Regional Stability Changes in Oxidized and Reduced Cytochrome *c* Located by Hydrogen Exchange and Mass Spectrometry

Kuruppu Dharmasiri and David L. Smith
Department of Chemistry, University of Nebraska-Lincoln, Lincoln, Nebraska, USA

Amide hydrogen exchange rates are highly sensitive to protein structure and may, therefore, be used to detect and characterize structural changes in proteins. Specific regions within folded proteins undergoing structural change can often be identified if localized amide hydrogen exchange rates are determined by nuclear magnetic resonance (NMR). The ability to measure localized amide hydrogen exchange rates by proteolytic fragmentation followed by mass spectrometric analysis opens the possibility to also identify localized structural changes in proteins by mass spectrometry. If successful, this approach offers considerable advantage over NMR in speed, sensitivity, protein solubility, and ability to study large proteins. This possibility has been investigated by determining the amide hydrogen exchange rates in oxidized and reduced cytochrome *c* by protein fragmentation/mass spectrometry. The fundamental difference in these forms of cytochrome *c* is the oxidation state of the iron, which other studies have shown results in only minor structural changes in the protein. In the present study, the largest differences in hydrogen exchange rates were found for peptide amide hydrogens located distant from the N- and C-termini, indicating that the structure in these regions is most affected by the oxidation state of the iron. These results are consistent with previous studies of oxidized and reduced cytochrome *c*, suggesting that hydrogen exchange and mass spectrometry may be generally useful for locating subtle changes in protein structure. (J Am Soc Mass Spectrom 1997, 8, 1039–1045) © 1997 American Society for Mass Spectrometry

Functional activity is one measure of whether a protein has achieved its native state. However, physical methods are required for elucidation of detailed structural information that may help understand protein function. Some of the most important analytical methods used to determine and detect changes in the three-dimensional structures of proteins include x-ray diffraction, nuclear magnetic resonance (NMR), circular dichroism (CD), UV (ultraviolet) difference spectroscopy, and tryptophan fluorescence spectroscopy. Despite the diversity and power of these methods, there remains a need for alternate methods to detect and characterize different protein conformations. Important criteria for assessing the utility of such analytical methods include the time and quantity of material required for analysis, as well as the ability to detect minor structural changes and to locate these changes within the three-dimensional structures of proteins.

Hydrogens located at peptide amide linkages in proteins incubated in D_2O may be replaced with deuterium [1–6]. The rates at which the hydrogens located at different peptide linkages are replaced with deuterium are critically dependent on whether the hydrogens are participating in intramolecular hydrogen bonds, and on whether the hydrogens have access to the deuterated aqueous solvent. Although detailed mechanisms through which amide hydrogens in folded proteins undergo isotopic exchange remain elusive, it is clear that isotope exchange rates are a sensitive probe of protein conformation. Because abundances at specific peptide linkages can be determined by NMR, it has become the principal tool for measuring amide hydrogen exchange rates [7–10]. For example, conformational changes that occur in cytochrome *c* when different antibodies are bound to different regions of cytochrome *c* have been described in detail using the combination of NMR and amide hydrogen exchange [11–13]. Likewise, the combination of NMR and hydrogen exchange has been one of our most important methods for identifying structural intermediates formed during protein folding [8, 10, 14, 15] and unfolding [16, 17]. Amide hydrogen exchange rates have also been determined by Fourier transform infrared spectroscopy (FTIR) [18, 19]. Though attractive for its experimental simplicity, FTIR is less

Address reprint requests to David L. Smith, Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588-0304. E-mail: dls@unlinfo.unl.edu

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useful for hydrogen exchange studies because it indicates only that a conformational change has occurred and not the location of the change.

Mass spectrometry has become another useful method for determining amide hydrogen exchange rates in peptides and proteins [20]. As with FTIR, mass spectrometry can be used to determine hydrogen exchange rates averaged over all peptide linkages in polypeptides [21–30]. Alternatively, amide hydrogen exchange rates in small regions of folded proteins can be determined by the protein fragmentation/mass spectrometry approach, which combines proteolytic fragmentation of the partially deuterated protein with directly coupled high-performance liquid chromatography (HPLC) mass spectrometry (MS) [31–39].

