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Mass spectrometric measurement of protein amide hydrogen exchange rates of apo- and holo-myoglobin

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Abstract

Measurement of backbone amide hydrogen exchange rates can provide detailed information concerning protein structure, dynamics, and interactions. Although nuclear magnetic resonance is typically used to provide these data, its use is restricted to lower molecular weight proteins that are soluble at millimolar concentrations. Not subject to these limitations is a mass spectrometric approach for measuring deuterium incorporation into proteins that are subsequently proteolyzed by pepsin; the resulting peptide masses are measured using a flowing-fast atom bombardment ionization source (Zhang Z, Smith DL, 1993, *Protein Sci 2*:522-531). In the current study, amide deuterium incorporation for intact apo- and holo-myoglobin was measured using liquid chromatography coupled directly to an electrospray ionization (LC/MS) source. Electrospray ionization provided a more complete coverage of the protein sequence and permitted the measurement of deuterium incorporation into intact proteins. Tandem mass spectrometry was used to rapidly identify the peptic peptides. It was found that within 30 s, the amides in apo-myoglobin were 47% deuterated, whereas holo-myoglobin was 12% deuterated. Peptic digestion and LC/MS demonstrated that regions represented by peptic peptides encompassing positions 1-7, 12-29, and 110-134 were not significantly altered by removal of the heme. Likewise, destabilized regions were identified within positions 33-106 and 138-153.

Keywords: hydrogen exchange; mass spectrometry; myoglobin; protein structure

It has been found that, within the native structure of proteins, hydrogen exchange rates can vary by several orders of magnitude. Solvent accessibility (Woodward et al., 1982) has been proposed to explain this variability, where proton or hydroxide catalysts penetrate the protein through transiently opened channels. Alternatively, the "local unfolding model" (Englander et al., 1988) postulates that segments of a protein transiently unfold and rapidly refold, and hydrogen exchange occurs during these unfolding events. In this model, amide hydrogens located within stable secondary structures are tightly hydrogen bonded and exhibit the lowest hydrogen exchange rates. Likewise, buried residues are unlikely to undergo local unfolding and therefore exhibit slow exchange kinetics.

Hydrogen exchange rates measured for whole proteins have been obtained in a variety of ways and have proven useful in providing global information about proteins and their interactions (Hvidt & Nielsen, 1966; Englander et al., 1979; Chiachiera & Kosower, 1992). Such studies do not, however, provide any detailed information about specific changes within the protein. For this level of information, hydrogen exchange rates for individual peptide bonds need to be measured. NMR has been particularly useful in this regard if sufficient material is available, if the protein is soluble at the required concentrations, and if proton assignments can be made, i.e., when the protein is sufficiently small (Englander & Mayne, 1992). An alternative procedure takes advantage of the fact that the half-life of hydrogen exchange for free amides can be extended to about 1 h at low pH (approximately 2.7) and reduced temperature. This technique employs peptic cleavage at pH 2.7 of partially tritiated proteins at 0 °C, followed by HPLC separation of the peptides. The separated peptides are then identified either by N-terminal sequence analysis or by amino acid composition, and the extent of tritium incorporation is determined by scintillation counting (Rosa & Richards, 1979; Englander et al., 1985).

A conceptually similar approach to the tritium exchange method employs a mass spectrometric method for the identification of peptic peptides and their level of deuterium incorporation. The potential of this technique was first demonstrated by Thevenon-Emeric et al. (1992), where it was shown that hydrogen exchange rates in the peptides Ile-Ser bradykinin and the 1-14 segment of β-hemoglobin as determined by mass spectrometry

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Abbreviations: ESI/MS, electrospray ionization mass spectrometry; FAB, fast atom bombardment ionization; LC/MS, coupled liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; pD, pH meter reading uncorrected for deuterium.

compared favorably with those determined by NMR. In a separate study, this same laboratory has coupled microbore reversed-phase HPLC with a mass spectrometer utilizing a flowing-fast atom bombardment ionization source (Zhang & Smith, 1993) (flowing-FAB) to demonstrate that hydrogen exchange rates can be determined for peptic peptides of cytochrome *c*. More recently they have examined human lens crystallins (Liu & Smith, 1994) and rabbit muscle aldolase (Zhang et al., 1993).

Whereas these initial mass spectrometric studies utilized a flowing-FAB interface, hydrogen exchange data can be readily acquired using an electrospray ionization interface (Covey et al., 1988; Fenn et al., 1989; Smith et al., 1991). The advantages of ESI over flowing-FAB include enhanced sensitivity, reduced susceptibility to suppression by coeluting compounds, and an increased molecular weight range (Hemling et al., 1990). Indeed, ESI/MS permits the measurement of hydrogen exchange rates for intact proteins. Katta and Chait (1993) have shown that deuterium incorporation into intact hen lysozyme and bovine ubiquitin can be determined by ESI/MS, and that denaturation by either disulfide bond reduction or addition of organic solvents increases the rate of incorporation. Furthermore, it was demonstrated that denatured equine myoglobin and bovine carbonic anhydrase were nearly completely labeled after 30 min of deuterium incorporation. More recently, ESI/MS was used in conjunction with hydrogen exchange to observe different populations of folding intermediates of lysozyme (Miranker et al., 1993), and Wagner and Anderegg (1994) have examined deuterium incorporation for different charge states of cytochrome *c*.

