Internal Protein Motions, Concentrated Glycerol, and Hydrogen Exchange Studied in Myoglobin†

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ABSTRACT: Experiments were carried out to measure the effect of concentrations of glycerol on H-exchange (HX) rates by using myoglobin as a test protein. Concentrated glycerol has only a small slowing effect on the HX kinetics of freely exposed amides, studied in a small molecule model (acetamide). Larger effects occur in structured proteins. The effect of solvent glycerol on different parts of the HX curve of myoglobin was studied by use of a selective "kinetic labeling" approach. Concentrated glycerol exerts an apparently reverse effect on protein H exchange; the faster exchanging "surface" protons are least affected, while the slower and slower amide NH is further slowed by larger and larger factors. These results seem inconsistent with solvent penetration models which generally visualize slower and slower protons as being placed, and undergoing exchange, farther and farther from the solvent–protein interface. On the other hand, the results are as expected for the local unfolding model for protein H exchange since concentrated glycerol is known to stabilize proteins against unfolding. In the local unfolding model, slower exchanging protons are released by way of higher energy and therefore generally larger, unfolding reactions. Larger unfoldings must be more inhibited by the glycerol effect.

It has long been appreciated that the hydrogen-exchange behavior of proteins is determined by some aspect of their internal motional character (Linderstrom-Lang & Schellman, 1959; Hvidt & Nielsen, 1966); the kind of motion at work is still a matter of debate, and a variety of models have been put forward. This issue is of importance since the effective utilization of H-exchange measurements to provide information on protein structure, structure change, and structural dynamics depends upon its resolution. A great deal of work directed at this issue has dealt with the effects of variables such as pH, temperature, organic cosolvents, and pressure, but these results have not resolved the central question of hydrogen-exchange mechanism [reviewed in Woodward & Hilton (1979), Barksdale & Rosenberg (1982), and Englander & Kallenbach (1984)].

A major distinction between proposed models for the protein H-exchange process relates to the solvent environment of the rate-limiting proton transfer event. Solvent penetration models suggest that the exchange process involves entry of solvent catalysts (water, hydroxide, and hydrogen ions) into the protein matrix, where the proton transfer event occurs. Unfolding models suggest that protein H exchange involves the transient cooperative unfolding of small regions of protein structure, so that H bonds are broken and the hydrogens are brought into H-bonding contact with bulk solvent, where the proton transfer event occurs.

Solvent viscosity might be expected to exercise recognizably different effects in these different cases. We set out to study the effect on myoglobin H exchange of solvent viscosity using concentrated glycerol solutions. As it turns out, the effects observed do not seem to relate to viscosity. Solvent glycerol has little effect on the H exchange rate of freely exposed protons. However, exchange rates of the structurally slowed hydrogens of proteins are quite sensitive to added glycerol, and a most interesting pattern of sensitivity is seen. The progressively slower hydrogens are more and more affected. Since this behavior cannot be explained by an effect of solvent glycerol on H-exchange chemistry, it appears to reflect the effect of concentrated glycerol on those protein fluctuations that determine protein H-exchange rate.

The present experiments show that those motions that occur most easily and determine exchange of the rather fast protons in myoglobin are hardly affected by solvent glycerol, while the more improbable fluctuations, which are responsible for the slowest protons, are rendered much more difficult. The implications of these results can be considered in terms of alternative models for the protein H-exchange process.

In this work it was desirable to experimentally distinguish the sensitivity to solvent glycerol of protein hydrogens according to their exchange rates. We utilized a "kinetic labeling" approach, which made it possible to differentially label protons according to their exchange rate and thus selectively study at increased resolution the graded effects of solvent character on these different rate groupings. It appears that the kinetic labeling approach may offer certain advantages for other studies of individual protons, or kinetic sets of protons, in macromolecules.

