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Effects of Denaturants on Amide Proton Exchange Rates: A Test for Structure in Protein Fragments and Folding Intermediates[†]

David Loftus,[‡] George O. Gbenle,[§] Peter S. Kim, and Robert L. Baldwin*

Department of Biochemistry, Stanford University, Stanford, California 94305

Received July 25, 1985

ABSTRACT: A method for detecting structure in marginally stable forms of a protein is described. The principle is to measure amide proton exchange rates in the absence and presence of varying concentrations of a denaturant. Unfolding of structure by the denaturant is reflected by an acceleration of amide proton exchange rates, after correction for the effects of the denaturant on the intrinsic rate of exchange. This exchange-rate test for structure makes no assumptions about the rate of exchange in the unfolded state. The effects of 0–8 M urea and 0–6 M guanidinium chloride (GdmCl) on acid- and base-catalyzed exchange from model compounds have been calibrated. GdmCl does not appear to be well-suited for use in the exchange-rate test; model compound studies show that the effects of GdmCl on intrinsic exchange rates are complicated. In contrast, the effects of urea are a more uniform function of denaturant concentration. Urea increases acid-catalyzed, and decreases base-catalyzed, exchange rates in model compounds. The exchange-rate test is used here to study structure formation in the S-protein (residues 21–124 of ribonuclease A). In conditions where an equilibrium folding intermediate of S-protein (I_3) is known to be populated (pH 1.7, 0 °C), the exchange-rate test for structure is positive. At higher temperatures (>32 °C) I_3 is unfolded, but circular dichroism data suggest that residual structure remains [Labhardt, A. M. (1982) *J. Mol. Biol. 157*, 357–371]. Under these conditions (pH 1.7, 45 °C) the exchange-rate test is negative, indicating that any residual structure in thermally unfolded S-protein does not have a detectable effect on amide proton exchange rates.

In native proteins, amide proton (peptide NH) exchange rates are often retarded by a factor of 10^6 or greater, compared to exchange rates from unfolded states. The large reduction in exchange rates is thought to be caused chiefly by hydrogen bonding (H-bonding) and solvent exclusion (Barksdale & Rosenberg, 1982; Englander & Kallenbach, 1983; Richards, 1979; Wagner & Wüthrich, 1982; Woodward et al., 1982). Amide protons are H-bond donors in α-helices and β-sheets; there is also a large reduction in solvent-accessible surface area when these structures are formed [reviewed by Richards (1977)].

Factors other than structure can also have significant effects on the rates of amide proton exchange. In unstructured model compounds, both nearest-neighbor inductive effects (Molday et al., 1972) and charge effects [Kakuda et al. (1971); Kim & Baldwin (1982); see also Matthew & Richards (1983)] are known to influence the intrinsic rate of amide proton exchange. This has made it difficult to correlate observed exchange rates with the presence of structure, especially when stability of the structure is low (i.e., the extent of protection from exchange is less than 100-fold).

Here we describe a method to detect relatively unstable elements of structure. We use denaturants to unfold the polypeptide; exchange from amide protons involved in structure is accelerated by unfolding. A principal advantage of this structure test is that no assumptions are made about the exchange rate constants in the unfolded state. It is necessary that the structure retards amide proton exchange rates and that the structure is unfolded by denaturant.

[†] This research was supported by a grant to R.L.B. from the National Institutes of Health (GM 19988-23). P.S.K. is a predoctoral fellow of the NIH Medical Scientist Training Program.

[‡] Present address: Medical Scientist Training Program, Washington University School of Medicine, St. Louis, MO 63110.

[§] Present address: Department of Biochemistry, College of Medicine, University of Lagos, Lagos, Nigeria.

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It is necessary first to calibrate the effects of denaturants on the intrinsic rate of amide proton exchange. We have measured the effects of urea and guanidinium chloride (GdmCl)[1] on exchange from the model compound, poly(DL-alanine) (PDLA). In addition, the effects of urea on exchange from a charged model compound, *N*-acetyllysine methyl ester (NALM), have been determined.

The exchange-rate test is used here to study structure in S-protein (residues 21–124 of RNase A). Structure can be observed by CD in S-protein at pH 1.7 below 32 °C, conditions in which S-protein has the properties of an equilibrium folding intermediate, denoted I_3 (Labhardt & Baldwin, 1979). I_3 combines rapidly with S-peptide to form native RNase S after a pH jump to 6.8. I_3 shows a thermal unfolding transition that is complete at 32 °C, pH 1.7. As judged by CD, however, residual structure remains after I_3 unfolds at 32 °C, pH 1.7 (Labhardt, 1982).

Thermal unfolding of I_3 gives CD changes in the far-UV that can be attributed to unfolding of the β-sheet: CD changes characteristic of α-helix unfolding are not observed (Labhardt, 1982). S-protein contains residues 24–35 and 50–60 that, in native RNase S, are H-bonded in helices (Richards & Wyckoff, 1973). Labhardt (1982) found that CD changes characteristic of α-helix unfolding are produced by adding 5 M GdmCl or 9 M urea to thermally unfolded S-protein at pH 1.7, 32 °C. Similar CD changes are produced by digestion with pepsin at pH 1.7 or with trypsin at pH 7.8. Raising the temperature above 32 °C at pH 1.7 does not produce a CD change characteristic of α-helix unfolding.

These results led Labhardt (1982) to suggest that the two helices in the S-protein moiety of RNase S are thermostable and do not unfold when I_3 is unfolded at 32 °C or at even higher temperatures. On the other hand, the α-helix formed in the isolated S-peptide is known to be thermolabile; only partial helix formation is observed near 0 °C, and the helix is almost entirely unfolded at 25 °C (Kim et al., 1982; Rico et al., 1983; Kim & Baldwin, 1984).

This curious situation prompted us to use an exchange-rate test for structure in S-protein at pH 1.7 both at 0 °C, where I_3 is well populated, and at 45 °C, where I_3 is unfolded but CD data suggest that residual structure remains (Labhardt, 1982).

Materials and Methods

PDLA was from Miles (lot A168), and NALM was from Sigma (lot 43C-2200). S-protein was prepared for us from RNase S (Sigma grade XII-S) by V. MacCosham using the method of Doscher & Hirs (1967). Urea and GdmCl were Schwarz-Mann ultrapure grade. GdmCl-d_6 was prepared by repeated lyophilization from D_2O (Sigma, 99.8% D). [^{3}H]-H_2O and [^{14}C]HCHO were from New England Nuclear. [^{14}C]S-protein was prepared by reductive methylation of S-protein with $NaCNBH_3$ and [^{14}C]HCHO (Jentoft & Dearborn, 1979). Urea and GdmCl solutions were prepared fresh daily. pH values reported are readings made with a glass electrode, without correction for isotope effects (Glasoe & Long, 1960).