Described below are deuterium exchange experiments performed on apo- and holo-myoglobin using a nebulization-assisted electrospray interface. Both the intact proteins and their peptic peptides were examined by LC/MS, and the peptic peptides were identified by tandem mass spectrometry (Hunt et al., 1986; Biemann 1990a).

Results

Early experiments were carried out by initiating deuterium in-exchange by dissolving dried protein in buffered D_2O, removing aliquots at various time points, quenching the exchange reaction by dropping the pD, and freezing the sample in liquid nitrogen. These aliquots were thawed and applied to a 1-mm bore C_8 column where a portion of the effluent was directed to a triple quadrupole mass spectrometer. Figure 1A depicts the "reconstructed" mass spectrum (Mann et al., 1989) for the 2-min time point of holo-myoglobin, where a major peak is observed at 16,987 Da, with 2 additional low-intensity potassium adducts. In contrast, the 2-min time point for apo-myoglobin exhibited a bimodal distribution (Fig. 2B), with 2 major components 30 Da apart. All samples representing other time points for apo-myoglobin exhibited broad or multiple peaks. It appeared that upon dissolution, apo-myoglobin is comprised of at least 2 populations interpreted as a more denatured state that rapidly incorporates deuterium and a less denatured form with a lower amide hydrogen exchange rate. To test this hypothesis, apo-myoglobin lyophilized from the same stock solution was first dissolved in a small volume of protonated buffer. After 30 min, D_2O was added to make the final solution 90% D_2O, and time points were sampled as described above. The mass spectrum of the 2-min time point of apo-myoglobin prepared in this manner is shown in Figure 1C, where a single peak of mass 17,009 Da was observed. All of the remaining aliquots sampled at various times exhibited major single peaks with low-intensity potassium adduct ions that were multiples of 38 Da higher in mass.

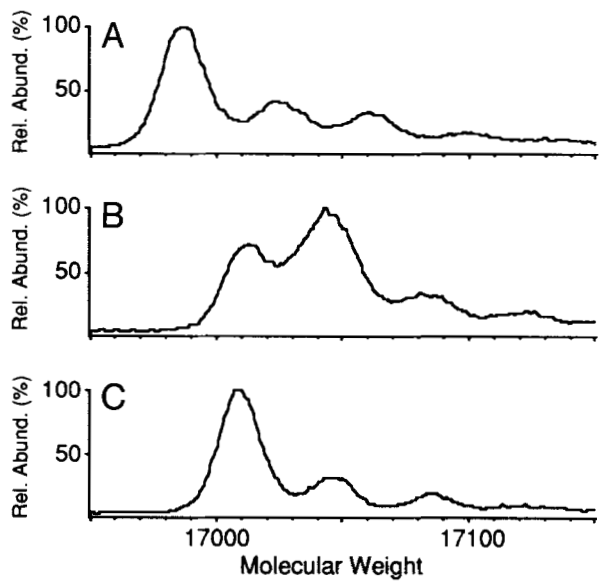


Fig. 1. Reconstructed mass spectra of intact holo- (**A**) and apo- (**B, C**) myoglobin after 2 min of deuterium incorporation. In (A) and (B), deuterium exchange was initiated by dissolving dried samples in deuterated buffers. In (C), deuterium exchange was initiated by the addition of D_2O to solubilized protein.

In subsequent experiments, holo- and apo-myoglobin were each dissolved in protonated buffers and deuterium exchange was initiated by the addition of D_2O. Time points were sampled, frozen in liquid nitrogen, and stored at −70 °C. Samples were individually thawed and immediately analyzed by LC/MS using a 10-min acetonitrile gradient, where both the injector and column were immersed in an ice bath. Because the proteins were

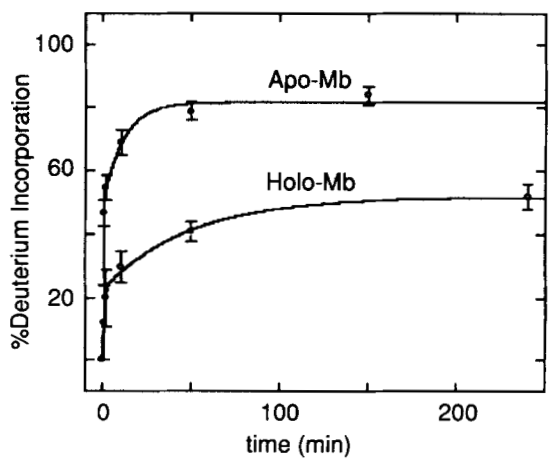


Fig. 2. Rates of deuterium in-exchange for intact apo-myoglobin (top curve) and holo-myoglobin (bottom curve). Error bars were derived from the multiple molecular weight measurements derived from the +13 to +20 charge states from single mass spectra obtained for each time point. Data were acquired and analyzed as described in the Materials and methods section.

thawed in a mixture of protonated and deuterated buffers (hydrogen exchange is quenched by the addition of protonated acidic buffer to the deuterated sample buffer), a fully protonated protein would suffer some deuterium in-exchange while thawing. Thus, a zero time point is necessary to compensate, and it is obtained by mixing the deuterated buffer with 0.5 M phosphate buffer, pH 2.7, before addition to the protonated protein. To measure the loss of deuterium that occurs during LC/MS, the completely deuterated protein is used, which is produced by dissolution of the protein in deuterated buffer at high pD under denaturing conditions. These controls established the 0% (M_r 16,969) and 100% (M_r 17,041) levels of deuterium incorporation. Equation 1 (see Materials and methods) was used to calculate percent deuterium incorporation for holo- and apomyoglobin at various time points as shown in Figure 2. Error bars represent standard deviations.