Materials and Methods

Sperm whale myoglobin was from Sigma Chemical Co. All exchange experiments used the cyanomet form in solution with 0.3 M NaCl at 12 °C. For full labeling, myoglobin was exchanged-in in solution at pH 10 with added tritiated water (ca. 10 mCi/mL) at 40 °C for 20 h (Englander & Staley, 1969). This was done in the absence of added buffer, so that the pH could subsequently be easily lowered to working values. To keep pH from dropping during the extended exchange-in period, the closed tube holding the myoglobin solution was itself kept in a tightly closed jar containing concentrated NaOH solution to trap incoming CO_2. After the exchange-in period, solution pH was lowered to the desired value by addition of tiny volumes of HCl, and exchange-out data were gathered by the gel filtration method (Englander & Englander, 1978).

For the kinetic labeling experiments, exchange-in proceeded at 12 °C for only 1.5 h in 0.3 M NaCl and 7 mM KCN, at various pH values as indicated. Passage through Sephadex

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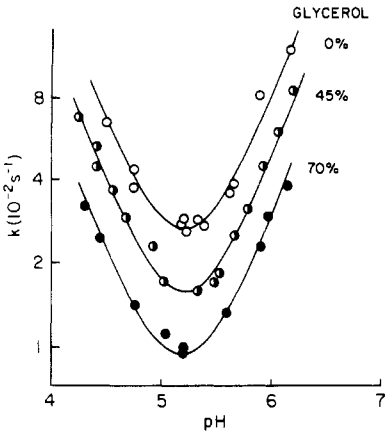

FIGURE 1: Effect of concentrated glycerol on H-exchange chemistry. The pH dependence of acetamide H–D exchange was measured by a spectrophotometric method in the glycerol concentrations indicated (w/w) at 12 °C. The theoretical specific acid–specific base curve (eq 1) was fit to the measured data.

columns then removed the free tritium and initiated exchange-out. These initial columns, at 0 °C, were washed with buffers containing 0.1 M NaCl, 10^{-4} M KCN, and 0.1 M pH buffer (acetate, phosphate, or β-alanine). Protein samples from the gel filtration columns were adjusted to the desired glycerol concentration by mixing preweighed amounts of glycerol with measured amounts of sample. The glass electrode read, with the various mixtures, pH 5.5, 7.4, and 9.8–9.9, respectively. In the first two, pH meter reading did not change with added glycerol. At the higher pH, runs with no glycerol were initiated at pH 9.8; samples to be diluted with glycerol were initiated at a pH reading of 10, since glycerol addition produced a drop of 0.1–0.2 pH unit. These samples were placed in a 12 °C bath and allowed to exchange out. Samples were taken from the solutions as a function of time, passed through Sephadex columns (0 °C) to remove free tritium, and then assayed for carried tritium in the usual way (Englander & Englander, 1978). Further details on H-exchange methodology can be found in Englander & Englander (1978) and earlier numbers in that series.

RESULTS

Calibration of Glycerol Effects. Amide HX rate depends primarily upon the exchange catalysts, hydrogen and hydroxyl ions. Thus, high concentrations of glycerol can influence HX rate through solvent viscosity but also may operate by affecting pH meter readings (Gelsema et al., 1977), the dissociation constant of water, and the activity of water, hydrogen ion, and hydroxide ion (Colvin, 1925). Figure 1 calibrates the summed effects of all these on the HX kinetics of freely exposed amides. Here acetamide was used as a convenient model system. Its amide protons respond to hydroxide and hydrogen ion activity and to solvent conditions in the same way as polypeptide NH, and the HX behavior of acetamide can be easily measured spectrophotometrically (Takahashi et al., 1978; Englander et al., 1979). Acetamide, initially in D_2O, was diluted 20-fold into H_2O or H_2O–glycerol mixtures buffered at the desired apparent pH, set with a standard glass pH electrode. The subsequent amide ND to NH exchange was followed in a Cary 118 spectrophotometer by means of the isotope-dependent absorbance change in the amide region (230 nm). Acetamide, having two protons per molecule, generates a conveniently large isotope-dependent absorbance change (~10% of total absorbance), and traces were accurately first order in all cases.

