mass spectrum, *t* is the time after initiation of the refolding reaction, $I(m/z, t)$ is the corresponding intensity of the signal, $T_1$ and $T_2$ are the exponential lifetimes with amplitudes of $a_1(m/z)$ and $a_2(m/z)$, respectively, and $a_3(m/z)$ is the amplitude spectrum for the nondecaying component. The latter reflects the mass spectrum for infinite time after initiation of the folding reaction. Instead of analyzing the time dependence of each curve $I(m/z, t)$ separately, we analyzed them all simultaneously with the method of "global analysis" (Beechem et al., 1985). The quality of the fit was judged by the value of $\chi^2$, defined as

$$\chi^2 = \frac{1}{nN}\sum_{m/z}\sum_{t}\frac{[Y(m/z, t) - I(m/z, t)]^2}{Y(m/z, t)^2} \quad (2)$$

where *n* is the number of experimental data for each value of *m/z* at time *t*, *N* is the number of *m/z* values for which the intensities have been recorded. and $Y(m/z, t)$ is the measured intensity for each value of *m/z* at time *t*. The basis of global analysis is the assumption that the different charge states, $I(m/z, t)$, all exhibit the same two lifetimes, $T_1$ and $T_2$. This reduces the number of parameters to be determined in the fitting procedure from 60 (three amplitudes and two lifetimes for each of 12 charge states) to 38 (three amplitudes for each of the 12 charge states and two lifetimes). This procedure increases the accuracy with which lifetimes $T_1$ and $T_2$ can be obtained because it extracts information from the entire spectrum rather than from a single peak (Beechem et al., 1985). The total error $\sigma$ for the measured lifetimes was calculated with $\sigma = (\sigma_1^2 + \sigma_2^2)^{1/2}$, where $\sigma_1$ is the error from the uncertainties in the i.d. of the reaction capillaries and $\sigma_2$ the uncertainty from the fit. An "exhaustive search" (Holzwarth, 1996) was used to estimate the latter for each lifetime. The change in lifetime that increased $\chi^2$ by 10% was used as an estimate of $\sigma_2$.

## RESULTS

Panels A and B of Figure 2 show the ESI mass spectra for cyt *c* in aqueous solutions of 5 and 0.45% acetic acid (v/v), corresponding to pH 2.4 and 3.0, respectively. At pH 2.4 (Figure 2A), cyt *c* is predominantly in the largely unfolded state II (Theorell & Åkesson, 1941; Goto et al., 1990) and shows a rather broad charge distribution with $16^+$ being the most intense peak. At a pH of 3.0 (Figure 2B), the conformational equilibrium is shifted toward the native state III (Theorell & Åkesson, 1941; Moore & Pettigrew, 1990; Drew & Dickerson, 1978). The mass spectrum now shows a rather narrow distribution of charge states which consists almost entirely of the $8^+$ and $9^+$ peaks. The relative contribution of higher charge states is about 10% and

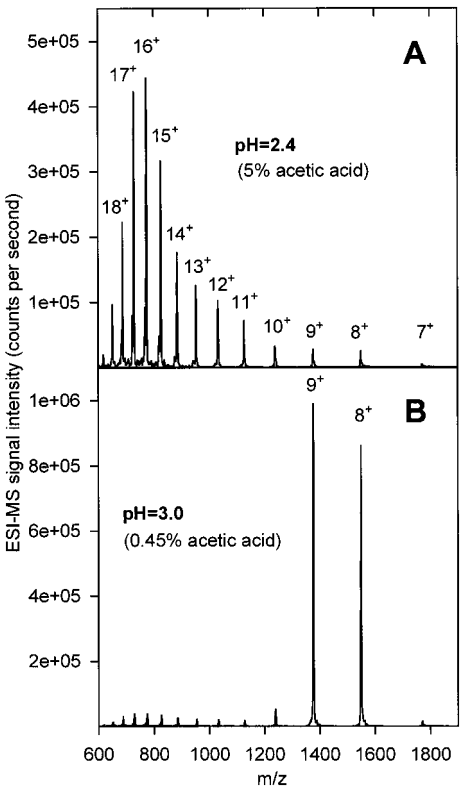


FIGURE 2: ESI mass spectra of cytochrome *c* in mixtures of acetic acid and water at pH 2.4 (A) and pH 3.0 (B). The notation $10^+$ is cyt $c + 10H^+$, etc.