For model compound studies, peptide group deuterium–hydrogen or hydrogen–deuterium exchange was monitored at 220 nm with the ultraviolet spectrophotometric technique (Englander et al., 1979). Solutions contained 0.1 M NaCl and 5 mM buffer salt equivalents (sodium oxalate, formate, cacodylate, phosphate, and glycine were used as buffers). First-order exchange rate constants were obtained from a Guggenheim plot, using Δt values greater than $t_{1/2}$ for the reaction (Guggenheim, 1926).

The pH dependence of exchange was fit with a nonlinear least-squares computer program to the equation

$$k_{obsd} = k_A[H^+] + k_B[OH^-] + k_C \quad (1)$$

where k_{obsd} is the measured rate of exchange; k_A, k_B, and k_C are the rate constants for acid-, base-, and water-catalyzed exchange, respectively. For measurements made in D_2O, $[H^+]$ and $[OH^-]$ in eq 1 were replaced by $[D^+]^*$ and $[OD^-]^*$. The asterisks refer to the fact that pH meter readings, uncorrected for glass electrode isotope effects, were used to calculate $[D^+]^*$. $[OH^-]$ and $[OD^-]^*$ were calculated by using pK_w values at 20.0 °C for H_2O and D_2O of 14.17 and 15.05, respectively.

S-protein was labeled with [^{3}H]H_2O at pH 1.7, 45 °C for 30 min. Trace amounts of [^{14}C]S-protein were included as an internal concentration standard (Schreier, 1977). Exchange-out was initiated by a 100-fold dilution of the labeling solution with nonradioactive buffer (0.1 M NaCl, 50 mM glycine, pH 1.7). This ensured that S-protein was already thermally unfolded when exchange was initiated at 45 °C. Exchange was monitored by using the filter assay described by Schreier (1977). After exchange-out was complete, aliquots were assayed to determine the background level of labeling, resulting from the presence of [^{3}H]H_2O in the exchange solution. All values have been corrected for this background and also for an ^{3}H:^{1}H isotope effect of 1.2 (Englander & Poulsen, 1969).

Results

Effects of Denaturants on the Intrinsic Rate of Amide Proton Exchange. The pH dependence of exchange from PDLA in the presence and absence of 6.0 M GdmCl (20.0 °C) is shown in Figure 1a. The data for 0 M GdmCl agree well with the results of Englander et al. (1979). The presence of 6.0 M GdmCl retards exchange in the acid-catalyzed pH region whereas there is little effect in the base-catalyzed region of exchange. Figure 1b shows that retardation of acid-catalyzed (pH 1.93) exchange rates by GdmCl is not a linear function of [GdmCl]. In the base-catalyzed (pH 4.00) region of exchange, the effect of [GdmCl] is biphasic; at low concentrations, GdmCl increases exchange rates, whereas exchange rates decrease at high GdmCl concentrations (Figure 1c).

The effect of 8.0 M urea on exchange from PDLA is depicted in Figure 2a. Urea accelerates acid-catalyzed exchange and retards base-catalyzed exchange in PDLA. As a result, the pH at which exchange is slowest (pH_{min}) is shifted to higher pH in the presence of urea (Figure 2a).

In contrast to GdmCl, the effects of urea on PDLA exchange rates are linear functions of urea concentration. In acid-catalyzed exchange (pH 1.93) from PDLA, there is a linear increase in rate as urea is added (Figure 2b); exchange is approximately 2 times faster in 8 M urea. Base-catalyzed (pH 4.50) exchange from PDLA decreases linearly with [urea]; exchange is approximately 5 times slower in 8 M urea (Figure 2c).

The effects of urea on exchange from *N*-acetyllysine methyl ester (NALM) were also determined. As with PDLA, 8.0 M urea causes a shift in pH_{min} to higher pH values (Figure 3a). The effects of urea on acid-catalyzed exchange (Figure 3b)

[1] Abbreviations: GdmCl, guanidinium chloride; PDLA, poly(DL-alanine); NALM, *N*-acetyllysine methyl ester; RNase A, bovine pancreatic ribonuclease A; RNase S, a derivative of RNase A cleaved at the peptide bond between residues 20 and 21; S-peptide, residues 1–20 of RNase S; S-protein, residues 21–124 of RNase S; pH_{min}, the pH at which proton exchange is slowest; CD, circular dichroism; NMR, nuclear magnetic resonance; BPTI, bovine pancreatic trypsin inhibitor.

Table I: Rate Constants for Exchange Reactions at 20.0 °C[a]

compound	denaturant	exchange reaction	k_A (M^{-1} s^{-1})	k_B (M^{-1} s^{-1})	k_C (s^{-1})
PDLA	none	D/H	0.45	7.4×10^7	7.0×10^{-4}
PDLA	8.0 M urea	D/H	1.8	1.4×10^7	2.8×10^{-4}
NALM	none	D/H	4.5	9.8×10^7	7.1×10^{-4}
NALM	8.0 M urea	D/H	13	1.2×10^7	1.4×10^{-4}
PDLA	none	H/D	0.21	1.9×10^8	8.8×10^{-4}
PDLA	2.0 M GdmCl	H/D	0.29	4.3×10^8	3.4×10^{-4}
PDLA	4.0 M GdmCl	H/D	0.18	4.7×10^8	2.4×10^{-4}
PDLA	6.0 M GdmCl	H/D	0.11	2.5×10^8	3.2×10^{-4}

[a] The rate constants k_A, k_B, and k_C refer to the acid-, base-, and water-catalyzed reactions, respectively. D/H exchange was measured in H_2O solutions and H/D exchange in D_2O solutions. All measurements were made in the presence of 0.1 M NaCl and 5 mM buffer salt equivalents. Corrections were not made for glass electrode isotope effects; reported values refer to apparent rate constants. See Materials and Methods for details.