Figure 1 shows exchange rate constants obtained in this way as a function of pH and glycerol concentration at 12 °C. The abscissa of Figure 1 gives the direct pH meter reading in these solutions, and the ordinate is the measured ^2H to ^1H exchange rate plotted on a logarithmic scale. Curves fit to the data in Figure 1 represent the theoretical U-shaped-specific acid-specific base catalysis curve determined, as shown in eq 1, by

$$k_{ex} = k_H[H^+] + k_{OH}[OH^-] \quad (1)$$

ambient hydrogen and hydroxyl ion activities, and their respective second-order rate constants. The results show that glycerol has a small effect, slowing both the acid- and base-catalyzed rates approximately equally. Glycerol at 45% concentration (w/w) slows HX rates by a factor of 1.7 and 70% glycerol by a factor of 2.9. Relative viscosity in these solutions is 5.3 at 45% glycerol and 28 at 70% glycerol (12 °C).

As with acetamide, the exchange of peptide group NH in polypeptides is also governed by eq 1, although somewhat different acid and base rate constants obtain due largely to inductive effects of neighboring peptide groups (Leichtling & Klotz, 1966; Molday et al., 1972). This places the U-shaped rate curve for polypeptides at a somewhat lower pH than found for acetamide, with the rate minimum below pH 3. Thus, the measurements made in this work on the peptide NH of myoglobin, at pH 5.5 and above, fall on the alkaline limb of the pH-rate curve. The effects of added glycerol on the chemical step of the H-exchange process, measured in acetamide (Figure 1), can be confidently expected to occur identically in polypeptides since they reflect general solvent effects. At the level of structured proteins, however, one should not be surprised to find additional effects operating through some influence of concentrated glycerol solutions on that part of the internal dynamical behavior of the protein that mediates the H-exchange process. It is just these effects that the present experiments were designed to study.

Myoglobin Exchange after Equilibrium Labeling. The slowly exchanging protons of myoglobin are widely spread out on the HX time axis, covering a scale about 8 decades wide (Englander & Staley, 1969; Abrash, 1970; Nakanishi et al., 1974). Conditions that bring different parts of the overall, multidecade H-exchange curve of myoglobin into a convenient time window are defined in Figure 2. In these experiments, myoglobin in the cyanomet form (Fe^{3+}·CN^-) was fully labeled in tritiated water so that even the slowest protons reach exchange equilibrium with solvent ^3H and ^1H. Then, tritium exchange-out data were gathered as indicated under Materials and Methods.

The uppermost curve in Figure 2, measured at pH 5.5 and 12 °C, displays the behavior of about 40 H's that exchange in the early time region of the overall myoglobin HX curve. The exchange-out curve, plotted on a linear time scale, then appears to level off because, in order to see a significant number of the next slower sites, it is necessary to scan another decade in time, e.g., out to perhaps 50 h. More conveniently one can speed all rates by ~100-fold by raising the pH by 2 units. The pH 7.4 exchange-out data (middle curve in Figure 2) monitors about 35 H's from the midpart of the overall myoglobin HX curve. At pH 9.8 about 25 of myoglobin's still slower protons are brought into a convenient time window. (Temperature could be used similarly.) The protons measured in these experiments represent almost wholly the internally H-bonded peptide NH of myoglobin (Englander & Staley, 1969), which are about 115 in number (Takano, 1977).

Myoglobin Exchange Studied after Kinetic Labeling. The data shown in Figure 2 serve to calibrate—in terms of pH, temperature, and time—convenient observational windows for the different NH of myoglobin. For example, Figure 2 shows