The purpose of the present study was to demonstrate that the protein fragmentation/MS approach can be used to detect and locate very minor structural changes in proteins. Specifically, this approach was used to identify regions in which the oxidized and reduced forms of cytochrome *c* differ. Cytochrome *c* was selected for this investigation because the structures of the oxidized and reduced states are particularly well characterized. Performing the isotope labeling step in a flow-quench system facilitated rapid characterization of these very similar conformers using less than a nanomole of protein.

Experimental

Isotope Exchange

Horse heart cytochrome *c*, pepsin, anhydrous monobasic potassium phosphate, anhydrous dibasic potassium phosphate, and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO); deuterium oxide (99.9 at. %) was purchased from Cambridge Isotope Lab. (Andover, MA). All chemicals were used without further purification. Cytochrome *c*, in which all exchangeable hydrogens had been replaced with deuterium, was prepared by incubating the protein in D_2O buffer (pD 6.8) at 70 °C for 3 h. Reduced cytochrome *c* was prepared by dissolving oxidized cytochrome *c* in 0.1 M sodium ascorbate solution. A semiautomated flow-quench apparatus was used to expose oxidized and reduced cytochrome *c* to D_2O for isotope exchange times from 0.2 to 120 s. As illustrated in Figure 1, 5 μL of oxidized or reduced cytochrome *c* (10 μg/μL) was injected into a flow (5 μL/min) of H_2O, which was diluted 20-fold with a phosphate buffered flow of D_2O (0.1 M phosphate, pD 6.8, 20 °C). Following deuterium exchange-in times of 0.2–120 s, isotope exchange was quenched by decreasing the pD to 2.3 and temperature to 0 °C. Pepsin (substrate:enzyme 1:1) was added with the quench acid (0.3 M phosphate, pH 2.2). After a 4-min digestion time, the digest was loaded into the sample loop (50 μL) of a capillary HPLC injector for immediate analysis by liquid chromatography/mass spectrometry (LC/MS). Details of the flow-quench apparatus have been described elsewhere [37]. For longer isotope exchange times (5–60 min), mixing was performed manually.

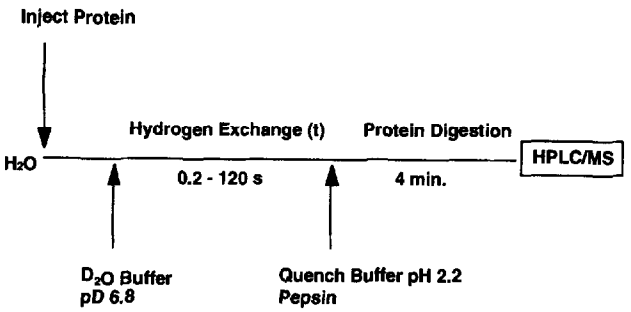

Figure 1. Flow-quench system used to exchange deuterium into cytochrome *c* for 0.2–120 s. Details of the system have been described elsewhere [37].

Isotopic Analyses

Peptides in the digest were fractionated by reversed-phase HPLC using a 150 mm × 0.32 mm column packed with POROS 10 R2 Perfusive media (PerSeptive Biosystems, Framingham, MA). Following a 3-min desalting period, peptides were eluted by a gradient of 0%–60% CH_3CN in 5 min. The flow rate of the mobile phases (H_2O and CH_3CN, both 0.05% trifluoroacetic acid) was 25 μL/min. The flow through the HPLC system was controlled by a Rainin pumping system. To minimize artifactual isotopic exchange during digestion, the digestion loop, the second HPLC injector, and the HPLC column were cooled to 0 °C. The molecular weights of partially deuterated peptides were determined by monitoring the HPLC effluent with a Micromass Autospec (Manchester, UK) high resolution mass spectrometer equipped with a focal plane detector and standard electrospray ionization source. The total flow from the capillary HPLC (25 μL/min) was directed to the electrospray ionization source, which was operated at 60 °C. The nebulizing and drying gas flows were 16 and 400 L/h, respectively. All mass spectra were acquired in the focal plane detection mode by scanning from 400 to 1600 u in ten steps where approximately 18% of the central mass-to-charge ratio was recorded in each step. Each scan required approximately 3 s for completion. The resolution with the array detector was 2000 full width at half maximum (FWHM). Mass spectra were recorded in the profile mode with an OPUS data system (Micromass, Manchester, UK). The mass spectrometer was calibrated with a mixture of peptides and myoglobin.

