Determinants of protein hydrogen exchange studied in equine cytochrome c

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Abstract
The exchange of a large number of amide hydrogens in oxidized equine cytochrome c was measured by NMR and compared with structural parameters. Hydrogens known to exchange through local structural fluctuations and through larger unfolding reactions were separately considered. All hydrogens protected from exchange by factors greater than 10³ are in defined H-bonds, and almost all H-bonded hydrogens including those at the protein surface were measured to exchange slowly. H-exchange rates do not correlate with H-bond strength (length) or crystallographic B factors. It appears that the transient structural fluctuation necessary to bring an exchangeable hydrogen into H-bonding contact with the H-exchange catalyst (OH⁻ ion) involves a fairly large separation of the H-bond donor and acceptor, several angstroms at least, and therefore depends on the relative resistance to distortion of immediately neighboring structure. Accordingly, H-exchange by way of local fluctuational pathways tends to be very slow for hydrogens that are neighbored by tightly anchored structure and for hydrogens that are well buried. The slowing of buried hydrogens may also reflect the need for additional motions that allow solvent access once the protecting H-bond is separated, although it is noteworthy that burial in a protein like cytochrome c does not exceed 4 Å. When local fluctuational pathways are very slow, exchange can become dominated by a different category of larger, cooperative, segmental unfolding reactions reaching up to global unfolding.

Keywords: cytochrome c; hydrogen exchange; protein dynamics; proton exchange

The measurement of protein hydrogen exchange (HX) now provides a major source of information on protein structure, energetics, interactions, and folding (Englander et al., 1996; Wand & Englander, 1996), especially when the exchange behavior of individual hydrogens is resolved by NMR analysis (Wagner, 1983; Wuthrich, 1986; Ernst et al., 1988). In spite of much effort over the years (Hvidt & Nielsen, 1966; Woodward et al., 1982; Englander & Kallenbach, 1984; Scholtz & Robertson, 1995) the interpretation of HX results is still limited by our incomplete understanding of the mechanism(s) of hydrogen exchange.

At the level of HX chemistry, one knows that exchange is catalyzed by acids and bases in well defined chemical reactions that require direct H-bonding contact between the catalyst and the exchanging group (Eigen, 1964). Structured proteins can block these processes and slow the exchange of many of their hydrogens. To properly interpret structural protection, it is necessary to understand the factors that block exchange and the mechanisms that relieve the structural block and allow exchange chemistry to proceed.

Recent studies have measured hydrogen exchange kinetics in various species of cytochrome c (cyt c) (Gooley et al., 1991, 1992; Timkovich et al., 1992; Marmorino et al., 1993) and other proteins (Goodman & Kim, 1991; Radford et al., 1992; Pedersen et al., 1993; Clarke et al., 1995; Kragelund et al., 1995) and have attempted to correlate HX rate and changes in rate with various aspects of structure. However, one now knows that some protein hydrogens exchange through a global unfolding event, some through subglobal unfolding reactions involving sizable cooperative units of structure, and others through small structural fluctuations (see below). The present work takes these distinctions into account in comparing a large data base of exchange rates in cyt c with various structural parameters.

Results
Data analysis
Quantitative hydrogen-deuterium exchange rates were obtained for 45 main chain peptide group NH hydrogens and 2 side chain
hydrogens in oxidized cyt c (pD 7, 20 °C). This includes all the NHs that exchange with protection factors between $10^3$ and $10^6$. Figure 1 illustrates the different kinds of HX behavior observed.