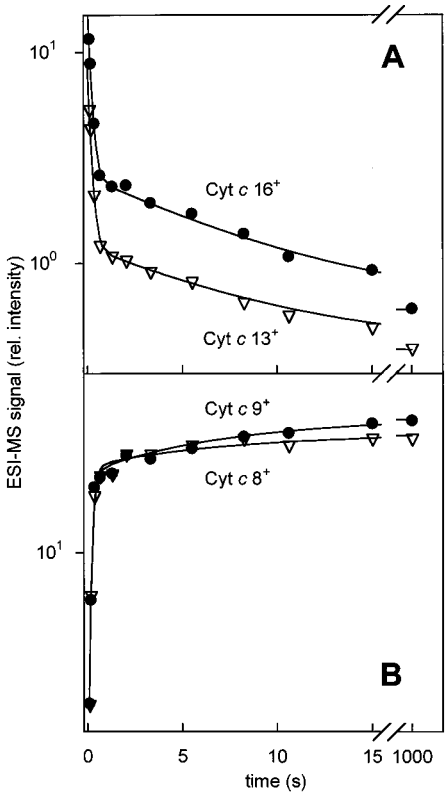


FIGURE 3: Time course of the signal intensity in the mass spectrum of cyt *c* for charge states $13^+$ and $16^+$ (A) and $8^+$ and $9^+$ (B). Intensities were measured at different times after changing the pH in the solution from 2.4 to 3.0. The first data point in each curve represents $t = 0.1$ s; the last one ("$t = 1000$ s") is taken from the stationary mass spectrum measured at pH 3.0. Data shown here are the average of three independent sets of experiments. The intensities of the data are normalized to the intensity of an internal standard. Solid lines are fits to the experimental data. The ordinates in this figure have a logarithmic scale.

suggests that at this pH some of the protein remains in state II. These pH-dependent changes in the mass spectrum are fully reversible (data not shown). It is noteworthy that the exact shapes of the spectra in Figure 2 depend on experimental details like the position of the sprayer.

The refolding of cyt *c* following a pH jump from 2.4 to 3.0 was monitored for the charge states $8^+-19^+$ by measuring the intensity of each charge state as a function of time (time-resolved ESI MS). Measurements were made from 0.1 to 15.5 s after mixing the solutions from the two syringes. Intensities for completion of the folding reaction were taken from the stationary mass spectrum of cyt *c* at pH 3.0. The intensities for each peak in the spectrum were normalized to the intensity of the internal standard $[N(CH_3)_4^+]$. The data used for this study represent the average of three independent sets of experiments. The normalized intensities of cyt *c* peaks $10^+-19^+$ decrease and the intensities of peaks $8^+$ and $9^+$ increase with time, reflecting the decay of the unfolded and formation of the folded state, respectively. Some typical data are depicted in Figure 3A,B.

A single exponential plus a constant was insufficient to fit the time course of the intensities in a global analysis and resulted in a $\chi^2$ of 0.128 (data not shown). Two exponentials and a constant gave a good fit to the experimental data, with a $\chi^2$ of 0.002 18. Typical fits are shown in Figure 3A,B. For the remaining data, the quality of the fits is similar to those shown here. The two lifetimes which were obtained from the global analysis are a $T_1$ of $0.17 \pm 0.02$ s and a $T_2$ of $8.1 \pm 0.9$ s, respectively. Approximately one-half of the uncertainties in the lifetimes comes from the scatter in the experimental data and the remainder from the uncertainties in the capillary diameters. The amplitude spectra corresponding to these lifetimes [sometimes referred to as "decay-associated spectra" (Beauregard et al., 1991)] are shown in Figure 4. Positive amplitudes correspond to a decrease and negative amplitudes to an increase in signal intensity with time. The shapes of the amplitude spectra for the fast and the slow lifetimes are very similar. The average ratio of the amplitudes $a_1(m/z)/a_2(m/z)$ shows that about 90% of the total intensity changes in the mass spectrum are associated with the lifetime $T_1$ and only about 10% with the lifetime $T_2$. The spectrum of the nondecaying component, $a_3(m/z)$, represents the mass spectrum for infinite time after initiation of the folding process and has a shape very similar to the stationary mass spectrum of Figure 2B.