The specific regions of myoglobin that change their amide hydrogen exchange behavior in the absence of heme were identified using the procedure described by Zhang and Smith (1993). Frozen aliquots representing time points ranging from 0.5 to 270 min were thawed, pepsin was added, and the mixture was digested for 5 min on ice before injection onto an ice-cold HPLC column. Peptides were separated in 20-min acetonitrile gradients. Zero time points and fully deuterated controls were treated similarly. For example, Figure 3A shows the +3 charge state of the undeuterated peptic peptide encompassing positions 110–134; the zero time point is shown in Figure 3B, demonstrating that some deuterium is incorporated during the brief peptic digestion. Figure 3C shows the increased peptide mass after 5 min of deuterium exchange and Figure 3D depicts the incorporation of 14 deuterons in the fully deuterated control.

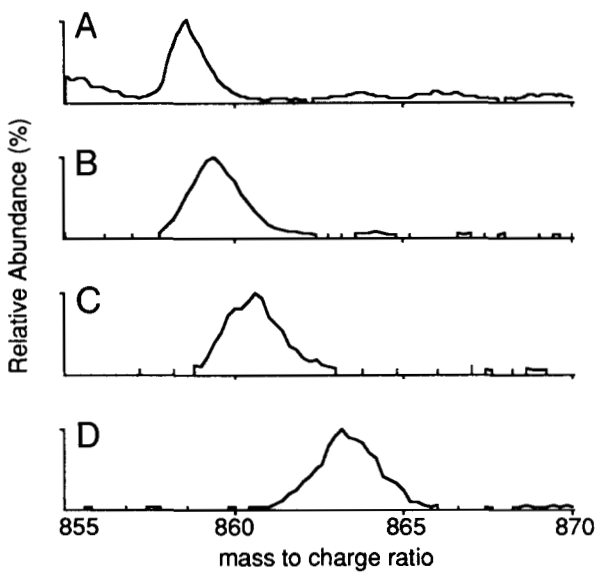


Fig. 3. The MH_3^{+3} ion of the peptide from holo-myoglobin of molecular weight 2,573.1 (encompassing positions 110–134) is shown at various levels of deuterium incorporation. **A:** The triply charged ion of the undeuterated peptide has a centroid at 858.7. **B:** The centroid of the zero time point is 859.49, which corresponds to an M_r of 2,575.5. **C:** The 5-min time point yields a triply charged ion with a centroid of 860.61, which corresponds to an M_r of 2,578.8. **D:** The fully deuterated peptide yielded a signal with a centroid of 863.33, corresponding to a peptide of mass 2,587.0 Da, and incorporation of 14 deuterons in this 25-residue peptide.

Because pepsin cleaves rather unpredictably, the peptide masses are not sufficient for their identification. For this purpose, the fully protonated peptides were fractionated by HPLC and their MS/MS spectra acquired. For example, Figure 4A shows the MS/MS spectrum of the peptide corresponding to positions 110–134 of myoglobin. Although such spectra are not easily interpreted a priori, in the context of the known amino acid sequence of the protein undergoing deuterium exchange, this interpretation is usually trivial. A computer program has been written in-house whereby a protein sequence is hypothetically cleaved in a random fashion, and all of the sequences that match the observed molecular weight of a peptic peptide are scored and ranked according to the degree to which each sequence accounts for the observed fragment ions in the MS/MS spectrum. Thus the MS/MS spectra of all of the undeuterated peptic peptides (including the larger peptides in excess of 3–4 kDa) were acquired within a few hours, and their interpretation was accomplished in a similar period of time. For example, Figure 4B shows the program output for the spectrum of Figure 4A. The undeuterated peptides used in these MS/MS analyses were obtained by collecting fractions from one of the time points and allowing them to back-exchange to the fully protonated form. The peptides identified using this procedure are indicated in Figure 5.

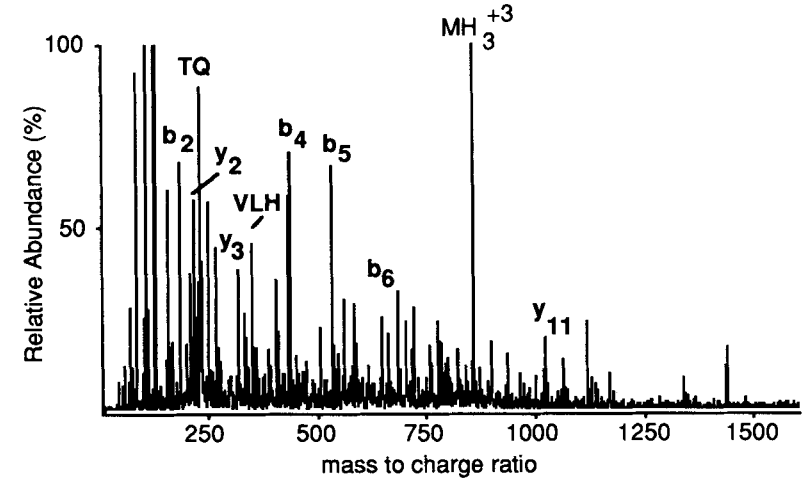
Figure 6 shows data for 3 peptides for which the deuterium incorporation rates did not differ significantly between holo- and apo-myoglobin. The zero time point and fully deuterated controls were measured twice, and 2 separate measurements were also made for the 270-min time point. Most of the peptides appeared in more than 1 charge state, thereby providing multiple mass measurements and better estimates of errors (confidence limit of 67%). In those cases where only single mass measurements were possible, the error was assumed to be ±0.25 Da.