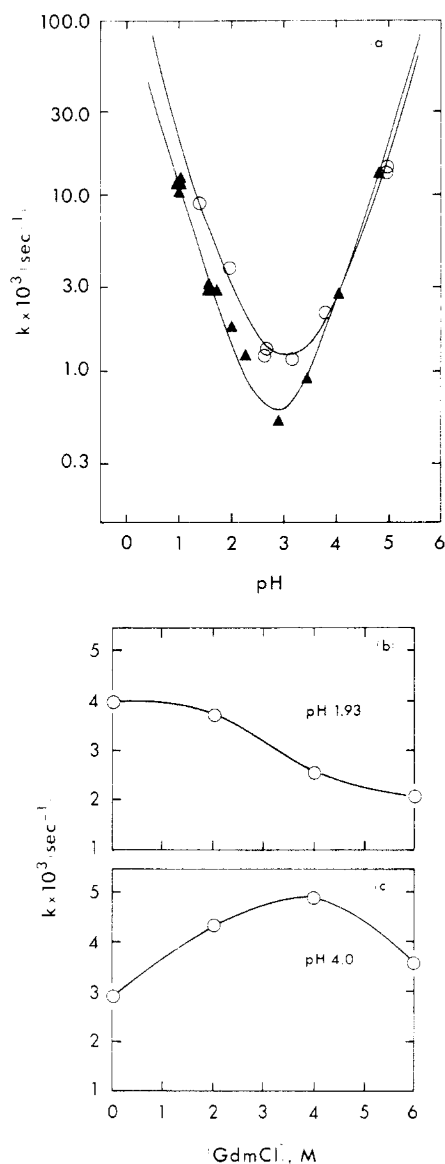


FIGURE 1: (a) pH dependence of amide proton exchange in PDLA (D_2O, 0.1 M NaCl, 5 mM buffer, 20.0 °C): (O) 0 M GdmCl; (▲) 6.0 M GdmCl. The lines are calculated by using rate constants listed in Table I. (b) Effect of GdmCl concentration on acid-catalyzed exchange of PDLA (pH 1.93, 20.0 °C). (c) Effect of GdmCl concentration on base-catalyzed exchange of PDLA (pH 4.00, 20.0 °C).

and base-catalyzed (Figure 3c) exchange from NALM are not, however, linear functions of [urea]. The difference in exchange rates in the presence and absence of 8 M urea is similar for PDLA and NALM (cf. Figures 2 and 3).

Table I lists the apparent rate constants for exchange from the model compounds in the presence and absence of denaturants (see Materials and Methods for details of the fitting

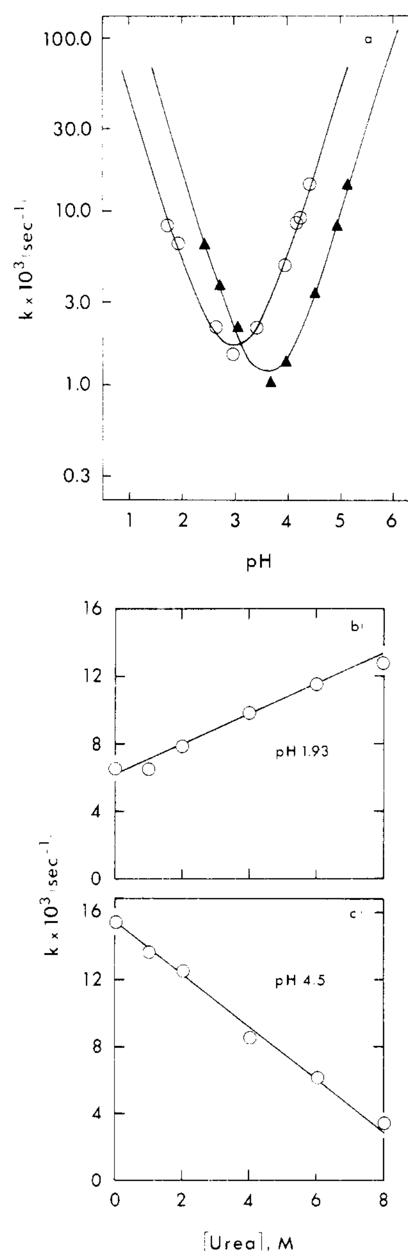


FIGURE 2: (a) pH dependence of amide proton exchange in PDLA (H_2O, 0.1 M NaCl, 5 mM buffer, 20.0 °C): (O) 0 M urea; (▲) 8.0 M urea. The lines are calculated by using rate constants listed in Table I. (b) Effect of urea concentration on acid-catalyzed exchange of PDLA (pH 1.93, 20.0 °C). (c) Effect of urea concentration on base-catalyzed exchange of PDLA (pH 4.50, 20.0 °C).

procedure). The rate constants determined here for acid, base, and water catalysis of exchange from PDLA in D_2O at 20.0 °C (Table I) are in good agreement with values obtained by Englander et al. (1979) and Gregory et al. (1983).

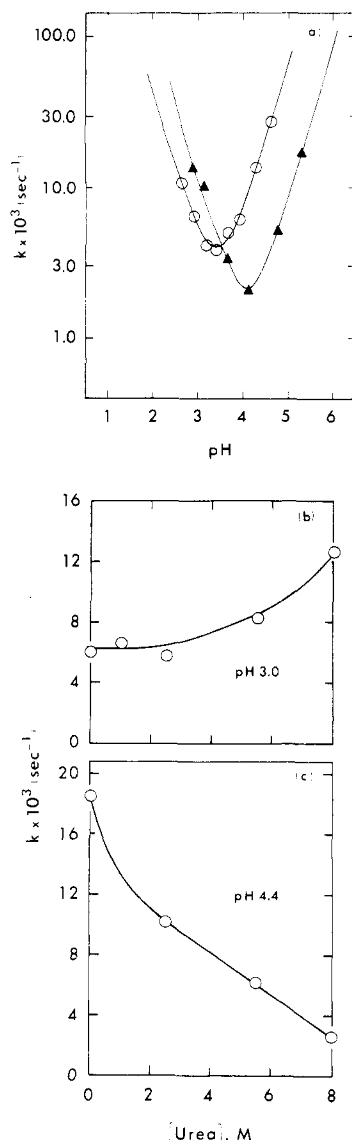


FIGURE 3: (a) pH dependence of amide proton exchange in NALM (H_2O, 0.1 M NaCl, 5 mM buffer, 20.0 °C): (O) 0 M urea; (▲) 8.0 M urea. The lines are calculated by using rate constants listed in Table I. (b) Effect of urea concentration on acid-catalyzed exchange of NALM (pH 3.00, 20.0 °C). (c) Effect of urea concentration on base-catalyzed exchange of NALM (pH 4.40, 20.0 °C).