HX rate data for each residue were fit to a monoexponential decay as in Figure 1 and expressed in terms of the free energy of the apparent opening reaction that dominates the exchange ($\Delta G_{\text{HX}}$), according to Equation 1.

$$\Delta G_{\text{HX}} = -RT \ln K_{\text{op}} = -RT \ln \left( \frac{k_{\text{ch}}}{k_{\text{h}}} \right)$$

The equilibrium constant for the rate-determining structural opening reaction, $K_{\text{op}}$, can be taken as the inverse of the HX protection factor, given by $K_{\text{op}} \approx 1/P = k_{\text{h}}/k_{\text{ch}}$, where $k_{\text{h}}$ is the measured exchange rate and $k_{\text{ch}}$ is the chemical exchange rate expected for the given hydrogen in the absence of structural protection, known from prior studies (Molday et al., 1972; Bai et al., 1993; Connelly et al., 1993). The approximation indicated is good to better than $1/10^3$ when $P > 10^3$, as is true for the present data. Equation 1 holds in the so-called EX2 (bimolecular exchange) limit where structural reclosing is faster than the chemical exchange rate of the exposed hydrogen (Hvidt & Nielsen, 1966). This condition has been verified for cyt c under the conditions used here (Bei et al., 1994).

$\Delta G_{\text{HX}}$ in Equation 1 can be understood in terms of a pre-equilibrium structural unfolding when exchange is dominated by large unfolding reactions that fully expose all the NHs measured. When exposure is less than complete, as may occur when exchange is dominated by local fluctuations, terms that include a rate for solvent species penetration and internal diffusion may then enter (Richards, 1979; Woodward et al., 1982). In this case some reinterpretation in terms of an activation energy contribution may also be required (see chapter 2 in (Englander & Kallenbach, 1984)).

**Measured rates**

Measured exchange rates and calculated protection factors and free energies (Equation 1) for the NHs measured here are in Figure 2 and Table 1 (pD 7, 20 °C). The open bars with up arrows in Figure 2 indicate the lower limit ($1 \times 10^{-3}$ h$^{-1}$) for the HX rate of the very slow Phe10, Leu88, and residues 94-99, which showed no decrease in NMR cross peak volume at the longest exchange time measured (400 h). The diagonally hatched bars represent NHs whose exchange was inaccurately determined as a result of being near the fast border of the experimental time window (see e.g., Fig. 1).

Two slowly exchanging side chain NHs were also measured, the indole NH of Trp59 which H-bonds to a buried heme propionate and the ring N$_{\mu}$H of the heme-ligated His18 which H-bonds to the buried Pro30 carbonyl. The protection factor and $\Delta G_{\text{HX}}$ for the His18 N$_{\mu}$H were calculated assuming that exchange is catalyzed by OD$^-$-ion at the diffusion-limited rate ($k_{\text{h}} = 10^{10}$ M$^{-1}$ s$^{-1}$). (Catalysis by phosphate buffer anion or water are alternative possibilities.) For the Trp59 indole NH, the $k_{\text{h}}$ value reported by Bai et al. (1993) was used.

**Structural correlations: H-bonding**

The availability of high resolution crystallographic structures (Bushnell et al., 1990; Berghuis et al., 1994) and solution NMR structures (Qi et al., 1994a, 1994b, 1996) for cyt c makes possible a direct comparison of site-resolved HX rates with various structural parameters. The correlation plots in Figures 3-5 distinguish hydrogens that exchange by way of large structural unfolding reactions and local fluctuations. This distinction was made on the basis of the dependence of HX rate on guanidinium chloride concentration (Bai et al., 1995), which relates to the amount of surface newly exposed in the transient structural distortion that allows exchange to occur.

All the hydrogens that exchange slowly enough to be measured under the present conditions (protection factor greater than $10^3$) are involved in recognized intramolecular H-bonds, including internal structural water molecules. Further, almost all the hydrogens known to participate in structural H-bonds (Bushnell et al., 1990;
Table 1. HX parameters for oxidized cyt c (pD 7, 20°C)*

<table>
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<tr>
<th>Residue</th>
<th>$k_{ox}$ (h⁻¹)</th>
<th>log P</th>
<th>$\Delta G_{HX}$ (kcal/mol)</th>
<th>Residue</th>
<th>$k_{ox}$ (h⁻¹)</th>
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*a Errors in reported values for $\Delta G_{HX}$ vary from one proton to another depending on cross peak intensity and overlap and on absolute rate but are generally less than 0.1 kcal/mol. Even large errors in $k_{ox}$ translate into small errors in $\Delta G_{HX}$ due to the logarithmic relationship (Equation 1). Errors in intrinsic $k_{ox}$ (Equation 1) are very small but one cannot guarantee that a protected NH on exposure will exchange at the calibrated free NH rate.