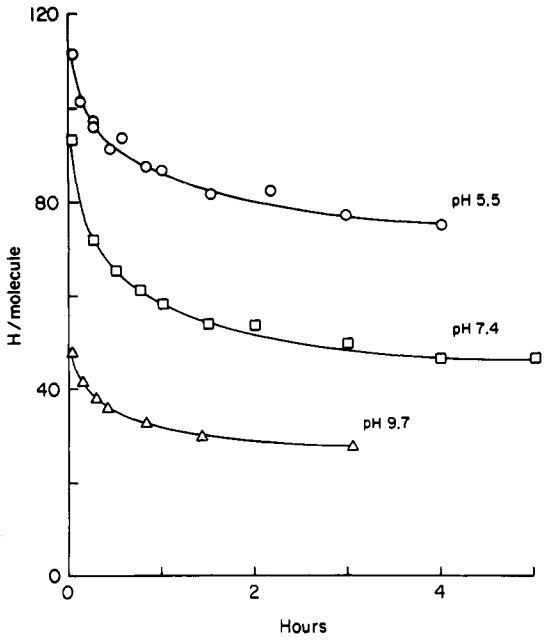

FIGURE 2: Exchange of fully labeled myoglobin at various pH. Cyanometmyoglobin, after labeling to equilibrium by rigorous exchange in tritiated water, was exchanged-out at 12 °C and various pH values to bring different parts of the multidecade HX curve into the laboratory time window.

Table I: Glycerol Slowing Factors[a]

pH	H range (approx)	H level	slowing factor t_{45}/t_0	slowing factor t_{70}/t_0
5.5	100–85	28	1.7	3.2
		23	1.8	4.1
		16	1.9	5.0
		10	2.8	6.8
7.4	80–60	30	3.1	6.5
		25	2.6	7.1
		20	2.4	8.3
		14	2.9	11
9.8	45–30	18	3.0	11
		16	3.3	12
		11	3.0	18

[a] Entries are read from the results in Figure 3, with each pH set covering the range of protons numbered by use of Figure 2 and 3. Slowing factors are given in terms of the extended time required for HX curves to fall to the value indicated in the added glycerol. The time ratios are keyed to the percent glycerol used (0%, 45%, and 70%). In 45% and 70% glycerol at 12 °C, where relative viscosity is 5.3 and 28, respectively, free amide HX rates are slowed by factors of 1.7 and 2.9.

that, in the time window between several minutes and 1.5 h at pH 5.5 and 12 °C, one will see the exchange of protons numbered approximately 110 to 80 in the myoglobin rate sequence, at pH 7.4 the protons 80 to 50, and at pH 9.8 protons 45 to 30. A kinetic labeling maneuver can produce protein samples with label largely on these sites, rendering them suitable for observation and all the rest essentially invisible. Here, one exchanges-in initially in tritiated water for only a limited time period, calculated to label the sites to be observed but not slower ones. Exchange-in conditions are selected by use of the calibrating curves (Figure 2); exchange-out of the selectively labeled protons can then be set to any convenient time scale by suitably adjusting the pH, temperature, etc.

Figure 3 shows some results obtained in this way. In the middle panel, for example, myoglobin was labeled in tritiated water (THO) for only 1.5 h at pH 7.4. According to Figure 2, this labels proton sites numbered approximately 50 and higher (faster) in the myoglobin rate sequence. The partially

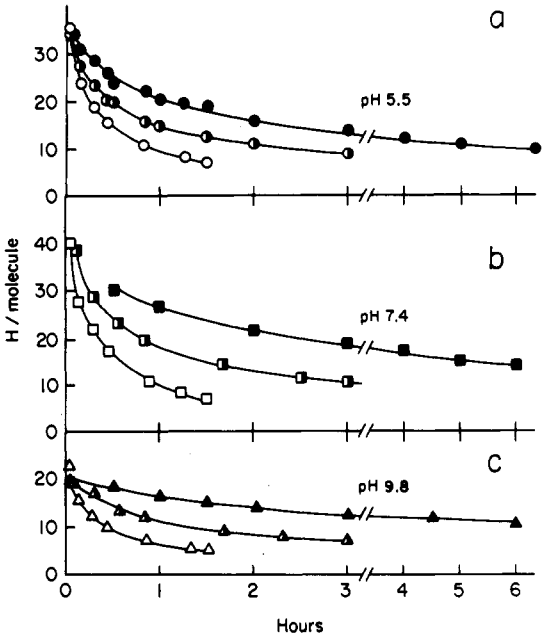

FIGURE 3: Exchange-out of partially labeled myoglobin. Cyanometmyoglobin was initially labeled for 1.5 h at the pH indicated and then exchanged out at the same pH (all at 12 °C). This kinetic labeling maneuver, defined in eq 3, exhibits at enhanced resolution the corresponding fractions (Figure 2) of the overall HX curve (eq 2). Myoglobin eluant from the first Sephadex column was diluted into glycerol (45% and 70% final concentration), and comparative HX data were gathered. Symbols indicate glycerol content as in Figure 1.

