Effect of Osmolytes on the Exchange Rates of Backbone Amide Protons in Proteins

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ABSTRACT: Osmolytes are small organic solutes produced by the cells of all organisms (except halobacteria) in high stress situations (e.g. extremes of salt concentration, high temperature, etc.) to stabilize their macromolecules and so conserve biological activity. They do not interact with the macromolecule directly but act by altering the solvent properties in the cellular environment, and so their presence indirectly modifies the stability of proteins. In this paper we examine the effect of a model osmolyte, glycine, on the stabilization of two proteins, chymotrypsin inhibitor 2 and horse heart cytochrome c. We have used NMR to monitor the effect of this osmolyte on amide hydrogen exchange rates, which allows a probe at discrete points within the protein structure. Hydrogen exchange rates of specific backbone amide protons provide information about the localized structural fluctuations that expose these amides to solvent and allow exchange to take place. We find that the presence of a high concentration of glycine osmolyte has a profound stabilizing effect on the proteins studied, which is accompanied by a large reduction of the exchange rate constants of most slowly exchanging amide protons. The spectra indicate that this arises without significant changes in the three-dimensional structure. However, the effects on individual amide protons within a single protein were not uniform, and a wide variation in the magnitude of the effects was observed. This ranged from no apparent change in the exchange rate, to decreases in the exchange rate constant by over 2 orders of magnitude. The osmolyte appears to alter a number of different processes that contribute to the observed exchange rates, and no simple generalization allows prediction of the extent of stabilization at any individual location. The results are discussed in light of the available structures of the proteins studied.

Osmolytes are small organic solutes that confer protection against denaturing stresses on macromolecules in the cellular systems of all bacteria (except halobacteria), plants, and animals. There are three main categories of osmolytes: polyhydric alcohols (polyols); free amino acids and their derivatives; methyamines (Yancey et al., 1982). These molecules stabilize proteins, not by interacting with them directly but by altering the solvent properties of the surrounding water and therefore the macromolecule—solvent interactions. This results in a shift in the unfolded ⇔ folded equilibrium to favor the folded species (Timasheff, 1992, 1993; Yancey et al., 1982). Their effect seems to be general to all proteins and has no inhibitory or enhancing effects on biological activity. The generalized nature of action of these compatible solutes in nature minimizes the need for adaptive changes in proteins that must retain biological activity under the extreme conditions (e.g., high salt or urea concentration, temperature, heat, dehydration, etc.) that can occur in cells.

Some of the most efficient osmolytes are amino acids and their derivatives, including glycine, α- and β-alanine, betaine, sarcosine and N,N-dimethylglycine (Yancey et al., 1982; Arakawa & Timasheff, 1983, 1985; Santoro et al., 1992; Taneja & Ahmed, 1994). Their presence in a solution is thought to result in enhanced solvent ordering leading to preferential exclusion of osmolyte from the protein structure (Gekko and Timasheff, 1981). A major driving force for protein folding is the hydrophobic effect, which is mediated via solvation and so is modulated by the presence of osmolytes. The most striking manifestation of the stabilization effect of osmolytes is a considerable increase in the $T_m$ of proteins in their presence. The degree of stabilization can be quite dramatic, for example, glycine-based osmolytes can raise the $T_m$ by as much as 22°C (Santoro et al., 1992).

All studies reported to date have examined the consequence of osmolyte addition on global thermodynamic parameters. A more selective indication of the stability of a protein is the rate at which individual backbone amide protons exchange with their surrounding solvent. The degree to which internal residues are accessible for hydrogen exchange is inversely related to the global stability of the fluctuating protein ensemble; i.e., there is a correlation between stability and exchange rates (Englander and Englander, 1983; Englander & Kallenbach, 1984; Woodward & Hilton 1979). Although exchange rate constants can only give an indirect measurement of stability, they can be determined at numerous positions throughout a protein structure and under a variety of experimental conditions. Stabilization of the structure is expected to decrease exchange rates. In the context of the present investigation, we can therefore ask two questions: (1) Do osmolytes, by stabilizing proteins, produce reduced rates of amide proton exchange?
(2) How are such changes distributed throughout the structure?