## DISCUSSION

The fact that denatured proteins show higher charge states in their ESI mass spectrum than native proteins is well-established (Loo et al., 1990; Chowdhury et al., 1990; Katta & Chait, 1991; Kebarle & Tang, 1993; Mirza et al., 1993; Loo, 1995; Przybylski & Glocker, 1996). The spectra in Figure 2 demonstrate that at pH 2.4 cyt *c* is in a largely unfolded state, whereas at pH 3.0, it predominately adopts a more tightly folded conformation. Previous experiments on cyt *c* in aqueous solution have shown that this protein can refold from the acid-denatured state (II) to its native conformation (III) with an apparent p*K* of roughly 2.5 (Theorell & Åkesson, 1941; Knapp & Pace, 1974; Moore & Pettigrew, 1990). We therefore attribute the pH-induced

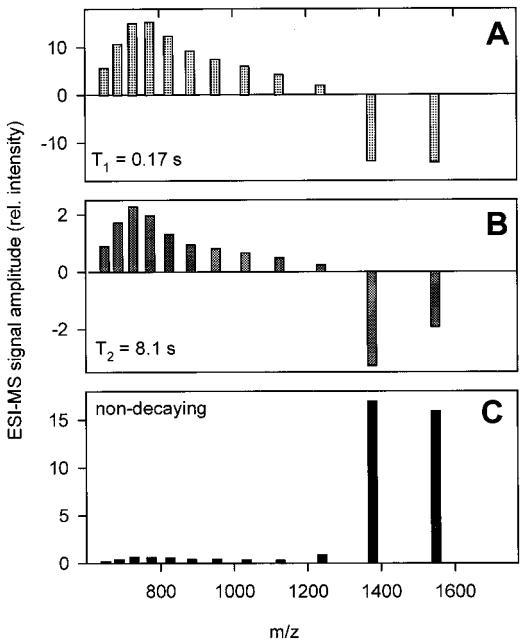


FIGURE 4: Amplitude spectra for the lifetimes associated with the refolding of cyt *c* for charge states $19^+-8^+$: $a_1(m/z)$ for a lifetime $T_1$ of 0.17 s (A), $a_2(m/z)$ for a lifetime $T_2$ of 8.1 s (B), and $a_3(m/z)$ for the nondecaying component (C).

differences in the ESI mass spectrum as depicted in Figure 2 to the transition between states II and III. These spectra are similar to literature data of Chowdhury et al. (1990) and Wagner and Anderegg (1994). The slight differences between our spectra and those of the previous studies may be due to the different type of cyt *c* (horse heart vs bovine cyt *c*) and to differences in the instrumentation.

Recently, van Berkel et al. (1995, 1996) have shown that electrolytic oxidation of water can lead to a decrease in the pH of the solution during ESI. Pronounced pH changes were found to occur only at low flow rates ($<1$ $\mu$L/min) and for an ESI ion source containing a platinum capillary. By using higher flow rates and a stainless steel capillary, as in our experiments, these effects were found to be suppressed. Alterations in the pH during ESI could complicate the relationship between charge states of gas phase protein ions and the protein conformation in solution if solutions are unbuffered and at near-neutral pH. When the acetic acid-induced equilibrium unfolding of cyt *c* is studied by ESI MS, the unfolding transition is found to occur as a two-state transition at about pH 2.7 (Wagner & Anderegg, 1994; L. Konermann, manuscript in preparation). This is in reasonable accordance with the value of about 2.5 found in optical experiments, given the varying types of cyt *c* and different buffer conditions used in these experiments (Theorell & Åkesson, 1941; Knapp & Pace, 1974; Moore & Pettigrew, 1990). We calculate, using the equations given by van Berkel et al. (1995, 1996), that for the conditions of our experiment the change caused by electrolytic processes during ESI is about 0.0002 pH unit at pH 2.5. In addition, ESI mass spectra of apomyoglobin obtained with our apparatus show the expected folding transition between pH 4.0 and 5.0 where any electrolytic processes would be expected to cause comparatively greater shifts (L. Konermann, manuscript in preparation). Thus, for the present study, changes of pH during the spraying process seem to be of minor importance.