labeled sample was then passed through a short Sephadex column to remove free THO and initiate exchange-out of the bound tritium label. At this point the experimenter has a sample of myoglobin with the faster half of its exchanging peptide NH labeled. In the present work we set the gel filtration run in Figure 3b at pH 7.4. Exchange-out measurements then focus on the same sites just mentioned, those numbered 80 to 50, seen to exchange at pH 7.4 in Figure 2. Here, these exchange out on a time scale of a few hours, conveniently for the present experiments. [Other conditions could be set: for example, at pH 5.5 the same protons would exchange far more slowly (Figure 1), e.g., conveniently for two-dimensional NMR studies.] Again, here these are the only sites labeled so that their behavior is effectively isolated and can be observed at higher resolution, as in Figure 3b. The other data in Figure 3 were obtained similarly; each panel focuses on a kinetically selected grouping of peptide NH which appears in a particular part of the overall myoglobin exchange curve.

In these exchange-out experiments, eluant samples obtained from the first gel filtration column were diluted into various glycerol solutions with pH chosen as just described, and subsequent HX behavior was measured. Comparative HX data could then be obtained for each kinetically selected grouping of protons in different concentrations of added glycerol (Figure 3).

Effects of Glycerol. Figure 3 shows, for each kinetic grouping, the exchange behavior observed in the absence of glycerol and in the presence of 45% and 70% glycerol. Added glycerol slows the exchange of all of myoglobin's hydrogens, but the different H's display differing sensitivity. Table I summarizes these results. For example, at pH 5.5 the level of 16 H's remaining is reached after 24 min in 0% glycerol (t_0 = 24 min), after 47 min in 45% glycerol (t_{45} = 47 min), and after 120 min in 70% glycerol (t_{70} = 120 min). Table I lists the slowing factor imposed by the glycerol concentrations

used in terms of the ratio t_{45}/t_0 and t_{70}/t_0. (In computing these, 100 s was subtracted from all measured times to account for the exchange-out time period before the samples were split into different glycerol concentrations).

These results display a pronounced trend. The fastest protons are slowed by a factor close to that found for free amides (Figure 1). Slower and slower hydrogens are slowed by ever larger factors, and this trend is emphasized at higher glycerol. A clear gradient through the entire exchange-out curve is obvious at 70% glycerol. At 45% glycerol, where the slowing effect is smaller, the point to point gradient is close to the noise level, but the overall effect is still evident when the sequential groupings are compared.

It can be somewhat difficult in the usual overall H-exchange curve to distinguish this kind of behavior—an increasing right shift of the curve along the time axis—from a selective effect on particular protons, which might, for example, raise the curve on the H axis. The selective kinetic labeling procedure used here makes clear the true situation.

Discussion

Kinetic Labeling. In this work we used a selective kinetic labeling approach that makes it possible to label and observe any predetermined fraction of the overall H-exchange curve of a protein while the major fraction of protons remains invisible and does not contribute to the measurement. Figure 2 shows that the usual H-exchange curve, because it is spread out over many orders of magnitude on the time axis, normally displays, at any given pH and temperature, the exchange of only a fraction of the exchanging protons [see also Woodward et al. (1975)]. In the usual representation (e.g., see Figure 2), the exchange behavior of these NH's must be measured in the presence of a large underlying fraction of background, slower exchanging label. This compromises the accuracy of the measurement and also makes ambiguous the effect of any experimental manipulation, since one may not know whether the response observed reflects the behavior of the protons apparently being observed or some of the much slower, background sites. These problems can be avoided by performing the experiment in the exchange-in/exchange-out mode (Figure 3). This produces samples in which the slow "background" protons are essentially unlabeled and so make no significant contribution to the exchange measurements.