Other apo- and holo-myoglobin peptides were found to have significantly different deuterium exchange rates; these encompassed positions 33–55, 56–69, 70–106, and 138–153 (Fig. 7A, B,C,D, respectively). Additional peptides overlapping those shown in Figures 6 and 7 were observed, and are indicated in Figure 5. Differences in deuterium exchange rates are more striking in the case of 3 tripeptides (Fig. 8A,B,C). Whereas the peptide encompassing positions 30–32 appears to incorporate more deuterium in the apo-protein, the deuterium incorporation rates of peptides 104–106 and 135–137 are more drastically altered. These smaller peptides contain only 2 amide hydrogens and therefore provide very specific information concerning particular amide hydrogens (i.e., increased spatial resolution of exchange sites). However, this is at the expense of increased measurement error. For the case of a tripeptide containing only 2 amides, 50% back-exchange of the deuterons during the peptic digestion and HPLC would result in a maximum mass increase of only 1 Da; thus, gradations between 0% and 100% deuterium incorporation must be inferred from very small changes in mass with increased error.

Discussion

Earlier studies utilizing ESI/MS for the measurement of deuterium incorporation infused proteins dissolved in D_2O directly into the mass spectrometer (Katta & Chait, 1993; Wagner & An-

A



B

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Myoglobin sequences:
Peptide molecular weight:  2573.1  Charge:  3
   .926  [D] 110- 134 [L]  AIIHVLHSKHPGNFGADAQGAMTKA
   .467  [D]  45-  66 [V]  KFKHLKTEAEBKASEDLKKHGT
   .430  [I]  31-  51 [E]  RLFTGHPETLEKFDKFKHLKT
   .330  [L]  50-  73 [G]  KTEAEBKASEDLKKHGTVVLTALG
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Fig. 4. Example of the identification of a peptic peptide from myoglobin. **A:** MS/MS spectrum of the MH_3^{+3} precursor ion of m/z 858.7. Some of the ions have been labeled according to the nomenclature of Biemann (1990b), and the single-letter amino acid code has been used to denote the more abundant internal fragment ions. Unlabeled ions below m/z 130 are amino acid immonium ions; the 2 small yet prominent ions at m/z 1,118.9 and 1,436.8 were not identified. **B:** Computerized interpretation of tandem mass spectral data. The MS/MS spectrum of A, the precursor ion mass-to-charge ratio, and the precursor ion charge were used as input. All sequences of molecular weight 2,573.1 that can be derived by random cleavage of myoglobin are identified and assigned scores (left column) based on the fraction of product ion intensity that can be accounted for by each sequence. The highest ranked sequence encompasses positions 110–134 of myoglobin and can account for 93% of the fragment ions present in the MS/MS spectrum of A. The other sequences of molecular weight 2,573.1 are less able to account for the MS/MS fragment ions.

deregg, 1994). This procedure, however, is not applicable for protein preparations containing exogenous components that are required for protein stability or solubility but incompatible with ESI, e.g., salts, nonvolatile buffers, or glycerol. For proteins that can be eluted from reversed-phase columns, LC/MS may be a more generally applicable method for the measurement of amide hydrogen exchange rates due to the chromatographic removal of contaminants. With the exception of the NδH proton of arginine, the deuterons on amino acid side chains are rapidly back-exchanged upon exposure to the protonated solvents used in LC/MS (Molday et al., 1972; Bai et al., 1993); however, the structurally informative amide hydrogens can still be measured.

In cases where measurement of amide hydrogen exchange by NMR is possible, this method of analysis is preferred because kinetics for individual amide protons can be measured (Englander & Mayne, 1992). However, the high sensitivity of LC/MS, especially when using microbore or packed capillary HPLC columns, should be of advantage when studying proteins available only in subnanomole quantities or those insoluble at millimolar concentrations. The LC/MS procedure could be carried out