Results and Discussion

Conformational changes in proteins may be large or small. Examples of large conformational changes include the formation of apocytochrome *c* and apomyoglobin from the respective holoproteins. Removing the heme from cytochrome *c* gives apocytochrome *c*, which has little or no secondary or tertiary structure [40, 41]. Two-dimensional NMR analysis of apomyoglobin indi-

cates that two of the seven α-helices are lost upon removal of the heme from the holoprotein [42]. Subsequent analyses by mass spectrometry and hydrogen exchange suggested that apomyoglobin consists of two or more rapidly interconverting structures that may be intermediates along the myoglobin folding path [29, 33]. The oxidized and reduced forms of cytochrome *c* illustrate how very minor changes in conformation may occur as a protein performs its function. Many studies of cytochrome *c*, which functions to transport an electron from the cytochrome b-c_1 complex to cytochrome oxidase, have sought to link structural differences in its oxidized and reduced forms to its ability to accept and donate an electron. Specifically, how do the relative positions of the approximately 104 amino acid residues present in most cytochromes *c* differ in the oxidized and reduced forms? The functional difference in the two forms is the oxidation state, Fe (III) or Fe (II), of the lone iron coordinated to the heme. Stability measurements performed using several different experimental methods indicate that the reduced form is several kilocalories more stable than the oxidized form.

High resolution x-ray diffraction [43–45] and NMR [46] have been used to determine the structural differences between oxidized and reduced cytochrome *c*. X-ray diffraction studies showed that the heme moves out of the pocket by 0.15 Å and that one of three water molecules moves 1 Å closer to the heme upon oxidation of the iron. The root-mean-square (rms) differences in corresponding atomic positions of oxidized cytochrome *c* and reduced cytochrome *c* is only 0.5 Å, indicating that oxidized and reduced cytochrome *c* have very similar structures. Analysis by high resolution NMR, which showed only 51 of 351 protons exhibiting redox-dependent chemical shifts, also points to "small structural adjustments" rather than large structural displacements or rearrangements. Despite the structural similarities of oxidized and reduced cytochrome *c*, hydrogen exchange rates at some peptide linkages, measured by high resolution NMR, are very different for oxidized and reduced cytochrome *c* [9, 47]. The present study was directed toward determining whether a semiautomated version of the protein fragmentation/MS approach for determining amide hydrogen exchange rates could be used to detect and locate the minor structural changes known to differentiate the oxidized and reduced forms of cytochrome *c*.

Hydrogen exchange analysis by protein fragmentation/MS, which has roots in pioneering experiments using tritium in place of deuterium [48, 49], has been used to determine the rates at which hydrogen located at peptide amide linkages in proteins are replaced with deuterium [31, 32, 34, 39]. As illustrated in Figure 1, the protein is exposed to D_2O for a time during which hydrogen located on peptide amide linkages may be replaced with deuterium. Whether these hydrogens are replaced is critically dependent on the structure of the protein. Following the deuterium exchange-in period, isotope exchange is slowed by a factor of approximately 10^{-5} by decreasing the pH to 2–3 and the temperature to 0 °C. Brief exposure to a high concentration of the acid protease pepsin fragments the protein into peptides whose molecular weights are determined by directly coupled capillary HPLC mass spectrometry. Since the HPLC step is performed using H_2O, deuterium located on functionalities exhibiting fast exchange (e.g., hydroxyl and primary amines) are replaced with protium [50]. As a result, the increase in molecular weight is due primarily to deuterium located on peptide amide linkages. Details of the protein fragmentation/MS approach have been described [31, 33, 39].