*b Extrapolated from HX at higher temperatures.

*c HX measured in 2D NOESY spectra.

*d The value of $k_{ox} = 224/s$ was used.

Berghuis et al., (1994) were seen to exchange slowly. Exceptions involve 5 residues (4 glycines and Glu 21) that were not seen in the spectra due to weak cross peaks and/or degeneracies with other cross peaks or water. Thus, there is essentially a one to one correlation between slow exchange and H-bonding (see also Gooley et al., 1992; Radford et al., 1992; Marmorino et al., 1993). H-bond length is generally assumed to correlate inversely with H-bond strength. Figure 3 compares the protection factor measured for individual NHs (Table 1) with their hydrogen bond length based on the NMR (Qi et al., 1994a, 1994b, 1996) (Fig. 3A) and X-ray (Bushnell et al., 1990; Berghuis et al., 1994) (Fig. 3B) structures. There is no significant correlation between HX slowing and the length (strength) of the protecting H-bond (in agreement with Radford et al., 1992), even for NHs that exchange through local fluctuations. At any given level of protection the H-bonds span the range of lengths.

Structural correlations: Rigidity and burial

It is often assumed that HX rates relate in some way to "looseness" or "rigidity" of structure. Figure 4 illustrates the absence of correlation between HX slowing and B factors obtained for the crystallographic structure (in agreement with Radford et al., 1992; Orban et al., 1995). Insofar as variations in the B factor from residue to residue reflects local molecular dynamics, these observations are against a role for very small atomic displacements in HX rate determination.

Figure 5A compares HX slowing of the different NHs with distance to the nearest molecular surface point. Hydrogens in H-bonded groups at the protein surface, with no protein atoms between the group and solvent water, are plotted at 0 Å. When all the hydrogens are considered, little correlation between HX rate and depth of burial is seen ($r = 0.2$). However there is a tendency for the hydrogens that exchange by way of large unfoldings (open symbols in Fig. 5) to be more deeply buried. For these hydrogens, a large unfolding dominates because alternative local fluctuation pathways, which must exist, provide even slower exchange. A tendency for the slowest exchanging hydrogens in a protein to occur in more buried or "core" positions has been noted before by many workers.

A strong correlation between HX protection and distance from the solvent interface ($r = 0.89$) is seen when only the NHs that are
Discussion

Requirements imposed by HX chemistry

The details of proton transfer chemistry impose stringent requirements on the structural processes that underlie protein HX behavior. Hydrogen exchange depends on proton transfer reactions which require direct steric contact and H-bond formation with HX catalysts (Eigen, 1964), specifically OH\(^{-}\) ion under the conditions usually used for protein studies (above pH ~ 3). Slowly exchanging hydrogens are slow because structure interferes with this attack. One wants to understand how structure blocks H-exchange and the structural and dynamic events that allow exchange to proceed.

Fig. 3. Correlation plot for HX protection and H-bond length. Hydrogen bond lengths are computed from the oxidized NMR (Qi et al. 1994a; panel A) and X-ray (Bushnell et al., 1990; panel B) structures. Filled symbols indicate N\(\text{Hs}\) that exchange via local fluctuations; open symbols are for larger scale subglobal and global fluctuations. When a given NH has more than one possible H-bond acceptor, the shortest bond with NHO angle greater than 135° is used. Computed correlation coefficients are (A) \(-0.05\) and (B) \(-0.2\).

Fig. 4. Correlation plot for HX protection and crystallographic B factors reported for the oxidized X-ray structure (Bushnell et al., 1990). The correlation coefficient is 0.2.