For PDLA, k_{OH^-} is less than k_{OD^-} (Table I). This difference, however, is nearly compensated by differences in pK_w for H_2O and D_2O: the observed rates of exchange for PDLA are approximately the same in H_2O and D_2O if corrections are *not* made for glass electrode isotope effects. This is consistent with the observation of Englander et al. (1979), who found that the rates of H/D exchange and ^3H/H exchange in PDLA are approximately the same if uncorrected pH meter readings are used. This convention also gives similar values in H_2O and D_2O for the ionization constants of model compounds (Bundi & Wüthrich, 1979). We follow this convention here, and the apparent rate constants given in Table I refer to apparent (D^+) activities based on this convention. The rates of H/D exchange in D_2O are slightly slower than those for D/H exchange in H_2O (compare 0 M GdmCl in Figure 1a with 0 M urea in Figure 2a). Similarly, the rates of H/D exchange are slightly slower than those for ^3H/H exchange [see Figure 3 of Englander et al. (1979)].

Exchange Rate Test for Structure: A Search for Residual Structure in Thermally Unfolded S-protein. Figure 4a depicts amide proton exchange from S-protein, 0 °C, pH 1.7, at

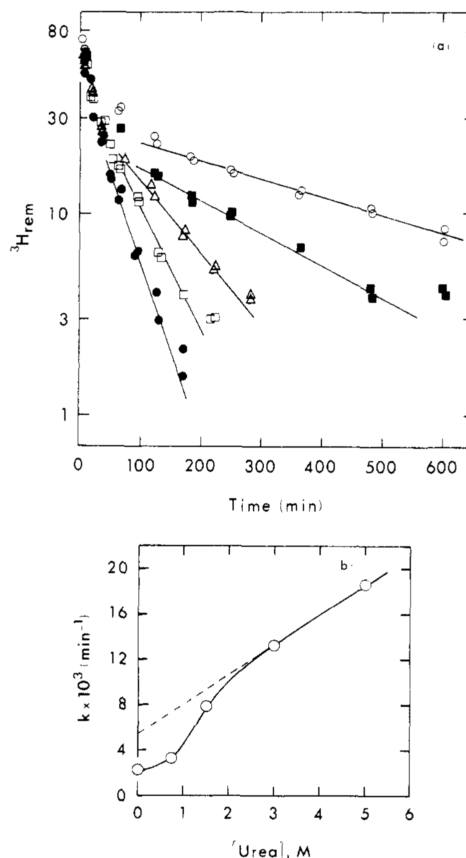


FIGURE 4: (a) Amide proton exchange of fully tritiated S-protein at 0 °C, 0.1 M NaCl, 50 mM glycine, pH 1.7: (O) 0 M urea; (■) 0.75 M urea; (△) 1.50 M urea; (□) 3.00 M urea; (●) 5.00 M urea. (b) Urea dependence of exchange rate for the slow phase in S-protein, 0 °C, pH 1.7.

different urea concentrations. In the absence of urea, the exchange kinetics are biphasic, with ~30 protons exchanging at a substantially slower rate. At higher urea concentrations, the two phases coalesce; the slow phase is accelerated more by urea than the fast phase.

When the exchange rate of the slow phase (pH 1.7, 0 °C) is plotted as a function of [urea], a nonlinear plot is obtained, with an apparent transition occurring between 0 and 2 M urea (Figure 4b). The exchange rate continues to increase with increasing [urea] after the transition between 0 and 2 M urea; this is expected, since urea accelerates intrinsic exchange rates in the acid-catalyzed region of exchange (cf. Figure 2b).

When S-protein is thermally unfolded at 45 °C, pH 1.7, the exchange kinetics are much more uniform than at 0 °C (Figure 5a). As expected, exchange is accelerated by increasing amounts of urea (Figure 5a).

There is a linear dependence of exchange rate on [urea] at pH 1.7, 45 °C (Figure 5b), in marked contrast to the results obtained at 0 °C, where I_3 is populated (Figure 4b). Urea enhances exchange rates in thermally unfolded S-protein (Figure 5b) by the same amount, within experimental error, as in acid-catalyzed exchange from PDLA (Figure 2b).

DISCUSSION

Exchange Rate Test for Structure. In the structure test used here, amide proton exchange rate measurements are made in the absence and presence of varying concentrations of a denaturant (e.g., GdmCl or urea). Those amide protons that have retarded exchange rates as a result of structure (e.g., H-bonding or solvent exclusion) will have an increased rate of exchange in the presence of adequate concentrations of

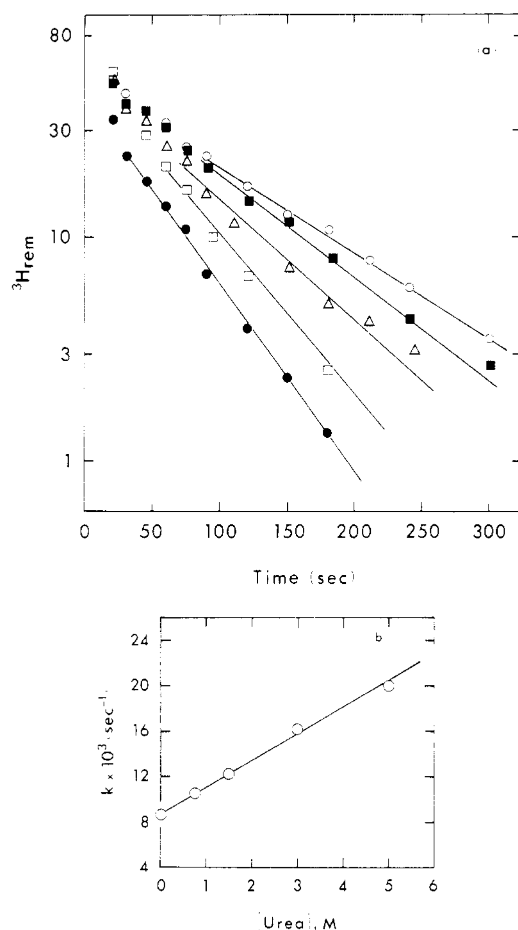
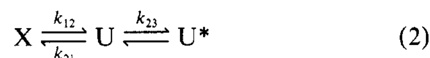


FIGURE 5: (a) Amide proton exchange of fully tritiated, thermally unfolded S-protein at 45 °C, 0.1 M NaCl, 50 mM glycine, pH 1.7: (○) 0 M urea; (■) 0.75 M urea; (△) 1.50 M urea; (□) 3.00 M urea; (●) 5.00 M urea. (b) Urea dependence of exchange rate for the slow phase in thermally unfolded S-protein, 45 °C, pH 1.7.

denaturant, since the denaturant will unfold the structure. Corrections must be made for the effects of denaturant on the intrinsic exchange rate. Urea seems to be better suited for use in the structure test than GdmCl; the effects of GdmCl on intrinsic exchange rates are complex compared to the effects of urea (cf. Figures 1 and 2).