Structural Changes Associated with the Oxidation State of Iron

The flow-quench system illustrated in Figure 1 was used to exchange deuterium into oxidized and reduced cytochrome *c* for 0.2–120 s, while manual mixing methods were used for longer exchange times of 5–60 min. Since rate constants for amide hydrogen exchange at neutral pH span a range from fractions of a second to months, this exchange-in time is suitable for determining rate constants for the most rapidly exchanging amide hydrogens. Longer exchange times would be required to determine the isotope exchange rates of the more slowly exchanging amide hydrogens. The present study has been directed toward the most rapidly exchanging amide hydrogens because our goal was to provide a rapid method, with the possibility for automation, for locating structural differences in protein conformers.

Peptic digestion of cytochrome *c* for 4 min at 0 °C gave approximately 20 peptides from which the deuterium levels in the corresponding segments of the intact protein could be determined. A set of eight peptides spanning the entire 104 residue backbone of cytochrome *c* was used to locate structural differences in the oxidized and reduced forms of the protein. The deuterium levels found in four segments, including amide linkages of residues 1–10, 11–21, 82–94, and 96–104, are presented in Figure 2 as a function of the time the intact proteins were incubated in D_2O. The solid line in each figure shows the deuterium levels these segments would have if the protein were completely unfolded, exposing the amide hydrogens to the aqueous solvent for the temperature and pH used in the present study [50]. Differences between the deuterium levels indicate the extent to which amide hydrogen exchange rates were diminished by the secondary and tertiary structure of the native protein.

Results in Figure 2 show the deuterium level in the segment including amide linkages 1–10 increasing to five when either oxidized or reduced cytochrome *c* was incubated in D_2O for 60 min. Fitting these data to a series of exponential functions [36, 39] indicates that one amide hydrogen in this segment has an isotope exchange rate constant of 2 s^{-1}, two have rate constants

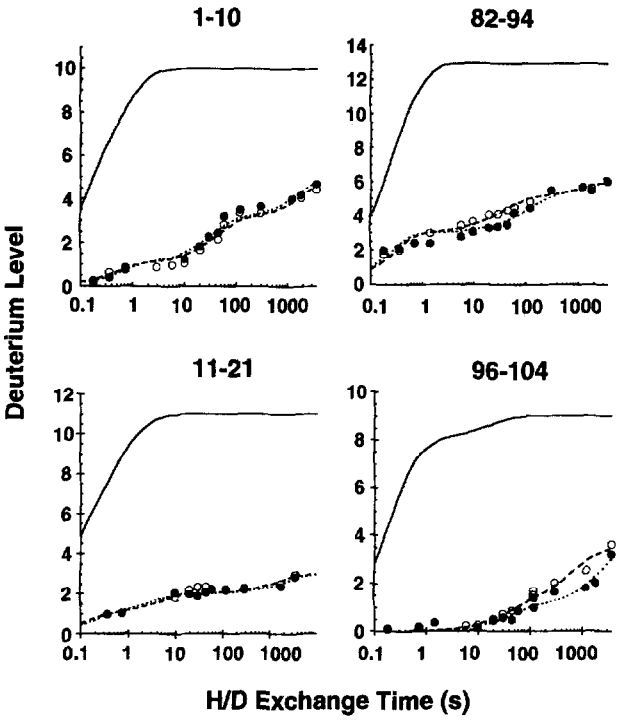

Figure 2. Deuterium levels in segments including residues 1–10, 11–21, 82–94, and 96–104 derived from oxidized (open circles) and reduced (closed circles) cytochrome *c* following incubation of the intact protein in D_2O. The solid line indicates the deuterium levels that are expected in these segments if there were no secondary or tertiary structure [50]; the dashed lines indicate the fitting of first-order rate constants to the experimental data.