Amide proton exchange is easy to measure by NMR, as in D$_2$O solution the NH resonances will exchange to give ND, resulting in a decay of the NH signal. The mechanism can be described by eq 1 (Richarz et al., 1979; Wagner & Wu ¨thrich, 1979), where O represents the “open”, solvent-exposed state and C the “closed” or folded solvent-inaccessible form. The exchange rate, $k_{ex}$, is then given by eq 2.

\[
C(\text{H}) = \frac{k_1}{k_2} O(\text{H}) = \frac{k_3}{k_4} \text{D}_2\text{O} = \frac{k_5}{k_6} C(\text{D})
\]

\[
k_{ex} = \frac{k_1 k_3}{k_2 + k_3}
\]

Under most conditions of practical interest, including those in the present investigation, EX2 kinetics prevail (Roder et al. 1985a) where $k_2 \gg k_3$ giving eq 3. In this equation, $K_{eq}$

\[
k_{ex} = K_{eq} k_3
\]

$= k_1/k_2$, and it thus dictates the position of the equilibrium between the solvent-exposed and solvent-hidden states of the exchangeable protons. $k_1$ is the intrinsic chemical exchange rate of individual protons, i.e., the rate at which that proton would exchange when solvent exposed. Although this has been seen to vary between individual residues in a protein structure (Roder et al., 1985b), it is the degree of solvent exposure ($K_{eq}$) that has the greater effect on exchange rates.

In the experiments described in this paper we have measured the effect of a model osmolyte on the exchange rates of individual amide protons. In this way, we should obtain information as to how the apparent solvent exposure equilibrium, a reflection of stability, is affected by the osmolyte at discrete points in the protein 3D structure. Our studies have involved examination of the effect of the osmolyte glycine on two proteins, namely horse heart cytochrome c and chymotrypsin inhibitor 2 (CI2). Cytochrome c is a well-characterized globular protein of 104 residues, (M$_r$ ~ 12 kD) involved in coupled electron transport. A high-resolution three-dimensional crystal structure has been elucidated (Bushnell et al., 1990) as well as a fully assigned proton NMR spectrum (Wand & Di Stefano, 1989). Some H-exchange work has also been described (Patel and Canuel, 1976; Roder et al., 1988), particularly on the N-terminal α-helix (Wand et al., 1986; Wand and Englander, 1986). CI2 is a small serine protease inhibitor found in barley seeds. There is a crystal structure of this protein (McPhalen et al., 1985; McPhalen & James, 1987), a fully assigned proton spectrum of the ordered portion (residues 22–83) (Kjær et al., 1987) and an NMR structure determination (Kjær and Poulsen, 1987; Ludvigsen et al., 1991).

**EXPERIMENTAL SECTION**

**Protein Purification.** Cytochrome c (horse heart, min. 95%) was obtained from Sigma Chemical Co. and was used without further purification.

CI2 was expressed from the plasmid pPO1 (Osmark et al., 1993) in E. coli K38 harboring the plasmid pGP1-2, which encodes RNA polymerase (Tabor & Richardson, 1985). Cells were grown overnight at 30 °C in 2TY medium supplemented with ampicillin (50 mg L$^{-1}$) and kanomycin (50 mg L$^{-1}$). Expressed protein was induced by heat shock for 1 h at 42 °C followed by further growth at 37 °C for 4 h. The cells were then harvested and sonicated, and the pH was adjusted to 4.8 with glacial acetic acid. After centrifugation at 15,000 rpm (Sorvall SS34 rotor) for 30 min, ammonium sulfate was added to the supernatant of the resultant cell contents mixture to 60% w/v. The precipitated proteins were recovered by centrifugation, redissolved and size fractionated on a gel filtration column (Sephadex G-75, Pharmacia). Fractions that inhibited hydrolysis of N-succinyl-al-a-la-pro-phenylalanine (Sigma) by subtilisin Carlsberg (Sigma) were identified, pooled, and lyophilized. The dry protein was used directly for denaturation studies and NMR.