The spectrum for pH 3.0 shows slightly lower charge states and is narrower than the spectrum of cyt *c* in pure water (pH $\approx$6, data not shown). Chowdhury et al. (1990) attributed these differences to a conformational change of the protein which could not be directly correlated to a known transition observed in solution. We note, however, that the ESI mass spectra of proteins also depend on parameters such as the conductivity and surface tension of the solution (Kebarle & Tang, 1993). Moreover, it is known that the presence of acetate anions can decrease the average charge state in the ESI mass spectrum of proteins (Mirza & Chait, 1994). Therefore, it seems likely that the above-mentioned changes are caused by "secondary" solvent effects even though a conformational change of the protein cannot be completely ruled out. It is noteworthy that the time-resolved data obtained with the method presented in this work are not prone to such secondary effects because the composition of the solvent is constant throughout the entire experiment.

With time-resolved ESI MS, measuring the kinetics for several values of *m/z* simultaneously is straightforward. This allows the application of global analysis for extracting the fitting parameters (exponential lifetimes and their corresponding amplitude spectra) from the data with an accuracy which is superior to a single curve analysis (Beechem et al., 1985). The kinetics of protein folding are commonly described as either a linear or a branched scheme of first-order reactions (Bycroft et al., 1990; Brandts et al., 1975; Tsong, 1973; Creighton, 1988; Johnson & Raushel, 1996). From the corresponding rate equations, the concentration of each species is described by a sum of exponentials (Ikai & Tanford, 1973; Beauregard et al., 1991). For the experimental data here, two exponentials and a constant term were sufficient to describe the data for all the charge states. Different charge states exhibit the same time constants but different amplitudes.

It is of interest to compare the kinetic data measured in this study with the results of stopped flow experiments on the refolding of cyt *c* where the protein conformation was monitored by tryptophan fluorescence, heme absorption, circular dichroism, and hydrogen−deuterium exchange in combination with two-dimensional NMR spectroscopy. Such experiments have been carried out under various conditions of denaturants and pH (Tsong, 1973; Brems & Stellwagen, 1983; Roder et al., 1988; Elöve et al., 1992, 1994; Sosnick et al., 1994, 1996; Colón et al., 1996). The refolding of guanidinium hydrochloride-denatured cyt *c* at pH 6.0 could be described by exponential lifetimes on the order of $<4$ ms, 20 ms, 0.2 s, and 10 s (Roder et al., 1988; Elöve et al., 1992, 1994). The processes occurring on the $<4$ ms time scale involve the formation of an early "molten globule"-like state which already contains a number of native-like tertiary interactions (Colón et al., 1996). A second intermediate is populated on the 20−200 ms time scale that exhibits a stable hydrogen-bonded structure in the two helical segments near the chain termini. The lifetime of about 0.2 s was found to be suppressed in refolding experiments carried out at pH 5.0 or below where the histidine residues in the unfolded protein are protonated so that non-native heme ligation which leads to misfolded intermediates does not occur (Tsong, 1973; Brems & Stellwagen, 1983; Elöve et al., 1994). Under these conditions, the refolding of cyt *c* appears to be a highly cooperative process and the majority of the proteins reach a native-like

conformation on the time scale of 20 ms (Sosnick et al., 1994, 1996). The slow (10 s) processes are thought to be brought about by a subpopulation of molecules for which the rate-limiting step in the refolding is a cis−trans isomerization of peptide bonds preceding proline residues (Brandts et al., 1975; Creighton, 1993).