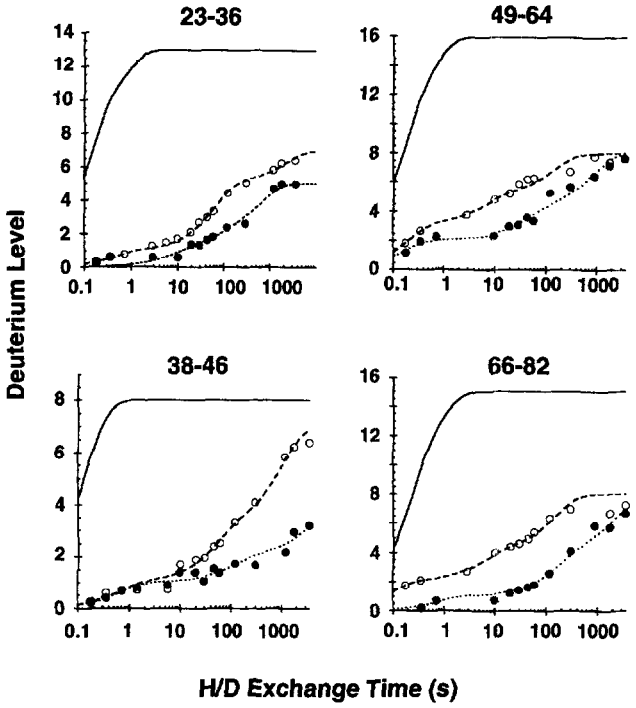

Figure 3. Deuterium levels in segments including residues 23–36, 38–46, 49–64, and 66–82 derived from oxidized (open circles) and reduced (closed circles) cytochrome *c* following incubation of the intact protein in D_2O. The solid line indicates the deuterium levels that are expected in these segments if there were no secondary or tertiary structure [50]; the dashed lines indicate the fitting of first-order rate constants to the experimental data.

of 0.02–0.03 s^{-1}, and two have rate constants of 0.0005–0.0006 s^{-1}. The remaining five peptide amide hydrogens in this segment have exchange rate constants less than 0.0001 s^{-1}. Results for the other three segments depicted in Figure 2 indicate a similarly wide range of isotope exchange rates. Although hydrogen exchange rates are different in each segment, they are independent of whether the cytochrome *c* was in the oxidized or reduced form. Since no difference in the amide hydrogen exchange rates in the regions including peptide linkages 1–21 and 82–104 was detected, these results suggest that the oxidation state of the iron has little effect on the cytochrome *c* structure in these regions. Though less likely, it is possible that isotope exchange rates within a specific segment both increased and decreased such that the net change in deuterium level was not detected.

Deuterium levels found in four segments derived from the interior of oxidized or reduced cytochrome *c* are presented in Figure 3. As in Figure 2, the solid line indicates the deuterium level expected if the protein were unfolded. It is significant that the deuterium level in these segments is greater when the segments were derived from the oxidized form of cytochrome *c*, suggesting that the structures of the two forms of the protein differ in these regions. Fitting the first order rate constants for isotope exchange to the time dependence of the deuterium levels in these segments provides a quantitative basis for expressing the effects of oxidation state on hydrogen exchange rates. Results for the peptide including amide linkages 23–36 when derived from oxidized or reduced cytochrome *c* are presented in Table 1. From these results, it is evident that the oxidation of the iron dramatically changes the rates of isotopic exchange at several peptide amide linkages in the 23–36 segment. The higher levels of deuterium found in these segments when derived from the oxidized protein indicate that redox driven structural changes have occurred throughout these regions.

The difference in deuterium level detected for peptides derived from oxidized and reduced cytochrome *c* may depend on the time used for deuterium exchange-in. For example, results for the segment including peptide linkages 38–46 indicate similar levels of deuterium for exchange times from 0.2 to 10 s. It is only for

Table 1. Distribution of isotope exchange rates of hydrogens located at peptide linkages 23–36 in oxidized and reduced cytochrome *c* determined by fitting data presented in Figure 3

Oxidation state	n^a	k (s^{-1})	n^a	k (s^{-1})	n^a	k (s^{-1})	n^a	k (s^{-1})
Oxidized	1	2	4	0.01	2	0.0005	6	<0.0001
Reduced	1	0.1	1	0.01	3	0.001	8	<0.0001

[a]Number of amide hydrogens with exchange rates of k (s^{-1}).