The simplest model of exchange, where exchange only occurs after unfolding of structure, can be described as



$$X \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} U \overset{k_{23}}{\rightleftharpoons} U^* \quad (2)$$

where k_{12} and k_{21} represent the unfolding and refolding rates, respectively, and k_{23} represents the intrinsic rate of amide proton exchange. X and U represent the structured and unfolded conformations, respectively, and U* represents the molecule after exchange. Exchange could also occur from intermediate states between X and U, and exchange from X may be nonnegligible at low denaturant concentrations. These considerations are not included in eq 2 for simplicity. Exchange from a folding intermediate, as in the case of the S-protein experiments discussed here, can be analyzed in the same manner.

Equation 2 has two limiting cases (Hvidt, 1964): (A) the X ⇌ U equilibrium is fast compared to exchange (k_{23}), or (B) intrinsic exchange (k_{23}) is fast compared to the X ⇌ U equilibrium. These limits are analogous to the EX2 and EX1 mechanisms of exchange from proteins, respectively (Hvidt & Nielsen, 1966).

(*i*) *Case A*: *X ⇌ U Equilibrium Is Fast*. This is the usual case, where $k_{12} + k_{21} \gg k_{23}$. The observed rate of amide proton exchange (k_{obsd}) depends on the equilibrium fraction of U and on k_{23}

$$k_{obsd} = \left(\frac{K_{12}}{1 + K_{12}}\right) k_{23} \quad (3)$$

where $K_{12} = k_{12}/k_{21}$. The structure test takes advantage of the fact that denaturants will increase K_{12} and therefore increase k_{obsd}.

The degree of protection from exchange for a given amide proton, $[\theta]_p$, is defined as the ratio of the exchange rate in the absence of structure, k_{23}, to the observed exchange rate, k_{obsd} [cf. Kuwajima & Baldwin (1983); Kim (1986)]:

$$[\theta]_p = \frac{k_{23}}{k_{obsd}} = \frac{1 + K_{12}}{K_{12}} \quad (4)$$

(*ii*) *Case B*: *X ⇌ U Equilibrium Is Slow*. If $k_{23} \gg k_{12} + k_{21}$, then biphasic exchange kinetics are predicted, even for exchange from a single site. The ratio of relative amplitudes for the two phases, α_f:α_s (fast and slow, respectively), will equal the U:X ratio. Exchange from U will proceed at the intrinsic exchange rate (k_{23}), and exchange from X will occur at a rate equal to the unfolding rate (k_{12}).

The predicted exchange kinetics for a given amide proton are

$$H_t = \alpha_f \exp(-k_{23}t) + \alpha_s \exp(-k_{12}t) \quad (5)$$

where H_t is the fraction of molecules with an unexchanged proton at time t, and the amplitudes are normalized ($\alpha_f + \alpha_s = 1$). The relative amplitudes are directly related to K_{12}, the equilibrium constant for X ⇌ U.

$$K_{12} = \alpha_f/\alpha_s \quad (6)$$

Denaturants will increase K_{12} and therefore increase the relative amplitude of the fast phase. In addition, denaturants are expected to increase the rate of unfolding, k_{12}, so that the exchange rate for the slow phase is predicted to increase.

Limitations of the Structure Test. Sensitivity of the exchange rate test for structure is different for the two limiting cases discussed above. When exchange from individual amide protons is monitored, the following estimates of sensitivity can be made.

(i) For the limiting case A (X ⇌ U equilibrium is fast compared to exchange), sensitivity is limited by errors in measurement of exchange rates (cf. eq 4) and by errors in estimates of the intrinsic exchange rate (k_{23}). Exchange rates obtained in the presence of denaturants (i.e., where the molecule is unfolded) are used to estimate k_{23} in the absence of denaturant; the major source of uncertainty is in evaluating the effects of denaturants on intrinsic exchange rates (see discussion below). Under optimal conditions, the exchange rate test for structure can probably be used to detect amide proteins with $[\theta]_p$ as low as ~2. In the simple model for exchange (eq 2), this corresponds to an equilibrium constant for structure formation of unity (cf. eq 4).

(ii) When exchange is fast compared to the X ⇌ U equilibrium (case B), the structure test is more sensitive. In this case, sensitivity of the structure test is limited by ability to detect the slow phase, α_s (cf. eq 6). It should be easy to distinguish the fast and slow phases since $k_{23} \gg k_{12}$. Assuming that it is possible to detect the slow phase when $\alpha_s = 0.2$, the structure test in case B can be used to detect amide protons with an equilibrium constant for structure formation, K_{21} ($=1/K_{12}$), of 0.25.

Equations 3–6 pertain to exchange from individual amide protons (e.g., as determined by ^{1}H NMR). When exchange from the entire molecule is measured (e.g., with ^{3}H methods), then the equations are replaced by a summation over all amide protons, with individual values of k_{23} and K_{12}. Only some amide protons in the molecule will be protected from exchange, and different amide protons will have different intrinsic exchange rates (Molday et al., 1972). These considerations will decrease the sensitivity of the structure test.

Urea Is Better Suited Than GdmCl for Use in the Structure Test. Denaturants might affect intrinsic exchange rates by binding to the polypeptide chain and/or by altering the bulk solvent. For the structure test, it is desirable to use a denaturant that has minimal and consistent effects on intrinsic exchange rates. We have investigated the effects of 0–6 M GdmCl and 0–8 M urea on acid- and base-catalyzed exchange from model compounds. Under all conditions studied, there was less than a 10-fold effect on the observed exchange rates.

GdmCl does not appear to be well suited for use in the structure test; the effects of GdmCl on exchange from PDLA are complicated (Figure 1). GdmCl decreases acid-catalyzed exchange for PDLA. Base-catalyzed exchange from PDLA is enhanced at low [GdmCl] but begins to be retarded at high [GdmCl] (Figure 1c). At all GdmCl concentrations, however, base-catalyzed exchange from PDLA is faster than exchange in the absence of GdmCl. In contrast, base-catalyzed exchange (pH 4.8) from S-peptide (residues 1–20 of RNase A) is slower in the presence of 6 M GdmCl (Schmid & Baldwin, 1979). Exchange from oxidized RNase near the pH_{min} (pH 3.15) is also retarded by 6 M GdmCl (Woodward & Rosenberg, 1970).