**Chemical Denaturation Experiments.** Guanidine hydrochloride (GdnHCl) (ARISTAR grade) was obtained from BDH Limited. Guanidine hydrochloride solutions were prepared by serial dilution from an 8 M stock solution prepared as described by Pace (1986). For each sample, 100 µL of protein solution (2–3 mg mL$^{-1}$, 50 mM sodium acetate buffer, pH 4.2) with or without 2 M glycine was diluted into 900 µL of the appropriate denaturant solution (±2 M glycine as required). The protein/denaturant solutions were preequilibrated at 21 °C for 1 h. Fluorescence spectra were collected using a Shimadzu RF-5001PC spectrofluorimeter. The excitation wavelength was 290 nm, and emission spectra were recorded at 356 nm. Data were analyzed as described in the Results by nonlinear regression using the program GraFit (Leatherbarrow, 1992).

**Two-Dimensional NMR Spectroscopy. Sample Preparation.** For the proton exchange measurements using cytochrome c, 30 mg of protein was added to 0.6 mL of H$_2$O or 0.6 mL of 2 M glycine-$d_5$ (Aldrich), reduced with excess sodium dithionite (Aldrich) with the pH adjusted to 4.75. For the NMR studies on CI2, 14 mg of purified protein was added to 0.6 mL of H$_2$O or 0.6 mL of 2 M glycine-$d_5$ and the pH adjusted to 7.0. After lyophilization overnight, in each case 0.6 mL of D$_2$O (Aldrich, 100%) was added and the respective sample was immediately transferred to the magnet. All pH values quoted are direct pH meter readings at room temperature and are uncorrected for isotope and temperature effects.

**Acquisition of Spectra.** NMR spectra were obtained on a Bruker AMX 600 MHz spectrometer (University of London Interdisciplinary Research Service) equipped with an Aspect 3000 data system. 2D J-correlated TOCSY spectra (Bax & Davis, 1985; Braunschweiler & Ernst, 1983) were recorded with a mixing time of 80 ms. For the cytochrome c samples the spectra were recorded at 40 °C and pH 4.75. For the CI2 samples, spectra were recorded at 30 °C and pH 7.0. The residual solvent peak was suppressed by presaturation. Spectra comprised 512 increments of 2K data points (16 scans with 4 dummy scans), and were zero filled to 2K points in the F1 dimension before processing. A series of spectra (approximate running time 2 h 45 min) were recorded over a 24 h period after addition of D$_2$O to the dried sample. This typically allowed seven time points from which the exchange rate constants could be derived.
RESULTS

Row, 1992). Nonlinear regression using the program first-order kinetics and rate constants were determined by acquisition and processing parameters. Exchange followed reference was needed as all spectra for any one rate the integral of radius 30 Hz centered over a peak of interest using peaks were determined by calculating the cross-peak volume software package (Bruker, 1995). No internal software package (Bruker, 1995). No internal

Analysis of Data. The degree of amide exchange was investigated as a function of time and was monitored by the decrease in intensity of the peaks corresponding to individual assignable residues. The intensities of the NH–C=H cross-peaks were determined by calculating the cross-peak volume integral of radius 30 Hz centered over a peak of interest using the Aurelia software package (Bruker, 1995). No internal reference was needed as all spectra for any one rate calculation were run on the same sample, with identical acquisition and processing parameters. Exchange followed first-order kinetics and rate constants were determined by fitting the peak intensities to a first-order decay curve by nonlinear regression using the program GraFit (Leatherbarrow, 1992).

RESULTS

Solvent-Induced Denaturation. The effect of 2 M glycine on the stability of both cytochrome c and chymotrypsin inhibitor 2 was studied by measuring their unfolding in solutions containing increasing concentrations of guanidine hydrochloride (Kellis et al., 1989). Unfolding was monitored by observation of the intrinsic protein fluorescence, which in cytochrome c arises from the sole tryptophan side chain Trp 59 and in CI2 from the sole tryptophan side chain Trp 24. The denaturation curves of these proteins, with and without 2 M glycine, are shown in Figure 1.