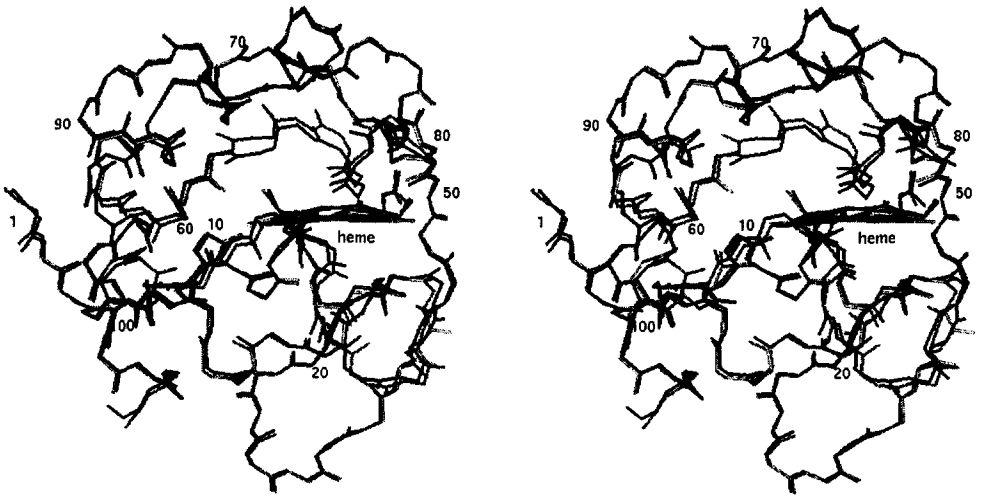

Figure 4. Stereoview of tuna cytochrome *c* backbone illustrating the regions in which the structures of oxidized (dark line) and reduced (light line) cytochrome *c* differ [43, 44].

isotope exchange times greater than 10 s that significant differences in deuterium levels were found. Curve fitting of these data indicates that this segment has one amide linkage with an isotope exchange rate constant of 1 s^{-1} for both oxidized and reduced cytochrome *c*. However, when derived from oxidized cytochrome *c*, this segment has two amide hydrogens with exchange rate constants of 0.02 s^{-1}, four with rate constants of 0.001 s^{-1}, and one with a rate constant of less than 0.0001 s^{-1}. The same segment derived from reduced cytochrome *c* has one amide hydrogen with an isotope rate constant of 0.008 s^{-1}, and six with rate constants of less than 0.0001 s^{-1}. It follows that some care in selecting isotope exchange times may be required to detect conformational changes in proteins. Although time course measurements enable determination of isotope exchange rate constants, as well as the distribution of rate constants within short segments of proteins, minor conformational changes can be detected from differences in deuterium levels detected for a single isotope exchange time.

Correlation with Other Studies

Amide hydrogen exchange rates determined in this study indicate that redox-induced structural changes in the N- and C-terminal regions (residues 1–21 and 82–104) are minor relative to those occurring in the intervening region (residues 23–82) of cytochrome *c*. These results are consistent with x-ray diffraction structures of oxidized and reduced tuna cytochrome *c* [43]. The main-chain structures for oxidized and reduced tuna cytochrome *c*, oriented for viewing the regions of greatest structural difference, are preseted in stereo in Figure 4. Visual inspection of these structures indicates that the largest redox-induced changes in the cytochrome *c* main chain occur for resiues 52–58 (directly above the heme in the orientation used in Figure 4). A more quantitative analysis of the x-ray diffraction results indicates that the largest structural changes in the cytochrome *c* backbone occur distant from the N- and C-termini. Oxidized and reduced forms of a closely related cytochrome *c*, iso-1-cytochrome *c*, have also been studied by x-ray diffraction [45]. Although all main-chain hydrogen bonds remain intact in the two forms of the protein, small structural differences were reported for several regions distant from the N- and C-termini. Similarly, Feng et al. reported the greatest redox-dependent NMR chemical shifts for residues 39–43 and 50–60 of horse heart cytochrome *c* [46]. Results of these studies, which form the basis for the most accurate assessment of redox-induced structural changes in cytochrome *c*, corroborate the hydrogen exchange results presented in Figures 2 and 3.