GdmCl reduces the OH^- activity in aqueous solutions (Nozaki & Tanford, 1967; Schrier & Schrier, 1977); base-catalyzed exchange is therefore expected to be slower in GdmCl solutions. Our observation that GdmCl increases the rate of base-catalyzed exchange from PDLA in a complex manner, and the previous observation that base-catalyzed exchange from S-peptide decreases in 6 M GdmCl, suggests that specific interactions between GdmCl and polypeptide chains also affect exchange rates. It is known that GdmCl interacts with polypeptides in a manner that depends on neighboring groups [see Bierzynski & Baldwin (1982) and references therein].

Urea increases acid-catalyzed exchange and decreases base-catalyzed exchange from PDLA; in both cases the effect is proportional to [urea] (Figure 2). Compared to the effects of GdmCl on exchange, the effects of urea depend more uniformly on denaturant concentration. Enhancement of the acid-catalyzed exchange reaction may be due to general-acid catalysis by ionized urea molecules. Urea is protonated with a p$K \sim 0.2$ (Warner, 1942; Weast & Astle, 1980); the predicted general-acid rate enhancement by ionized urea with this pK_a is in good agreement with the results in Figure 2b (D. Loftus, unpublished results).

With NALM, which contains a single amide proton, the effects of urea are not linear functions of [urea] (Figure 3). This suggests that a linear extrapolation of rates will not be valid for all amide protons. Nevertheless, the effect of urea is in the same direction for PDLA and NALM, in both the acid- and base-catalyzed reactions. In addition, the difference in rates between 0 and 8 M urea is approximately the same for the two model compounds.

If the effect of urea on acid-catalyzed exchange is caused predominantly by ionized urea acting as a general-acid catalyst, then the effect is predicted to be proportional to [urea]. If, however, urea is influencing exchange rates through a general solvent effect, then one might expect log k to be proportional to [urea] [cf. Nozaki & Tanford (1967); Schellman (1978)]. For both acid- and base-catalyzed exchange from PDLA (Figure 2b,c), the data are fit best as linear functions of [urea], although a logarithmic proportionality is not outside experimental error (plots not shown). Acid-catalyzed exchange from NALM (Figure 3b) is not a linear function of [urea]; it also does not show a logarithmic proportionality (plot not shown). Base-catalyzed exchange from NALM, however, can be fit reasonably well by a straight line if log k vs. urea is plotted (not shown). Further work is required to determine whether urea effects on exchange are fit best in a linear or logarithmic manner: the results may be different for acid- and base-catalyzed exchange as well as for different amide protons. The urea dependence of exchange rates for the slow phase in thermally unfolded S-protein at pH 1.7 (Figure 5b) is fit best with a linear proportionality constant; again, however, a logarithmic proportionality is not outside experimental error.

Woodward & Rosenberg (1970) report that 3 M urea has no effect on the rate of exchange from oxidized RNase. Their measurement, however, was made near the pH_{min} (pH 3.15), where the effects of urea are small (cf. Figure 2a). The effects of urea on exchange from PDLA have been measured as a function of pH by Englander [reported in Figure 14 of Englander & Kallenbach (1983)]; her results agree satisfactorily with ours. Retardation of base-catalyzed exchange by urea has also been reported by Swenson & Koob (1970).

Comparison of exchange rates from folded proteins in the presence and absence of urea has been used as a test of models for amide proton exchange from native proteins [Hilton et al. (1981) and references therein]. The effects of urea on the intrinsic rate of exchange are often significant, and correction for urea effects could affect the interpretation of exchange from native proteins in the presence of urea. For example, although the base-catalyzed exchange rates in BPTI are approximately the same in the presence or absence of 8 M urea (Hilton et al., 1981), base-catalyzed exchange from PDLA is $\sim$4.5-fold slower in the presence of 8 M urea (Figure 2c) [see also discussion by Englander & Kallenbach (1983)].

The Test for Structure Applied to I_3 and to Thermally Unfolded S-protein. We have measured the urea dependence of exchange from S-protein at 0 °C, pH 1.7. At this pH and temperature, in the absence of urea, I_3 represents $\sim$65% of the population (the remainder is unfolded). Measurements were also made at 45 °C, pH 1.7, where I_3 is not detectable (Labhardt & Baldwin, 1979; Labhardt, 1980, 1982).

The test for structure is negative in conditions where I_3 is thermally unfolded (45 °C, pH 1.7). Urea accelerates exchange from S-protein, as expected since urea increases the intrinsic rate of exchange in the acid-catalyzed pH region. Over half the amide protons in thermally unfolded S-protein exchange at approximately the same rate, as depicted by the lines in Figure 5a. This observed rate of exchange from thermally unfolded S-protein is proportional to urea concentration (Figure 5b). A linear urea dependence of exchange rates is also found with PDLA and implies that nonlinear urea effects (e.g., observed with NALM) are small and/or cancel each other in unfolded S-protein. The difference in exchange rate between 0 and 5 M urea is approximately the same for PDLA and thermally unfolded S-protein. The biphasic kinetics seen in Figure 5a probably reflect the broad distribution of intrinsic exchange rates found in dipeptides (Molday et al., 1972).