Fig. 5. Correlation plot for HX protection and distance of the exchanging hydrogen from the nearest point on the molecular surface. Zero on the abscissa represents direct contact of the H-bonded group with solvent. Panel A shows all the measured hydrogens (\(r = 0.2\)). Panel B shows the hydrogens in the amino-terminal and 60s helices (\(r = 0.89\); compare Fig. 2). The molecular surface was computed using the oxidized X-ray structure (Bushnell et al., 1990) and a 1.4 Å radius probe (Connolly, 1983). A similar picture is obtained for the NMR structures (Qi et al., 1994a, 1996).
Hydrogen exchange determinants

The results found here agree with prior information indicating that slowly exchanging hydrogens are almost always involved in H-bonding with other protein groups. H-bonding slows hydrogen exchange because the acceptor atoms (O, N, and S) are large enough to essentially envelop the smaller H atom and sterically block effective H-bonding with and proton transfer to OH− catalyst. Burial alone without H-bonding would presumably suffice to slow exchange but the uncompensated burial of even a single polar hydrogen costs roughly 2 kcal/mol in free energy (Honig & Yang, 1995; Pace et al., 1996). The typical protein, stabilized only about 10 kcal/mol altogether, cannot support many such hydrogens. Cyt c provides no such NHs for study.

Local fluctuations and large unfoldings

Under native conditions, most hydrogens in cyt c and other sufficiently stable proteins exchange by way of local fluctuations. Local openings can be recognized by their near-zero dependence on added denaturant (i.e. value; Bai et al., 1995), signifying little new surface exposure in the exchange competent conformation. Also the observation that neighboring NHs show very different protection factors (Fig. 2, Table 1) can indicate that exchange depends on local motions. Other criteria such as dependence on temperature (to be discussed elsewhere) and protein modifications (Perrett et al., 1995; Betz et al., 1996; Neira et al., 1997) can help to distinguish local and global motions.

When local pathways are sufficiently slow, exchange may become dominated by large, usually high energy fluctuations that have the character of sizeable unfolding reactions. The possibility that some hydrogens might exchange through global unfolding was first raised by A. Rosenberg (Rosenberg & Chakravarti, 1968; Rosenberg & Enberg, 1969), was considered analytically by W.P. Bryan in connection with possible solvent penetration pathways for protein HX (Bryan, 1970), and has been pursued especially by C.K. Woodward and her colleagues (Woodward & Hilton, 1979; Woodward et al., 1982; Woodward, 1994). Exchange by way of transient global unfolding has now been shown in many proteins (listed in Englander et al., 1996; Arrington & Robertson, 1997). Hydrogens that exchange through a global unfolding pathway display a $AG_{HX}$ value close to the $AG_{unf}$ value extrapolated from equilibrium melting analysis and often account for the slowest exchanging hydrogens in any given protein. Alternative demonstrations that only compare $\Delta AG_{HX}$ and $\Delta AG_{unf}$ imposed by specific mutations without considering the absolute value of $\Delta G_{unf}$ may be less definitive (e.g., see Betz et al., 1996).

Large but still subglobal unfoldings have recently been shown to determine the exchange of some hydrogens in cyt c (Bai et al., 1995; Bai & Engleander, 1996), ribonuclease H (Chamberlain et al., 1996), and a hyperthermophile rubredoxin (Hiller et al., 1997). Exchange by way of large unfolding reactions can be recognized by the sharp dependence of rate on denaturant concentration, which relates to surface exposure in the unfolding reaction. Moreover, when the exchange of neighboring and non-neighboring NHs is dominated by the same large unfolding pathway, the different NHs exhibit common protection factors, independently of their local structural situation in the native protein.

The behavior to be expected for HX that is determined by large unfolding has been discussed (Qian et al., 1994; Perrett et al., 1995; Loh et al., 1996) in terms of the phenomenological EX1 and EX2 behavior considered in classical HX work (Hvidt & Nielsen, 1966). Hydrogens that exchange by way of global unfolding may exhibit EX1 behavior when the reclosing rate is sufficiently slowed (Kiefhaber & Baldwin, 1995; Perrett et al., 1995) or when $kch$ (Equation 1) is made sufficiently fast (Arrington & Robertson, 1997). Local fluctuations appear always to produce EX2 exchange. The more mechanistic issues including the parameters that determine local fluctuations and large unfolding remain to be understood. The present work considers some possible determinants.