Table 1: Effect of Glycine on the Stability of Cytochrome c and CI2

<table>
<thead>
<tr>
<th>Parameter (± std. error)</th>
<th>Cytochrome c</th>
<th>CI2</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta G_{H2O} ) (kJ mol(^{-1}))</td>
<td>26.74 (±1.16)</td>
<td>20.42 (±1.05)</td>
</tr>
<tr>
<td>( \Delta G_{H2O} ) in the presence of 2 M glycine (kJ mol(^{-1}))</td>
<td>44.02 (±1.17)</td>
<td>28.16 (±1.80)</td>
</tr>
<tr>
<td>( \Delta \Delta G_{U} ) (kJ mol(^{-1}))</td>
<td>17.28 (±1.65)</td>
<td>7.74 (±2.08)</td>
</tr>
<tr>
<td>( m )</td>
<td>2.58 (±0.26)</td>
<td>1.20 (±0.14)</td>
</tr>
</tbody>
</table>

The free energies for unfolding for cytochrome c and CI2 in the presence and absence of 2 M glycine were derived from analysis of the variation of intrinsic protein fluorescence as a function of GdnHCl concentration as described in the text. Data in the absence and presence of osmolyte were fitted simultaneously to yield a common m value (eq 5). Standard error values (in brackets) were derived from the data fitting.

The free energy of unfolding in the presence of a denaturant such as guanidine hydrochloride is linearly related to the concentration of denaturant (Pace, 1986) (eq 1).

\[
\Delta G_{U} = \Delta G_{H2O} - m[\text{denaturant}]
\]

Here, \( \Delta G_{H2O} \) is the apparent free energy of unfolding in the absence of denaturant and \( \Delta G_{U} \) is the free energy of unfolding in the presence of denaturant. This allows denaturant-induced unfolding to be used to measure protein stability (Kellis et al., 1989). For a two-state unfolding transition, it is possible to fit the data to eq 5, which incorporates pre- and post-denaturation slopes that account for experimentally observed effects on fluorescence that are not due to unfolding (Santoro & Bolen, 1988; Pace et al., 1990; Horovitz et al., 1992; Clarke & Fersht, 1993).

\[
F = (\alpha_N + \beta_N[D]) + (\alpha_U + \beta_U[D]) \times \frac{\exp[(m[D] - \Delta G_{H2O})/RT]}{1 + \exp[(m[D] - \Delta G_{H2O})/RT]}
\]

In this equation, \( \alpha_N \) and \( \alpha_U \) are the slopes of the baselines at low (N) and high (U) GdnHCl concentrations, and D is the denaturant GdnHCl. The data sets were then analyzed by multiple nonlinear regression. For each protein, both data sets were fitted simultaneously to eq 5. The values of both m and the slope of the denaturation curve were held to be the same in the presence of denaturant. This allows denaturant-induced unfolding to be used to measure protein stability (Kellis et al., 1989). For a two-state unfolding transition, it is possible to fit the data to eq 5, which incorporates pre- and post-denaturation slopes that account for experimentally observed effects on fluorescence that are not due to unfolding (Santoro & Bolen, 1988; Pace et al., 1990; Horovitz et al., 1992; Clarke & Fersht, 1993).

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\[
F = (\alpha_N + \beta_N[D]) + (\alpha_U + \beta_U[D]) \times \frac{\exp[(m[D] - \Delta G_{H2O})/RT]}{1 + \exp[(m[D] - \Delta G_{H2O})/RT]}
\]
proline (no peaks). Complete assignment information is available for both cytochrome c (Wand & Di Stefano, 1989) and CI2 (Kjær et al., 1987) and was used to identify the resonances monitored in this study.

The rate of hydrogen exchange is strongly dependent on pH and temperature (Patel & Canuel, 1976), so conditions were found empirically that gave measurable exchange rates both in and out of glycine for as many residues as possible, over an experimentally accessible time period (24 h). As will be seen, this is not straightforward as the addition of osmolyte greatly slows amide exchange. In the case of chymotrypsin inhibitor 2 the conditions used were pH 7 and 30 °C and for cytochrome c, pH 4.75 and 40 °C. These conditions allow a large number of exchange rates to be measured both in the presence and absence of osmolyte. The spectrum of either protein in 2 M glycine under these conditions was essentially the same as the native protein (compare Figure 2a with Figure 3a, and Figure 4a with Figure 5a), showing that glycine at the concentration used has negligible effect on the relative chemical shifts of the protons.