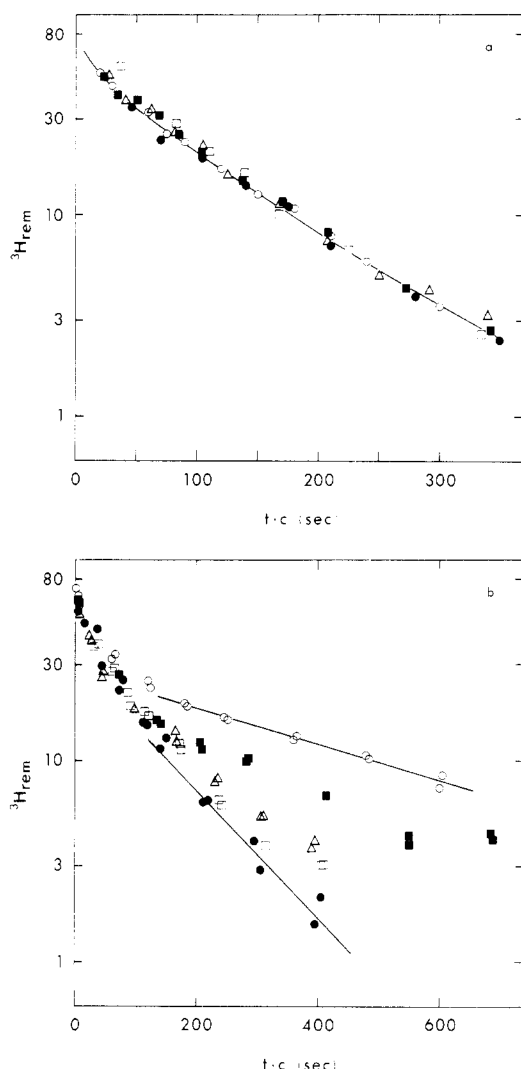


FIGURE 6: (a) Time-normalized plot of amide proton exchange from thermally unfolded S-protein, 45 °C, pH 1.7. The curves were normalized with a time-expansion coefficient, c (see text). (○) 0 M urea, c = 1.00; (■) 0.75 M urea, c = 1.14; (△) 1.50 M urea, c = 1.39; (□) 3.00 M urea, c = 1.86; (●) 5.00 M urea, c = 2.33. (b) Amide proton exchange from S-protein, 0 °C, pH 1.7, using the time-expansion coefficients obtained at 45 °C [see legend to (a)]. (○) 0 M urea; (■) 0.75 M urea; (△) 1.50 M urea; (□) 3.00 M urea; (●) 5.00 M urea.

A more sensitive analysis can be made if one looks at the shape of the exchange curve. In Figure 6a, we have replotted the exchange kinetics for thermally unfolded S-protein at different [urea]; the time axis has been normalized so that the curves at each [urea] are superimposable. To normalize, we substitute ct for t, where c is a coefficient for expansion of the time axis; c is dependent on urea concentration (see Figure 5b). The absence of structure capable of retarding exchange from thermally unfolded S-protein is indicated by (i) superposition of the time-normalized exchange curves at all urea concentrations and (ii) linear dependence of the expansion coefficient, c, on urea concentration (data not shown, but evident from Figure 5b). There is no evidence for H-bonded or solvent-excluding structure capable of retarding exchange in thermally unfolded S-protein.

At 0 °C, pH 1.7, exchange from S-protein is clearly biphasic. As urea is added, exchange is accelerated and the kinetics become almost monophasic (Figure 4a). Above 3 M urea, the shape of the exchange curve at 0 °C is nearly identical with that observed at all urea concentrations in thermally unfolded S-protein (T = 45 °C). This suggests that at low urea concentrations and low temperature (0 °C), the slow phase is due to structure in the S-protein that protects protons from exchange.

When the rate of the slow phase at 0 °C is plotted against urea concentration, a transition is evident between 0 and 2 M urea (Figure 4b). It is not possible to normalize the time axis for the exchange curves at 0 °C, since the shape of the exchange curve changes with [urea]. When the expansion coefficients obtained at 45 °C are used at 0 °C, the slow phase of exchange is still clearly evident (Figure 6b). The difference in exchange rate for the slow phase between 0 and 5 M urea is ~4.5-fold larger at 0 °C than at 45 °C. We conclude that the structure test is positive at 0 °C and that I_3 protects amide protons from exchange.

An estimate of the number of protons protected from exchange in I_3 can be obtained by assuming that the slow phase at 0 °C, pH 1.7, in the absence of urea, is caused by structure in I_3. This may overestimate the number of protected protons in I_3, since some amides may have low intrinsic exchange rates. Measurements of exchange from unfolded S-protein (either at 45 °C or at high [urea], 0 °C) indicate, however, that most amide protons in unfolded S-protein have comparable exchange rates at pH 1.7. The apparent number of protons that exchange in the slow phase at 0 M urea, 0 °C, pH 1.7, is 27. Assuming that 65% of the molecules are I_3 under these conditions (Labhardt & Baldwin, 1979b; Labhardt, 1980, 1982), the number of protected protons per I_3 molecule is 42. RNase A contains ~48 protons that are stable to exchange for 6 h at pH 6, 10 °C (Schmid & Baldwin, 1979); the S-peptide moiety of RNase S protects eight amide protons from exchange (Kuwajima & Baldwin, 1983). Thus, I_3 appears to protect as many protons as the S-protein moiety in RNase S. It is likely that I_3 is a low-pH folded form of S-protein.

Although exchange from I_3 must be at least a few-fold slower in I_3 than in U, it is not possible to estimate the stability of structure in I_3 from these measurements. If the $I_3 \rightleftharpoons U$ equilibrium is fast compared to exchange (limiting case A), then the maximum retardation of the observed exchange rate will be ~3-fold, since 35% of the S-protein molecules are unfolded at 0 °C, pH 1.7 (Labhardt & Baldwin, 1979). On the other hand, if the $I_3 \rightleftharpoons U$ equilibrium is slow compared to exchange (limiting case B), then the stability of I_3 will be reflected in the exchange rate of the slow phase ($=k_{12}$). The rate constants of the $I_3 \rightleftharpoons U$ equilibrium have not been measured; this prevents us from placing an upper limit on the degree of protection from exchange in I_3.

The exchange rate test fails to detect structure in thermally unfolded S-protein at pH 1.7, 45 °C. In contrast, CD results suggest that the two helices in S-protein are thermostable (Labhardt, 1982). The number of H-bonded peptide NH protons in the two helices is large enough (15) that the two helices should be detected readily if the H-bonded NH protons are protected by a factor of 3 or more. For comparison, in native RNase S the seven H-bonded amide protons of the S-peptide α-helix are protected by a factor of about 5000 at pH 5, 20 °C (Kuwajima & Baldwin, 1983). Either the two α-helices of the S-protein are not formed at 45 °C, pH 1.7, and the CD data are misleading, or else the two helices are present but are not very stable. In the latter case, it is puzzling to understand why they are not unfolded completely by raising the temperature.

Applications of the Exchange Rate Test for Structure. Since sequence-specific effects of urea on intrinsic exchange rates might be significant, the structure test that we describe here is best suited for use with individual amide protons that

can be resolved by ^{1}H NMR. The number of systems that fall into this category will increase as 2D NMR methods continue to improve and more peptides and small proteins are assigned. Use of ^{3}H-exchange methods with the structure test is also feasible, as demonstrated by our study of I_3. It is desirable to have a negative control; in our study of I_3, thermally unfolded molecules provide this control. Urea has a moderate effect on intrinsic exchange rates, and the extrapolation to low urea concentrations appears to be almost linear. The usual precautions should be taken to avoid cyanate formation in urea solutions (Stark et al., 1960; Hagel et al., 1971). The main advantage of the structure test is that no assumptions are made about the intrinsic rates of exchange.