The rates of amide hydrogen exchange in oxidized and reduced cytochrome *c* have also been determined by high resolution NMR. Wand et al. found the exchange rates at Cys 14 and Ala 15 in oxidized cytochrome *c* larger than in the reduced form by factors of 22 and 12, respectively [9]. However, much smaller differences were found for amide hydrogens located on residues 8–13. Subsequent measurements of approximately half of the amide linkages in cytochrome *c* showed that the exchange rate in the oxidized form is consistently larger than in the reduced form [51]. It is important to note that isotope exchange rates were measured for amide hydrogens with exchange rate constants of less than approximately 0.0001 s^{-1} in the NMR study, while isotope exchange rates for amide hydrogens with exchange rate constants of greater than approximately 0.0001 s^{-1} were measured in the present study. Because the NMR study used slowly exchanging amide hydrogens, while the present study used rapidly exchanging amide hydrogens to detect structural changes, the amide hydrogens used to detect redox-induced structural changes in cytochrome *c* were not the same in the two studies. Because of this difference, it is not possible to compare quantitatively the results of Wand et al. with those of the present study. However,

results of both studies do agree that hydrogen exchange is generally faster in the oxidized form of cytochrome *c*.

Conclusions

Structural differences in oxidized and reduced cytochrome *c* are among the most carefully studied of all conformational changes in proteins. X-ray diffraction and NMR studies show that the positions of atoms located in the backbones of oxidized and reduced cytochrome *c* differ by less than 1 Å, with the N- and C-terminal regions changing less than internal regions [43, 45, 46]. The present study demonstrates that amide hydrogen exchange rates are higher in the internal regions of oxidized cytochrome *c*, consistent with the notion that this region is less stable when the heme iron is in the +III state. When combined with previous studies using the protein fragmentation/MS approach, the present results indicate that minor conformational changes in a wide range of proteins can be detected and located using amide hydrogen exchange and mass spectrometry.

Although amide hydrogen exchange studies do not lead to the specific locations of atoms in three-dimensional space, as do studies using x-ray diffraction and high resolution NMR, amide hydrogen rates are exquisitely sensitive to the three-dimensional structures of proteins. Amide hydrogen exchange information is complementary to circular dichroism, UV absorbance, tryptophan fluorescence, and differential scanning calorimetry, and has the advantage of specifying the region in which a conformational change has occurred. When compared with high resolution NMR for determining amide hydrogen exchange rates, the protein fragmentation/MS approach offers considerable advantage in speed, sensitivity, protein solubility, and ability to study large proteins. Of course, NMR continues to be the only generally viable method for determining the isotopic exchange rates of individual amide hydrogens.

Potential applications of the protein fragmentation/MS method for detecting conformational changes in proteins are numerous. Ligand binding is often accompanied with structural changes in the vicinity as well distant from the binding site. Such structural changes can be detected by changes in hydrogen exchange rates [13]. If these changes in hydrogen exchange rates are viewed as a fingerprint for a specific type or location of binding, the protein fragmentation method may be used to rapidly screen for ligands binding to specific regions of proteins. Sample throughput could be increased to several samples per hour by targeting the analysis to specific segments of the protein. The protein fragmentation/MS approach may also be particularly useful for studies of large proteins that are often studied by NMR only as fragments or functional domains. A high level of correlation between amide hydrogen exchange features in the isolated domains and the intact protein would justify extrapolation of structural information based on NMR analyses of the domains to the intact protein.

Acknowledgments

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