Representative spectra illustrating the effects of the osmolyte on exchange are shown in Figures 2–5. Figure 2 shows spectra of cytochrome c, pH 4.75, 40 °C, after exchange times of 12, 342, and 1002 min; Figure 3 shows spectra of cytochrome c in 2 M glycine under otherwise identical conditions, after approximately the same time
periods (40, 366, and 1018 min). Figure 4 shows spectra of chymotrypsin inhibitor 2 at pH 7, 30 °C, after exchange times of 188, 524, and 1028 min; Figure 5 shows spectra of chymotrypsin inhibitor 2 in 2 M glycine under the same conditions, after the similar exchange times of 195, 533, and 1040 min. It is immediately obvious from comparing the first and last spectra in both series run in the absence of the osmolyte that over the time period of the experiment significant exchange of the slowly exchanging amide protons has occurred, and some of the cross-peaks have disappeared. In comparison, very little exchange has occurred in the presence of 2 M glycine over the same period. Thus this osmolyte greatly slows down the average rate of amide proton exchange throughout the protein structure, and this effect is seen for each of the model proteins studied.

After assignment of individual cross-peaks based on previously published work (Wand & Di Stefano, 1989; Kjær et al., 1987), their volumes were integrated and the first-order exchange rate constants were calculated. Figures 6 and 7 show graphs of peak intensity against time for several representative residues in cytochrome c and chymotrypsin inhibitor 2, respectively. The calculated exchange rate constants of the various assigned backbone amide protons in the absence and presence of osmolyte for cytochrome c and chymotrypsin inhibitor 2 are listed in Tables 2 and 3.

DISCUSSION

A protein can be dissolved in up to 2 M glycine, a powerful osmolyte, without its structure or function being altered (Gopal and Ahluwalia, 1993; Matthews & Leatherbarrow, 1993). By comparison of denaturation curves, using increasing concentrations of GdnHCl, of each protein in water and in 2 M glycine (Figures 1), it is clear that a much higher concentration of GdnHCl is needed to denature the proteins in the presence of the osmolyte. Hence, the presence of the osmolyte at this concentration confers a quite dramatic increase on the stability of the proteins used in the present study. The stabilization is consistent with the well-documented effects of osmolytes on protein stabilization and confirms that the protein systems described are suitable models for further analysis.

Two-dimensional NMR provides a method of measuring exchange rates of individual labile protons, both in water and in 2 M glycine. Glycine up to 2 M concentration has negligible effect on the intrinsic quality and nature of the NMR spectra of these proteins (see Figures 2–5). As 1H chemical shifts are a sensitive probe of protein conformation, this is consistent with the protein structure being unaltered by the presence of the osmolyte at this concentration (Matthews & Leatherbarrow, 1993). Perdeuterated glycine was used to minimize spectral interference.

Hydrogen exchange of backbone amide protons in a protein consists of two main stages, the first involving fluctuations that render the individual protons solvent accessible and the second, the actual base-catalyzed exchange event. The overall rate of exchange of individual amide
protons with the solvent thus depends on two factors: the proportion of time that any proton spends solvent-exposed and in the correct orientation for exchange ($K_{eq}$, eq 3) and the intrinsic exchange rate of that proton ($k_3$, eq 3). Although $k_3$ will vary between individual residues in the protein structure (Roder et al., 1985b), it is $K_{eq}$ that has the greater effect on amide exchange rates, resulting in rates ranging over 10 orders of magnitude (Woodward et al., 1982). In the context of the current work, it seems reasonable to assume that the effects observed also reflect changes to $K_{eq}$, consistent with the increased stability of the protein when in the presence of osmolyte.