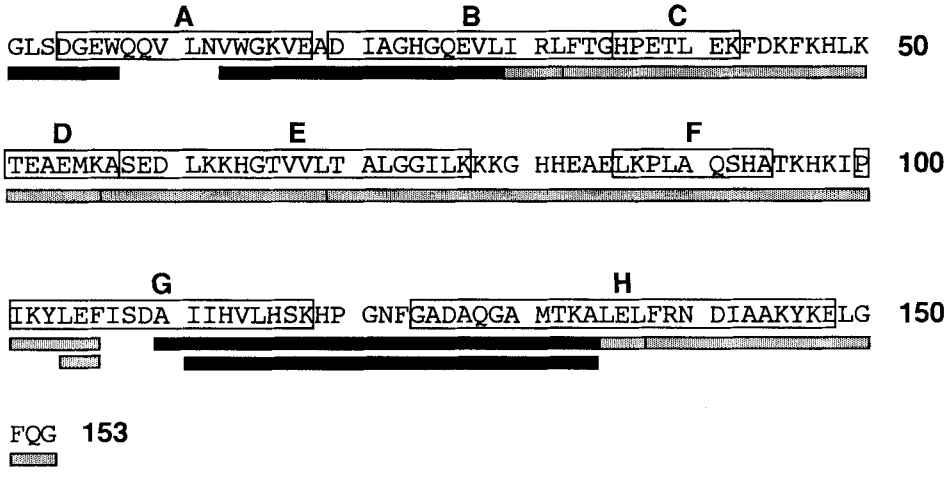

Fig. 5. Peptic peptides from equine myoglobin, identified by the tandem mass spectrometric procedures of Figure 4, are indicated by shaded bars beneath the sequence and encompass approximately 95% of the protein sequence. Filled bars denote regions defined by peptic proteolysis where deuterium exchange rates were nearly identical for apo- and holo-myoglobin; stippled bars denote regions where exchange rates were substantially different. Regions of the sequence that encompass the A–H helices are labeled and enclosed in boxes.

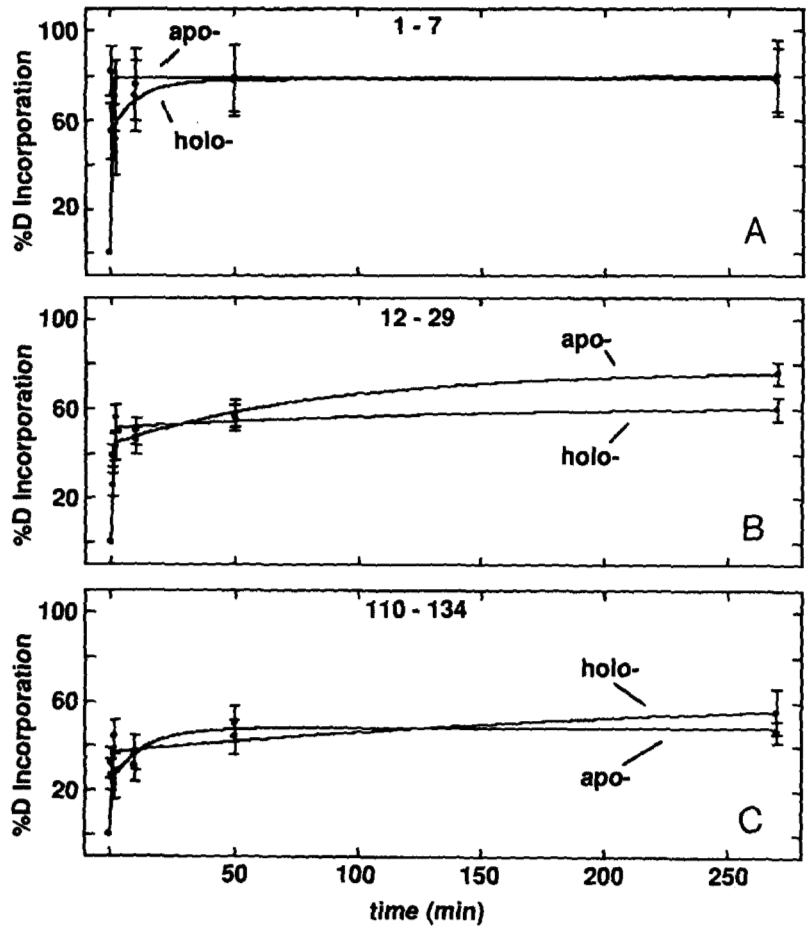

Fig. 6. Peptides from apo- and holo-myoglobin that exhibit similar deuterium in-exchange curves encompassed residues 1–7 (**A**), 12–29 (**B**), and 110–134 (**C**). Data were acquired and analyzed as described in the *Materials and methods* section.