Kinetic labeling excises a slice of protons from the overall H-exchange curve according to the following equations. Equation 2 describes the overall multiexponential exchange-out

$$H_{out} = \sum H_i e^{-k_i t_{out}} \quad (2)$$

curve generated by exchange-out of a sample initially fully labeled by long incubation in tritiated water. If the protein is initially tritiated for only a limited exchange-in period, the subsequent exchange-out curve is described by eq 3. As

$$\begin{aligned} H_{in/out} &= \sum H_i(1 - e^{-k_i t_{in}})(e^{-k_i t_{out}}) \\ &= \sum H_i e^{-k_i t_{out}} - \sum H_i e^{-k_i(t_{in}+t_{out})} \end{aligned} \quad (3)$$

$$H_{in/out}(t_{out}) = H_{out}(t_{out}) - H_{out}(t_{in} + t_{out}) \quad (4)$$

indicated in eq 4, the exchange-in/exchange-out curve (eq 3) represents a moving difference taken from the fully labeled exchange-out curve (eq 2) [see also Englander & Rolfe (1973)]. That is, the value for H remaining unexchanged, measured in the exchange-in/exchange-out mode ($H_{in/out}$) at time t, should be equal to the difference between the numbers of H measured in the fully equilibrated molecule (eq 2) at times t_{out} and $t_{in} + t_{out}$. The effect of kinetic labeling is to perform this transformation automatically and unambiguously with a consequent gain in specificity, resolution, and accuracy. It can be seen that the sites measured in exchange-in/exchange-out experiments (Figure 3 and eq 4) do not exactly correspond in a one to one relationship with those measured in full labeling/exchange-out experiments (Figure 2 and eq 2), especially at long exchange-out times, but this does not reduce the advantages of the method.

The so-called "functional labeling" strategy (Englander & Englander, 1983; Englander et al., 1983), which takes advantage of HX rate changes resulting from functional protein interactions, is by no means equivalent to and should not be confused with kinetic labeling [see equations in Englander & Rolfe (1973)]. Functional labeling produces a sample labeled only at sites that *change* their exchange rates significantly in the functional interaction being studied.

This discussion was phrased in respect to measurement of H exchange by ^3H–^1H labeling methods, detected by radioisotopic methods. It should be clear that an analogous approach can be used with other isotopic combinations, for example, with ^2H–^1H labeling, which would adapt this approach to NMR methods. In that case, one could study the behavior of a small set of selectively labeled amides in the H form, while the large majority of normally obscuring amides, in the ^2H form, are invisible. This approach can allow one to resolve individual amide NH, even in sizable proteins having many amides, in a way that would be impossible in the fully protonated protein. Further, one has here the ability to bring different parts of the overall multidecade exchange curve into any preselected time window by judicious choice of the pH and temperature.

Effect of Glycerol on Protein H Exchange. The results obtained here show that concentrated glycerol has little effect on the HX rate of freely exposed protons. In 70% glycerol where relative viscosity is 28, the acid- and base-catalyzed rates are slowed by only 3-fold. Contributions to this behavior include effects on water activity and ionization constant and artifacts in the pH meter reading in addition to solvent viscosity. These effects are small, both individually and in sum.

The structurally slowed, H-bonded NH's in myoglobin were separated by use of the kinetic labeling method into three groupings, placed in the fast rate, the intermediate rate, and the slow parts of the myoglobin exchange curve. In the presence of 45% and 70% glycerol, these are all significantly slowed. The faster NH's display a slowing factor about that found for the small molecule model. The slower and slower protons exhibit slowing factors that increase smoothly to quite high values. The group to group trend can be seen in the data at both 45% and 70% glycerol (Table I). In 70% glycerol, the trend is clear even within each grouping studied. This indicates that the apparent trend is not due to some unsuspected artifact based, for example, on some pH anomaly.