H-bond separation

Much prior work has tended to focus on burial itself as the cause of slow exchange (Lumry & Rosenberg, 1975; Richards, 1979; Woodward et al., 1982) with the assumption that contact with solvent is sufficient to accomplish exchange. All the slowly exchanging hydrogens measured here are involved in H-bonding, including some side chains and water molecules as acceptors. The results show that even continuing contact of an H-bonded group with water is insufficient to permit the proton transfer reaction. H-bonded NHs, essentially at the cyt c surface, exchange more slowly than the free peptide rate by large factors, up to $10^{9}$ (Fig. 5A). Similarly H-bonded hydrogens in small molecule models are slowed by many orders of magnitude even though the group is in direct contact with water (Eigen, 1964; Haslam & Eyring, 1967; Rose & Stuehr, 1968). The present results indicate that direct abstraction of an H-bonded hydrogen on contact with OH− catalyst without H-bond separation has an efficiency less than $10^{-11}$ per collision (from the $10^{9}$ protection factor for Ala96NH and the fact that the efficiency for fully exposed NHs is $<10^{-2}$ (Englander & Kallenbach, 1984)).

These considerations, together with the known chemical requirements for exchange of unprotected hydrogens, indicate that the exchangeable hydrogen itself must be unmasked and made accessible for H-bonding contact with the catalytic solvent species. The larger nitrogen and oxygen atoms in the donor and acceptor groups within an H-bond block this steric contact. Evidently, it is necessary to transiently separate the H-bond donor and acceptor in order to allow the exchange process to proceed. The large unfavorable free energies found even for NHs at the protein surface, in the range of 4 kcal/mol and higher, indicate that the structural distortions required, although local, are difficult to achieve.

The present results show no correlation of local fluctuational exchange with local structural parameters including H-bond strength and X-ray B factors. The small free energy of the H-bond, or rather the even smaller variation from one H-bond to another, cannot contribute significantly to the large protection factors found here, greater than $10^{7}$ (4 kcal/mol). Also the facile and frequent H-bond breakage inferred in molecular dynamics simulations appears to be insufficient to account for exchange, as are the fluctuations that dominate measured X-ray B factors. One can infer that the fluctuations that matter must significantly displace the proton and/or the acceptor atom, by 3 Å at minimum, making it possible to interpose and H-bond with a OH− ion. (For more discussion, see chapter 6 in (Englander & Kallenbach, 1984).)

It is interesting that the most exposed helical NHs approach a minimal protection factor of 4,000 (see Fig. 5B). This value is above the inverse Zimm-Bragg parameter, l/\sigma, given as ~400 for a polyalanine-based helix (Chakrabartty et al., 1994), which expresses the statistical-mechanical equilibrium constant for the opening of H-bonds in the middle of a helix. The relation between these values remains to be examined.
Burial, H-bond breakage, and solvent access

In amphipathic helices, H-bonded hydrogens that are removed from the protein surface tend to exchange more slowly than their neighboring NHs on the solvent-exposed surfaces (Fig. 5B). Also NHs that are relatively deeply buried, i.e., in the range of 2 to 4 Å, have very slow local fluctuational pathways (Fig. 5A).

Why does burial slow H-exchange? One apparent possibility is that H-bonded groups that are buried are more difficult to separate sufficiently due to the resistance to distortion of immediately neighboring structure. An illuminating example is provided by Ala96NH which exchanges exceedingly slowly even though it happens to be essentially at the protein surface. Ala96 is in the C-terminal helix which runs more or less orthogonally between two other roughly parallel helices (the N-terminal and the 60s helices). Its large protection against exchange appears to be due to the resistance to motion of neighboring structure. Both of its immediate neighbors, Ile95 and Tyr97, are anchored in interhelical contacts. The H-bond acceptor, the Glu92 carbonyl, appears to be similarly protected. In the algorithm of Hilser and Freire (1996) such local factors occur within a window size of 3.