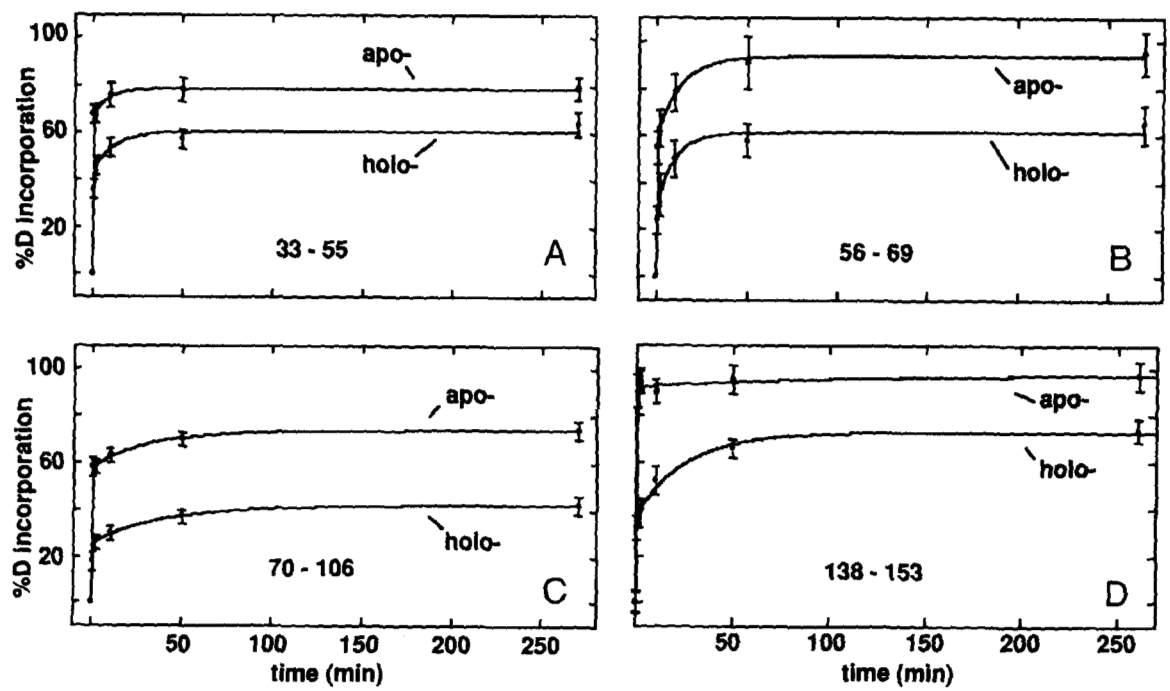

Fig. 7. Peptides from apo- and holo-myoglobin that exhibit distinctly different deuterium in-exchange curves encompassed residues 33–55 (**A**), 56–69 (**B**), 70–106 (**C**), and 138–153 (**D**).

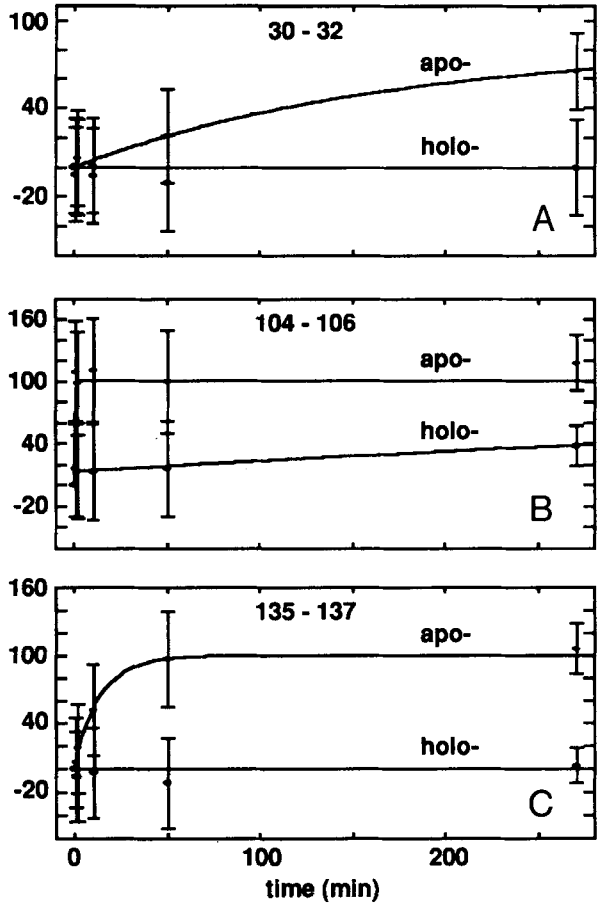

Fig. 8. Compared to larger peptides, tripeptides from apo- and holo-myoglobin provide more focused information concerning exchange rates, but the smaller mass differences between fully deuterated and undeuterated tripeptides are subject to increased error. The apo-myoglobin tripeptide 30–32 (**A**) (top curve) may be slightly destabilized relative to its counterpart in holo-myoglobin (bottom curve). In contrast, the peptides 104–106 (**B**) and 135–137 (**C**) seem to be drastically altered in apo-myoglobin (top curves) compared to holo-myoglobin (bottom curves).

on large glycoproteins, protein aggregates, or other large complexes as long as portions of the protein can be digested with pepsin or other acid proteases.

The HPLC-FAB instrumentation used in the studies of Zhang and Smith (1993) has been reported to be subject to suppression effects due to coeluting compounds (Hemling et al., 1990). Moreover, this technique is usually less sensitive than ESI/MS, and has a lower mass range. It is not surprising, therefore, that the current study yielded peptides covering 95% of the myoglobin sequence, whereas the flowing-FAB results on cytochrome *c* provided considerably less. Of course, it can be argued that the more extensive coverage of the myoglobin sequence is a result of the ability to measure higher molecular weight peptides than is possible with flowing-FAB, and that measurements on large peptides are less useful because they provide less definitive identification of amide hydrogens than small peptides.

Deuterium incorporation measurements of intact apo-myoglobin were useful in quickly revealing that solubilization of the lyophilized protein resulted in at least 2 populations exhibiting different exchange rates (Fig. 1). One explanation may be that the dried protein was composed of a mixture of both native and denatured apo-myoglobin. Although the denatured protein folds within 4 s (Jennings & Wright, 1993), it would rapidly incorporate deuterium during this brief period. Similarly, electrospray mass spectra of intact lysozyme that had been pulse labeled using high pD D_2O over time points ranging up to 2 s have been interpreted as evidence of multiple transient folding populations (Miranker et al., 1993). An alternative explanation for the bimodal peaks is that the dried apo-myoglobin had been unwittingly contaminated such that a second peak comprised of an unresolved mixture of potassium and sodium adducts was observed 30 Da higher in mass. This possibility is unlikely because apo-myoglobin from the same stock solution that had first been solubilized prior to addition of D_2O did not result in bimodal peaks (Fig. 1). In all cases, the hydrogen exchange was quenched by the same potassium phosphate buffer (500 mM), and the potassium adduct ions for holo-myoglobin and solubilized apo-myoglobin were always less than 50% intensity relative to the unpotassiated peak. Thus, the consistently broad bimodal peak shapes observed when deuterium incorporation is initiated by the addition of deuterated buffer to dried apo-myoglobin must not be due to alkali metal adduction. Most of the alkali metal contamination (including the 500 mM potassium phosphate quench buffer) is largely eliminated by the HPLC step prior to ionization.