A similar effect appears to occur in HX data presented by Knox & Rosenberg (1980) for lysozyme in glycerol. Under the conditions used by Knox and Rosenberg, the slowest NH's in lysozyme are released by way of transient denaturative unfolding of the protein. That part of the curve exhibits a strikingly large effect, about 200-fold slowing, in 60% glycerol (see Figure 6 of their work). This result reflects the ability of glycerol to stabilize against reversible, whole protein denaturation, which is well-known to mediate exchange of the slowest protons when a protein is relatively destabilized (Rosenberg & Enberg, 1969; Woodward & Hilton, 1979). Exchange of the earlier protons is determined by smaller motions, the exact nature of which is the subject of the various models that have been put forward for the protein HX process.

These protons in lysozyme exhibit a graded effect of added glycerol similar to that observed here for myoglobin. The effect can be seen most directly in Figure 5 of Knox & Rosenberg (1980), which shows straightforward exchange-out curves in varying glycerol, though the effects seen are somewhat less apparent than those observed here since a lower concentration of glycerol was used and the overall HX curve was measured rather than resolved subfractions of it as in the present work.

We assume that the glycerol effect observed with myoglobin and lysozyme will occur fairly generally and does not represent some unusual characteristic peculiar to just these proteins.

Glycerol Effect and H-Exchange Mechanism. The observations made here bear on a central problem in the protein H-exchange area, concerning the fluctuational mechanism that determines the protein HX process. A variety of models for protein H exchange have been put forward. These can generally be distinguished in terms of local unfolding and solvent penetration models.

In penetration models, water molecules and ion catalysts (OH^-, H_3O^+) are imagined to enter the protein and to catalyze the rate-limiting exchange event within the protein matrix. The various models available are couched in quite different terms, but very generally the factors suggested to determine HX rate involve depth and extent of burial and/or ease of forming channels or other pathways to reach protons that are otherwise inaccessible to solvent [reviewed in Englander & Kallenbach (1984)]. The motions responsible for solvent penetration have generally been visualized in terms of the small, rapid, picosecond fluctuations studied, for example in molecular dynamics simulations (Karplus & McCammon, 1981).

The local unfolding model [reviewed in detail in Englander & Kallenbach (1984)] proposes that slowly exchanging protein hydrogens are slow in general because they are involved in intramolecular H bonding and that in order for these to exchange with solvent protons, the H bonds must be broken and re-formed to solvent. H-bond breakage in regions of regular secondary structure is throught to involve sizable cooperative segments of structure in a denaturation-like unfolding event; for example, in an α-helix one or more turns of α-helix may transiently unfold as a unit. In this view, HX rate has no necessary correlation with distance from the protein surface but rather is determined by individual unfolding equilibria. Recent work appears to define such unfolding units in four different proteins [see sections 9.3 and 9.4 of Englander & Kallenbach (1984)].

The effects on protein behavior of solvent additives like glycerol have been dealt with by a number of workers. Concentrated solutes like glycerol are well-known to stabilize a large number of proteins against denaturative unfolding [listed in Gekko & Timasheff (1981)]. This effect has been studied in some detail by Timasheff and co-workers, who find that glycerol and similar agents are preferentially excluded (relative to water) from the protein surface so that denaturative unfolding, which would add new surface, is disfavored [see Arakawa & Timasheff (1982) and earlier papers in that series]. The mechanistic basis for this phenomenon has so far proven elusive, but the thermodynamic result is clear. The presence of concentrated glycerol must tend to minimize protein surface area.

In the local unfolding model for protein H exchange, unfolding reactions that labilize slower and slower protons to exchange are likely to be progressively larger in size. Since diverse factors play a role in stabilizing segments against unfolding, there will be no strict relationship here, but a fair correlation between energy of the opening reaction and size of the opening unit seems fairly certain. Thus, the well-known ability of glycerol to stabilize proteins against unfolding seems able to explain the HX trends in Table I in a quite natural way in terms of the local unfolding model for protein H exchange; slower protons which experience generally larger unfoldings will be more affected.