To understand the way in which osmolytes could affect exchange rates, it is important to consider the nature of the motions that render the backbone amide protons accessible to solvent. Under the conditions used for these studies, low activation energy exchange from the native state, involving small fluctuations in the protein structure, predominates. The two main models that describe this process are the local unfolding model (Englander & Kallenbach 1984; Englander et al., 1992) and the solvent penetration model (Hvidt & Nielson, 1966; Woodward & Hilton, 1979). More specific noncooperative fluctuations include cavities created by mobile defects in protein packing (Lumry & Rosenberg, 1975) and transient formation of channels from bulk solvent to interior sites (Richards, 1979). Cooperative unfolding models range from the breakage of single hydrogen bonds (Linderstrom-Lang, 1955), concerted local unfolding of small protein segments (Englander, 1975; Englander et al., 1992), “regional melting” (Kossiakoff, 1982), “limited structural fluctuation” (Mayo & Baldwin, 1993), and larger (but still sub-global) unfolding processes (Bai et al., 1995). Although the hydrogen-bonding structure is dominant in governing amide exchange (Kossiakoff 1982; Woodward et al., 1982), other factors, such as burial from the solvent within the protein core, can also determine how protected a proton is from exchange (Radford et al., 1992). In addition, the presence of distinct folding domains is also likely to influence amide exchange (Kim et al., 1993; Dobson et al., 1994).

The Effect of Osmolytes on Exchange. The interpretation of NH exchange rates from individual protons within a protein is complex, as several processes may contribute to the exchange mechanisms (Dempsey, 1986; Delepine et al., 1987). However, as the experiments described here compare exchange rates for the same protons under different solvent conditions, the effect can be investigated without requiring detailed interpretation, at an atomic level, of the exchange mechanism itself.

The relative effect of the osmolyte on the exchange rate, i.e., the ratio of the exchange rate with and without osmolyte, serves to indicate the degree to which this stabilization is manifest at the location of each specific amide (Tables 2 and 3). If the osmolyte was stabilizing the protein to the same extent throughout, this factor would be expected to be the same for each residue. The results in this study, however, show that the magnitude of the effect that glycine has on individual exchange rates varies enormously (from 0.38 to >326). There appears little correlation between primary sequence and the extent of the effect of the osmolyte. Neither is there any correlation between the amount that the osmolyte affects amide exchange and the type of amino acid involved. The data show no simple correlation with static solvent accessibilities (data not shown) or with the temperature factors from X-ray crystallographic studies on these proteins (Bushnell et al., 1990, McPhalen & James, 1987).

The most likely explanation is that these effects depend on the location of the particular residue within the 3D structure, the secondary and tertiary structure present at that point and the fluctuation(s) required for hydrogen exchange to occur. A detailed description of the effects, considering the protein structure, is given below.

Cytochrome c. The results of Patel & Canuel (1976) and Wand et al. (1986) support local cooperative unfolding reactions, as opposed to solvent penetration, as determinants of protein hydrogen exchange reactions for cytochrome c under conditions such as those used here. Most of the slowly exchanging amide protons are hydrogen bonded (Bushnell et al., 1990), although those that are not are not necessarily the fastest exchanging.

The N-Terminal α-Helix. Among the slowest amide protons to exchange are those positioned in the N-terminal α-helix, i.e., residues 2–15. Of these residues studied, N2 exchanges much faster than do any of the others in this helix, presumably because it is very near to the terminus and the most exposed. It is also stabilized to the greatest extent by 2 M glycine. Resides 9 and 11–14 exchange at very similar rates and in general the slower the exchange rate constant, the less the effect of the osmolyte. This helix is
docked via contacts involving residues 6 and 10 to the C-terminal α-helix, leaving K8 the most exposed residue, with no hydrophobic contacts. It seems likely that this residue would need least fluctuation to become accessible.
to solvent, which could explain why addition of glycine does not retard the exchange rate.

The C-Terminal α-Helix. The next slowest exchanging set of residues is those in the C-terminal α-helix, i.e., residues 87–102. Within this group, all residues except R100 have similar exchange rates, with R91 and I95 exchanging the fastest and also being the most affected by glycine. The C-terminal helix could well be part of the same cooperative unfolding unit as the N-terminal helix, both helixes being docked together, and their melting is the final transition to the unfolded state (Bai et al., 1995).