In the present work, the folding process was found to exhibit two exponential lifetimes ($T_1 = 0.17 \pm 0.02$ s and $T_2 = 8.1 \pm 0.9$ s) which are associated with roughly 90 and 10% of the total changes in the intensities of the mass spectrum, respectively. These are in good agreement with the lifetimes of roughly 0.2 and 10 s found in the studies cited above. Faster processes could not be detected in this work due to the limited time resolution of the current experimental setup. Consistent with all the earlier studies, our data show an amplitude of about 10% for the ∼10 s component. Previous work showed that the amplitude for the lifetime of 0.2 s is largely suppressed at a pH of 5.0 and below. Under these conditions, the refolding was dominated by a process on the 20 ms time scale. In the data of Elöve et al. (1994), the ∼0.2 s component showed an amplitude of only 12% at pH 5.0, whereas Tsong (1973) and Brems and Stellwagen (1983) did not find this component at all when refolding was carried out between pH 3.0 and 5.0. Compared to these earlier results, the amplitude of roughly 90% for the 0.2 s component found in the current study seems to be surprisingly high. However, it is possible that in comparison to other techniques ESI MS is more sensitive to the 0.2 s component. When these results are compared, it should also be pointed out that all the refolding experiments cited so far have been carried out under substantially higher salt concentrations (by a factor of 50−600) and/or at concentrations of guanidinium hydrochloride of up to 1.5 M. These differences in the solvent composition might very well influence the kinetics of the refolding process. It is difficult to carry out ESI MS experiments under such conditions due to the strong interference of salts with the ESI process (Przybylski & Glocker, 1996). On the other hand, to our knowledge, the refolding of cyt *c* has not yet been studied by stopped flow techniques under conditions similar to those of the ESI MS experiments presented here. In order to better characterize the relationship between stopped flow and time-resolved ESI MS data, future experiments should be carried out under identical solvent conditions. In this context, it would also be desirable to improve the time resolution of the ESI MS measurements (see below).

Even though there may be some discrepancies with regard to the amplitudes of the kinetic components, the data presented in this study can be interpreted in a way which is consistent with previous data from the literature. The dominant fraction of cyt *c* molecules achieves a native-like conformation on the time scale of 0.2 s or faster. In addition, there is a slow folding subpopulation which refolds on a much slower time scale of 10 s. Under the conditions used in this study, the folding appears to be a highly cooperative process since there are no indications of the presence of kinetic folding intermediates which give a charge state distribution different from that of the unfolded state. It is interesting to note also that the fast and the slow refolding subpopulations apparently give very similar charge state distributions since the amplitude spectra $a_1(m/z)$ and $a_2(m/z)$ show only minor differences.

The present work introduces a method for time-resolved ESI mass spectrometry which allows monitoring of the kinetics of (bio)chemical reactions in solution. The refolding of acid-denatured cyt *c* could be followed because the acid-unfolded state II and the native state III differ in their charge distributions. It should also be possible to apply this technique in cases where reactants and products show the same charge distribution but differ in their masses, e.g. for a protein which loses a cofactor or binds a substrate. The shortest time that could be measured with the current experimental setup is roughly 0.1 s. A further improvement to shorter times should be possible by (1) using higher flow rates and/or reaction capillaries with a smaller i.d., (2) using a sprayer with a different geometry allowing shorter reaction capillaries, and (3) modification of the tee where the three capillaries are connected to achieve a shorter mixing time. The limit for the time resolution is likely to be in the range of some milliseconds which is the time required for the formation of gas phase ions from molecules in solution (Kebarle & Tang, 1993). An extension to longer times could easily be achieved by lowering the flow rate in the reaction capillary. The uncertainties in the measured lifetimes, introduced by the uncertainties in the capillary diameter, could be reduced by calibrating each mixing capillary separately.

Time-resolved ESI mass spectrometry provides information about the folding kinetics of proteins which is complementary to the results of stopped flow experiments where optical properties of the protein or the protection against hydrogen−deuterium exchange is monitored. It does not give information on a residue-specific basis and is limited to the study of conformational changes which produce changes in the charge state distributions during ESI. However, an advantage of this technique is the fact that it does not rely on the presence of intrinsic chromophores in the protein. It is also easily implemented on electrospray mass spectrometers.

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