It can be noted that structure stabilization by glycerol is not a kinetic, viscosity-dependent effect but rather an equilibrium thermodynamic one. Glycerol stabilizes against unfolding by disfavoring the unfolding equilibrium. Similarly, in the unfolding model, HX rate in almost all cases proportions to the unfolding equilibrium constant. Thus, the HX slowing due to added glycerol must here also be considered an equilibrium effect, not dependent on viscosity.

In respect to the parameters pertinent for HX penetration models, the effects of external solvent and solvent viscosity on small internal motions and on the migration of small molecules like CO and O_2 within the protein matrix have been considered both experimentally and theoretically. One can conclude from all this work that the motions of solvent-exposed groups are likely to be significantly affected by added glycerol and the buried ones much less so (Van Gunsteren & Karplus, 1982; Gavish, 1980). In studies of O_2 penetration into proteins, Jameson et al. (1984) found that concentrated solvent sucrose hardly affects the quenching, by entering O_2, of porphyrin fluorescence in globin. Calhoun et al. (1983) noted a similar lack of effect in studies of the quenching of room temperature tryptophan phosphorescence by O_2 in alcohol dehydrogenase. Beece et al. (1980) concluded that solvent viscosity affects the outermost barrier in myoglobin–CO recombination but not at all the innermost one. Certainly there has been no indication that protein sites effectively more and more remote from the solvent would experience increasing effects, and this seems a most unlikely result. It can be expected then, from the point of view of penetrational models, that added glycerol either would have a larger effect on the faster, more surface-related protons or might affect all the structurally slowed protons to a similar extent. It seems unlikely that slower and slower exchanging protons would be more and more affected. Nevertheless, that is the result we find.

One cannot assert that the glycerol effect observed here is inconsistent with all possible penetration models. Models describing hypothetical penetration processes that will have the desired kinetic behavior might be considered. In trying this exercise one should be aware of certain simple ground rules. (1) Most of the exchangeable amide protons of myoglobin (and other proteins) are not buried but lie essentially *at the protein surface* [close to 70% in myoglobin according to calculations of Richards (1979); see Englander & Kallenbach (1984, p 539)]. (2) It is very unlikely that any significant amount of glycerol will reside inside a protein, either in an equilibrium solvation sense or in transiently formed channels that could account for water entry; channel entry will be exceedingly sensitive to molecular size (Richards, 1979), and glycerol is far larger than H_2O. (3) Several cases are now known in which hydrogens sequentially located along a length of helix, some on the protein surface and some "inside", exchange at the same rate [see, e.g., section 9 of Englander & Kallenbach (1984)]. So far as we can see, penetration-based models that might explain the glycerol-dependent gradient described here seem inconsistent with well-known facts of protein structure, with effects observed in the case of O_2 and CO penetration into proteins (Beece et al., 1980; Calhoun et

al., 1983; Jameson et al., 1984), and with available information concerning the distribution of exchange rates among protons in real proteins (Englander & Kallenbach, 1984).

In summary, the present results with myoglobin and earlier results for lysozyme (Knox & Rosenberg, 1980) show that the more slowly exchanging hydrogens are more affected by concentrated solvent glycerol. If the HX mechanism involves protein unfolding reactions, then these results seem perfectly reasonable. The slower protons relate to more difficult unfolding reactions, which presumably correlates with unfolding unit size. Added glycerol, which makes surface exposure energetically unfavorable (Gekko & Timasheff, 1981), would then have generally greater effect for the slower protons. No such relationship appears in the case of solvent penetration models, certainly not as these have so far been described. Here one can expect either that the structurally slowed protons would be affected approximately equally or that the faster protons would be more affected. It is difficult to see how a penetration-dependent H-exchange mechanism would produce the kind of reverse gradient in glycerol effect that is observed, with the slowest protons being the most affected.

Registry No. H_2, 1333-74-0; acetamide, 60-35-5; glycerol, 56-81-5.

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