The deuterium exchange curves for intact apo- and holo-myoglobin (Fig. 2) indicate that, within 30 s, apo-myoglobin incorporates 52 more deuterons (corresponding to an additional 35%) than the holo-protein. This large and rapid increased incorporation suggests that the absence of heme exposes many amides or that less hydrogen bonding (i.e., secondary structure) is present in the apo-protein than in the holo-protein. The latter interpretation is in agreement with CD studies showing a difference of 25% in α-helical character (Harrison & Blout, 1965).

Only an incomplete set of NMR-derived hydrogen exchange rates for individual residues within apo-myoglobin has been determined (Hughson et al., 1990), making it difficult to make a complete assessment of the data presented here. Deuterium incorporation for individual residues within CO-myoglobin have also been measured by neutron diffraction after the protein crystal had been soaked in D_2O for several months (Cheng & Schoenborn, 1991). However, the difference in deuterium in-exchange time between this and the current study (several months versus a few hours) makes this comparison unrealistic. Recent 2-dimensional NMR experiments on apo-myoglobin have demonstrated the presence of secondary structure in the N-terminal A, B, and C helices (positions 3–18, 20–35, and 36–42, respectively) and the E and G helices (positions 58–77 and 100–118) (Cocco & Lecomte, 1994). No secondary structure could be identified in the regions corresponding to the D and F helices in holo-myoglobin or in the C-terminal region following the G helix. This report is consistent with the current mass spectral data (summarized in Fig. 5) in that the peptides exhibiting the least differences between apo- and holo-myoglobin in deuterium incorporation rates encompassed the N-terminus (positions 1–7 and 12–29) and a peptide containing parts of the G and H helices (110–134). Although separated in sequence, these 2 regions are juxtaposed in the crystal structure of holo-myoglobin, suggesting that this domain remains intact within apo-myoglobin. In contrast, within 50 min, the apo-myoglobin tripeptide 135–137 was fully deuterated, whereas the same peptide in holo-

myoglobin incorporated no deuterium after 270 min. Likewise, the C-terminal peptide of apo-myoglobin (positions 138–153) was completely deuterated within 30 s, but the same peptide in the holo-protein was only 70% deuterated after 270 min. The rapid incorporation of deuterium into these C-terminal peptides of apo-myoglobin indicates a loss of secondary structure in this region compared to holo-myoglobin. In addition to amides that exhibit altered exchange rates, the other segments shown in Figure 7 most likely contain some amide hydrogens that maintain a similar degree of protection in holo- and apo-myoglobin but cannot be differentiated within the regions defined by peptic proteolysis.

Although hydrogen exchange measurements for peptides typically cannot provide information on individual residues, a comparison of mass differences between holo- and apo-myoglobin at various times allows a few inferences to be made. For example, the time course of mass differences (uncorrected for deuterium loss during analysis) for peptide 56–69 (Fig. 9A) is relatively constant over the period extending from 30 s to 270 min. The simplest explanation is that, within this region of holo-myoglobin, there are 4 amides (correcting for back-exchange) with very slow exchange rates that are dramatically deprotected in the absence of heme. In contrast, Figure 9B shows the same analysis for the peptide 33–55, where the largest mass difference is observed at the earliest time points, suggesting destabilization of amides with intermediate exchange rates. Finally, Figure 9C shows a tripeptide where slowly exchanging protons are slightly deprotected in apo-myoglobin relative to the holo-protein.

In conclusion, LC/MS with ESI can be used to measure amide hydrogen exchange rates for intact proteins or peptic peptides derived from native proteins. Differences in hydrogen exchange rates can be measured for proteins with ligands bound and unbound if the ligand binding affects secondary structure. The comparison of holo- and apo-myoglobin suggests that an N-terminal region and a portion of the G and H helices were unaffected by the removal of the heme, whereas other features of the structure exhibit increased hydrogen exchange rates that may relate to loss of secondary structure.

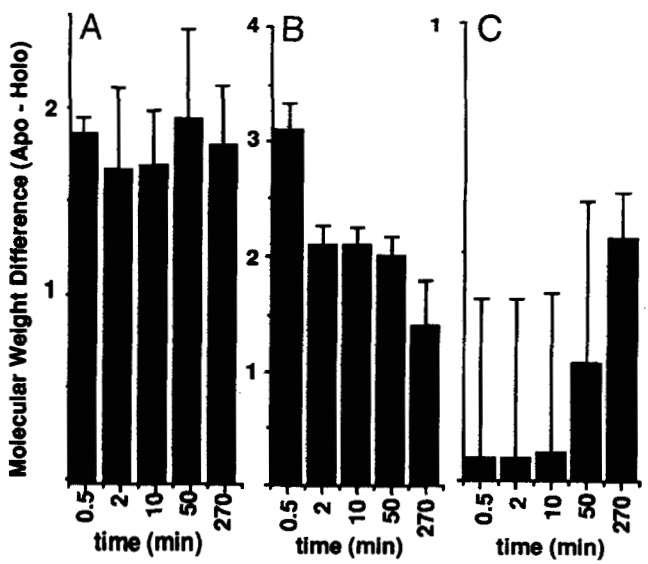

Fig. 9. Molecular weight differences (uncorrected for back-exchange) between apo- and holo-myoglobin peptides 56–69 (**A**), 33–55 (**B**), and 30–32 (**C**) that have not been corrected for hydrogen back-exchange. Panel A depicts a peptide that maintains approximately the same mass difference over 270 min, whereas panel B shows a region where the greatest difference in mass occurs early and diminishes over time. Panel C shows a tripeptide where the greatest molecular weight differences occur after longer time periods.