The “60s” Helix. The other main helical section of cytochrome c is the 60s helix, involving residues 60 to 69 and possibly extending to W59. In this relatively fast exchanging helix, L68 is the slowest of those observed to exchange, and it is also affected by glycine to a considerably greater degree than the other residues. This helix is packed against the hydrophobic core of the protein, the greatest area of contact involving this leucine residue. The whole helix seems to fall into a pattern where, moving outward in both directions from L68, the exchange rates increase and the effect of glycine on these rates decreases. Bai and coworkers (1995) suggest that this helix forms a cooperative unit which is tightest in the center. All the amides in this helix are reported to exchange by local fluctuations except for L68, which requires more extensive unfolding. L68 may then be most affected by glycine because glycine has a greater effect on transitions involving global unfolding reactions, and has a lesser effect on small fluctuations.

The Omega Loop. The group of amides most affected by glycine is H18, G29, and L32. These amides are also among the fastest to exchange. They all lie on the ω loop made up of residues 18–33 (Leszczynski & Rose, 1986) which pack closely together underneath the heme group as part of the hydrophobic core. The side chains of these residues are fully buried in the 3D structure of cytochrome c (Dickerson et al., 1971). The fact that they all have similar exchange rates and that these rates are slowed so remarkably by glycine seems to imply that they are part of one cooperative unfolding unit, which, once it has unfolded, exposes all three residues to solvent for exchange. This is consistent with the suggestion by Bai et al. (1995) that there is a cooperative binding loop involving residues 20–35. The unfolding of this loop would break up the hydrophobic core of the protein, which could be the reason that addition of glycine has such an extreme effect on the exchange stability of this loop.

General Data. Bai et al. (1995) suggested that the hydrogen exchange behavior of cytochrome c in low concentrations of denaturant reveals a sequence of metastable, partially unfolded forms that occupy free energy levels reaching up to the fully unfolded state. The step from one to another is accomplished by the unfolding of one or more of these cooperative units, which are made up of entire ω loops or mutually stabilizing pairs of whole helixes and loops. The partially unfolded forms that they detected by hydrogen exchange appear to represent major intermediates in reversible, dynamic unfolding reactions that occur even at native conditions. The same cooperative units are also consistent with the results of these studies. The 60s helix displays contrary behavior to the other cooperative units discussed. This may indicate that exchange of the slower residues in the 60s helix involves more extensive disruption of the hydrophobic core.

Chymotrypsin Inhibitor 2. CI2 forms a single cooperative folding unit (Li & Daggett, 1996; Itzhaki et al., 1995; Jandu et al., 1990). The α-helix is in strong hydrophobic contact with the β-sheet region, the hydrophobic core involving the side chains of W24, L27, A35, V38, I39, A46, F48, V66, L68, V70, I76, and P80 (McPhalen et al., 1985). The extended loop is also strongly held against this protein core by two hydrogen bonds to Arg65 and Arg67. Many of the residues exchange too slowly in glycine for it to be possible to calculate the precise effect that glycine has on the exchange rate constants, although limits can be estimated.

The α-Helix. Of the α-helix residues studied, K43 and K37 exchange the fastest. K37 is solvent accessible but K43 is not directly solvent exposed. While the presence of glycine retards exchange by a factor of only 15 for K37, exchange of K43 is slowed by a factor >300. This suggests that different unfolding processes expose this latter residue, and these are more greatly affected by the presence of glycine.

The β-Sheet Region. Residues D71 and N75 lie at the top of strands 4 and 5 respectively of the β-sheet region. Both residues are buried and D71 exchanges at about 4 times the rate of N75. The exchange rates of these residues in the presence of glycine are similar, with D71 having a retardation factor of 32. It seems possible that these protons are exchanging due to the same substructure fluctuation, which is stabilized by the osmolyte.

R81 and V82 lie on strand 6 of the β-sheet. V82 exchanges about 3.5 times faster than R81, and its retardation factor in 2 M glycine is also about 3.5 times greater than that of R81. This would be consistent with glycine retarding a common fluctuation that exposes both residues to solvent.