Another possible determinant is solvent access after opening. Once a buried H-bond is separated, further motional distortions may be necessary to obtain solvent access and expose the hydrogens to HX catalyst. On the other hand, it is noteworthy that most NHs are very close to the protein surface. NHs on the buried surface of an α-helix are removed from solvent by only a few angstroms, essentially the diameter of a single atom. The most deeply buried NHs in cyt c are only 4 Å from the surface (Fig. 5). Thus the motional distortion necessary to sufficiently separate H-bonded groups, 3 Å or more, may in itself often provide satisfactory access to bulk solvent.

In summary, results available suggest that HX slowing is most directly connected with H-bonding, that a large separation of the H-bond donor and acceptor is necessary for exchange to occur, and that the detailed motions involved are inhibited by the resistance to distortion of immediately neighboring structure.

Materials and methods

Horse heart cytochrome c was from Sigma Chemical Co. (highest grade). D₂O was from Isotec (>99.9% D). All chemicals were reagent-grade. A stock solution of cyt c (13 mM, in 0.1 M sodium phosphate buffer in H₂O at pH 7.0) was prepared containing excess potassium ferricyanide to ensure complete oxidation. For each hydrogen exchange time point, a 0.5 mL aliquot was transferred into D₂O exchange buffer (50 mM potassium phosphate, pH 7.0) by centrifugal gel filtration (3 mL spinning gel filtration columns, Sephadex G-25) into D₂O exchange buffer (50 mM phosphate buffer, pH 7.0) by centrifugal gel filtration (3 mL spinning gel filtration columns, Sephadex G-25 (Jeng & Englander, 1991)). Sixteen samples were incubated at 20°C in a thermostatted water bath for time periods ranging from 2 min to 400 h in increments of ~2-fold. The exchange reaction was quenched by adding 15 μL of a 1.0 M l-ascorbic acid solution at pH 5.0 and 0°C, resulting in a reduced cyt c solution at pH 5.3. The protein was then transferred (centrifugal gel filtration) into NMR analysis buffer (D₂O, pH 5.3, 20 mM potassium phosphate, 20 mM deuterated acetic acid, 12 mM l-ascorbic acid, with TSP).

To follow H-D exchange in time, proton NMR spectra (magnitude mode COSY; Aue et al., 1976; Nagayama et al., 1980) were recorded for each quenched cyt c sample (reduced protein, pH 5.3, 20°C) on a Bruker AM 500 spectrometer (64 transients plus 4 dummy scans of 1,024 complex data points for each of 512 time increments, spectral width 9,090 Hz in both dimensions, water suppression by weak presaturation for 1.2 s). Data were processed on a Silicon Graphics workstation using the Felix software package (Biosym Technologies, San Diego, CA), with an unsnipped sine bell window function and 2 Hz line broadening in both dimensions. The HX analysis used resonance assignments of the reduced form (Wand et al., 1989).

H-D exchange was measured by the time dependent decrease in integrated volumes for resolved NH-C-H cross peaks. Raw cross-peak volumes were measured. An appropriately scaled baseline footprint was subtracted (average of baseline volumes in empty areas of the fingerprint region, scaled to the size of each crosspeak footprint). Volumes in each spectrum were normalized to the non-exchanging heme bridge 4 CH₂CH crosspeak in the fingerprint region. The volume of cross peaks at zero exchange time was obtained by initially spinning samples directly into low pH NMR buffer. Exchange rate was determined from a non-linear least-squares exponential fit with a residual term which may be due to residual water in the exchange buffer sample or spectral artifacts. Where crosspeaks overlapped (e.g., Lys8 and Lys73 or Glu12 and Thr102), a single footprint was used and the data were fit to biexponential decays.

Structural comparisons made here utilized the crystal structure of oxidized horse cyt c refined to 1.9 Å (Bushnell et al., 1990) (PDB#1HRC) and the solution NMR structures (Qi et al., 1994a, 1996) (PDB#1FRC and A.J. Wand, pers. comm.). Distance from an exchanging NH to the protein molecular surface was determined by the Connolly algorithm (Connolly, 1983) using a 1.4 Å probe radius and is graphed after subtraction of 1.2 Å so that an H-bonded group at the protein surface is shown at zero distance.

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References

Hydrogen exchange determinants