Materials and methods

Horse skeletal muscle apo- and holo-myoglobin were obtained from Sigma Chemical Company.

For exchange experiments, myoglobin (5 nmol) was first solubilized in 20 μL of 200 mM potassium phosphate, pH 7.2, in H_2O (buffer A) at room temperature for 30 min. Deuterium in-exchange was initiated by the addition of 380 μL D_2O to the solubilized protein. Aliquots of 20 μL were quenched at various times with 20 μL of 500 mM potassium phosphate, pH 2.8, in water (buffer B). Addition of a high concentration buffer ensured that the hydrogen exchange reaction was quenched at the appropriate pH. Phosphate buffer was chosen because one of its pKs (pK_1 = 2.15) is in the range of the desired pH. Quenched samples were immediately frozen in liquid nitrogen and stored at −70 °C until analyzed by LC/MS. Mass measurements of samples prepared 1 week or 1 day prior to analysis gave identical results indicating that back-exchange of deuterium during storage was undetectable.

The actual number of deuteriums incorporated into peptides (or percent deuterium incorporation) was calculated by comparison with 2 controls – a zero time point and the completely deuterated protein. The zero time point was obtained by dissolving 5 nmol myoglobin in 20 μL of buffer A, and adding D_2O (380 μL) previously acidified with 400 μL buffer B. Aliquots of 40 μL were removed and frozen. The completely deuterated protein was produced by incubation in deuterated buffer at pD 7.2 for 5 h at 80 °C. The percent incorporation of deuterium for either peptides or the intact protein, D%, was calculated as follows (Zhang & Smith, 1993):

$$D\% = (m_t - m_{0\%})/(m_{100\%} - m_{0\%}) \times 100, \qquad (1)$$

where m_t is the peptide (or protein) mass at time t, $m_{0\%}$ is the mass of the peptide at time zero, and $m_{100\%}$ is the peptide mass at infinite time (completely deuterated). For data presentation, $\%D$ was plotted against time and fit to a sum of 2 exponentials.

LC/MS was performed on an API-III triple quadrupole mass spectrometer (PESciex, Thornhill, Ontario) equipped with a nebulization-assisted electrospray ionization source. An Applied Biosystems, Inc. (ABI) syringe pump (model 140A) was used to generate HPLC gradients of 0–60% acetonitrile containing 0.03% trifluoroacetic acid at 75 μL/min through a 1 × 30-mm C_8 column. The eluting solvent was directed through a coil of narrow bore stainless steel tubing that was immersed in an ice bath in tandem with a Rheodyne injector and the column. A post-column splitting tee was used to divert approximately two-thirds of the effluent to an ABI model 785A UV detector, with the remainder directed toward the mass spectrometer. Mass spectra were acquired using a step size of 0.2 Da with a dwell time of 0.75 ms at unit resolution. No efforts were made to reduce back-exchange during the electrospray process, although the 100% controls generally showed an overall back-exchange of about 50% during peptic digestion, HPLC, and ionization.

Because D_2O is washed away with the buffer salts, peptides are being electrosprayed only in the presence of H_2O, acetonitrile, trifluoroacetic acid, and any coeluting peptides. Thus, water is in a vast excess over any coeluting partially deuterated peptide and intermolecular scrambling of label during ESI would lead predominantly to a loss of deuterium, i.e., the 100% and 0% controls would approach identity.

Partially deuterated samples were individually removed from the freezer, thawed, and immediately proteolyzed by the addition of pepsin with an enzyme-to-substrate ratio of 1:1 by weight. After 5 min of peptic digestion on ice, the sample was injected onto the C_8 column, and the acetonitrile gradient started after 5 min of desalting. In early experiments, the phosphate buffer was allowed to pass through the electrospray needle, which was directed away from the entrance of the mass spectrometer so as to avoid fouling of the instrument. However, it was found that the phosphate buffer contaminated the needle tip, thereby interfering with the ionization of peptides long after elution of the salts. To avoid this in subsequent experiments, the column was disconnected from the mass spectrometer for the duration of the desalting period and reconnected prior to initiation of the acetonitrile gradient. The molecular weights of partially deuterated peptides were determined by calculation of centroids of the peak envelope.

MS/MS used a step of 0.25 Da and a dwell time of 1 ms with a resolution in the first and third quadrupoles sufficient to transmit 2-3-Da-wide windows. Precursor ions were accelerated to kinetic energies ranging from 15 to 25 eV and collisionally activated with argon in the second quadrupole. The instrument control software reports the collision gas pressure as a "gas thickness," which was typically set to 2.5×10^{14} atoms of argon/cm^3.

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