A77 and Q78 constitute the half-turn in the β-sheet region. These residues are at very similar distances from solvent but Q78 exchanges at a greatly accelerated rate. In 2 M glycine, the rate of exchange of Q78 is lowered dramatically, while the rate of A77 is lowered by the moderate factor of 5.8. In this instance, adjacent residues are affected to greatly different extents, suggesting different processes being involved in their solvent exposure, with consequently differing effects of the osmolyte.

R65 and F69 are on the same strand of the β-sheet structure. R65 exchanges at a moderate rate, even though it is very buried in the protein structure. F69 exchanges at a much slower rate, even though it lies on the protein surface and is solvent accessible. It seems likely that no major structural fluctuation is required in order to exchange this amide proton. The effect of glycine on the exchange of F69 is small. This result is consistent with glycine having an effect on the unfolding/folding equilibrium responsible for exchange of R65 but having relatively little effect on the smaller fluctuation required for exchange of F69.

Thermodynamic Effects of the Osmolyte. The Gibbs free energy change associated with the exchange process can be expressed as eq 6. Here, \( R \) is the universal gas constant (\( R = 8.31 \text{ J K}^{-1} \text{ mol}^{-1} \)) and \( T \) is the Kelvin temperature. The difference in free energy change resulting from addition of
osmolyte is then given by eq 7. This can also be expressed

$$\Delta \Delta G_{\text{ex}} = -RT \ln \Delta K_{\text{eq}}$$  (7)

in terms of the exchange rates in water ($k_{D,O}$) and in glycine ($k_{gly}$) (eq 8). Stabilization by solvents will alter the exchange

$$\Delta \Delta G_{\text{ex}} = -RT \ln(k_{D,O}/k_{gly})$$  (8)

rate by a factor proportional to the $K_{\text{eq}}$, but under the conditions used here (i.e. where the protein is not denatured), this refers to the $K_{\text{eq}}$ of each individual amide from the folded state. If the osmolyte was stabilizing the protein to the same extent throughout the protein structure, the factor by which it affects the exchange rate, and therefore the $\Delta \Delta G_{\text{ex}}$, would also be the same for each residue. The results in this study, however, show that the magnitude of the effect that glycine has on individual exchange rate constants varies across a range of 0.37 to > 326.

Each individual $K_{\text{eq}}$ refers to the equilibrium constant for the sum of any unfolding events or structure fluctuations that render the proton in question solvent accessible, however large or small a contribution that event may be making.

Mechanism of Action of the Osmolyte. Various conclusions can be drawn about the effect of this osmolyte on amide proton exchange.

1. If the exchange happens by virtue of an unfolding equilibrium affected by the glycine osmolyte, then the rate of exchange will be altered. The results of this study suggest that most slowly exchanging amide protons do show a significant alteration in their exchange rates in the presence of 2 M glycine.

2. If glycine has no affect on the processes that expose the amide to solvent, then glycine will not affect the exchange rate. A number of amide protons are found to have broadly similar exchange rate constants in the presence and absence of the osmolyte (in cytochrome c these are K8, Q12, C14, I57, K100, and A101). The moderate increase of exchange rate constant for a few protons (by a factor of up to ~3) may be due to the glycine acting as a mild base.

3. If exchange occurs from a form approaching the denatured state of the protein, then the effect of glycine will be maximal and the lowering of the exchange rate constants will reflect the thermodynamic stabilization afforded by the osmolyte. The maximum stabilization factors found in this study are for the extremely buried protons within the ω loop of cytochrome c (H18, G29, and L32). It is likely that the exposure of these residues will require extensive unfolding, which explains the large effect of the stabilizing agent on these exchange rates.

4. If the exchange can occur through various fluctuations, some of which are affected by glycine, then the osmolyte will have an intermediate effect. Most of the slowly exchanging amides from the two model protein systems studied here fall into this intermediate category.

We presume that the exchange stabilization produced by the presence of the glycine osmolyte results predominantly from a shift in $K_{\text{eq}}$ (eq 3) that reflects the thermodynamic stabilization of the protein structure. This in turn alters the exchange rate constant as described by eq 8. For both model proteins, the mechanism by which buried protons exchange appears consistent with local unfolding processes. Particu-