Although our studies of S-protein were done at pH 1.7 (since I_3 has been characterized at this pH), the exchange rate test for structure is best applied at pHs above 4, where exchange is base-catalyzed. In this pH region, urea slows down the exchange rate of a fully exposed amide proton. Consequently, acceleration of the exchange rate by [urea] indicates that urea is causing structural unfolding.

Previous Tests for Structure in Thermally Unfolded Proteins. Aune et al. (1967) observed that the addition of GdmCl to any of three thermally unfolded proteins (hen lysozyme, RNase A, or bovine chymotrypsinogen) causes a small but apparently cooperative change in optical rotation. This observation raised the possibility that thermally unfolded proteins might retain some elements of native structure that could be important in directing the refolding reaction. A puzzling feature of the phenomenon demonstrated by Aune et al. (1967) is that the change occurs at rather high GdmCl concentrations, 3–5 M, whereas residual elements of native structure in thermally unfolded proteins might be expected to show only marginal stability. Later, Tanford & Aune (1969) found that the thermodynamic properties of structure in thermally unfolded lysozyme can be attributed plausibly to hydrophobic clusters. Pfeil & Privalov (1976) observed that the enthalpy and ΔC_p of thermally unfolded lysozyme are the same as those of the GdmCl-unfolded protein when extrapolated to the same conditions, and they pointed out that structure in the thermally unfolded state cannot be stabilized by bonds whose formation involves a significant change in enthalpy. Because interaction of a polypeptide with GdmCl involves a substantial heat of interaction (Pfeil & Privalov, 1976), a sizable extrapolation is needed to take account of this effect in comparing the enthalpies of a heat-unfolded and GdmCl-unfolded protein.

Matthews & Westmoreland (1975) note that ^{1}H NMR can be used to demonstrate a structural difference between thermally unfolded and GdmCl-unfolded RNase A: there is a pK difference between two pairs of histidine residues in the heat-unfolded protein, and the difference disappears in 3 M GdmCl. Brown & Klee (1971) point out that residual α-helix formation can be observed by CD for peptides containing the residues (3–13) that form an α-helix near the N-terminus of RNase A. The helix shows only marginal stability in water and is quite thermolabile, being practically undetectable above 25 °C. The contrast between this behavior and the apparently thermostable behavior of the CD-detected structure in thermally unfolded RNase and S-protein (Labhardt, 1982) provided one of the reasons for undertaking this study. Our results indicate that amide proton exchange rates are not affected significantly by CD-detected structure in thermally unfolded S-protein.

Acknowledgments

We thank Drs. A. Bierzynski, N. R. Kallenbach, and A. M. Labhardt for discussions of this work.

Registry No. PDLA, 25281-63-4; NALM, 6072-02-2; GdmCl, 50-01-1; H_2, 1333-74-0; S-protein, 9001-99-4; urea, 57-13-6.

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Halo Enol Lactones: Studies on the Mechanism of Inactivation of α-Chymotrypsin†

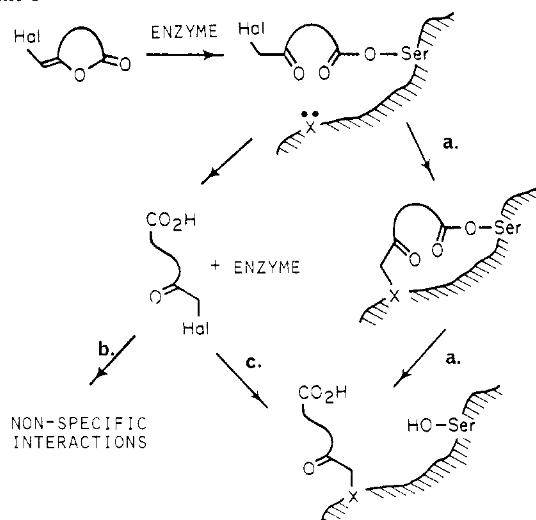
Scott B. Daniels and John A. Katzenellenbogen*
Department of Chemistry, University of Illinois, Urbana, Illinois 61801
Received August 2, 1985

ABSTRACT: In a previous investigation [Daniels, S. B., Cooney, E., Sofia, M. J., Chakravarty, P. K., & Katzenellenbogen, J. A. (1983) *J. Biol. Chem. 258*, 15046–15053], we demonstrated that α-aryl-substituted five- and six-membered ring halo enol lactones were effective inhibitors of chymotrypsin, and we proposed that they reacted by an enzyme-activated mechanism: acyl transfer to the active site serine generates a halomethyl ketone that remains tethered in the catalytic site until it alkylates an accessible nucleophilic residue. In this study, we have investigated in greater detail the process of chymotrypsin inactivation by an α-naphthyl-substituted five- and six-membered bromo enol lactone. Inactivation by both compounds appears to be active site directed, since the time-dependent inactivation is retarded by competing substrate. The possible involvement of a paracatalytic mechanism for inactivation (generation of a free, rather than active site bound, inactivating species) was investigated by comparing the inactivation efficiencies of the lactones with that of the bromomethyl keto acid hydrolysis products. The bromomethyl ketone derived from the five-membered lactone is ineffective, whereas that derived from the six-membered lactone is highly efficient. However, the possible involvement of the free keto acid in chymotrypsin inactivation by the six-membered lactone is ruled out by experiments involving selective scavenging. The long-term inactivation of chymotrypsin requires the presence of the bromine substituent and appears to involve an alkylation rather than an acylation reaction (hydrazine resistant). Furthermore, a 1:1 lactone:enzyme stoichiometry is demonstrated with the ^{14}C-labeled six-membered lactone. These results are consistent with the mechanism-based inactivation process previously presented.

In previous studies, we described the preparation of halo enol lactones (Krafft & Katzenellenbogen, 1981) and their activity as active site directed irreversible inhibitors of chymotrypsin (Chakravarty et al., 1982; Daniels et al., 1983). On the basis of their structure, we proposed that these lactones were acting as mechanism-based inactivators: Acyl transfer to the active site serine would generate a halomethyl ketone that would remain tethered in the catalytic site and, during the lifetime of the acyl enzyme, would alkylate an accessible nucleophilic residue; this covalent attachment, at or near the active site, would then impair the catalytic activity of the enzyme, even after deacylation (Scheme I, pathway a).

In order for this mechanism-based or "suicide" inactivation process to achieve high efficiency and selectivity, it is important that the activated inhibitor undergo covalent attachment more rapidly than it departs from the active site. Otherwise, the released halomethyl keto acid can also react with components outside of the catalytic site, compromising the selectivity of its inactivation (Scheme I, pathway b). The assessment of the

Scheme I



†Support of this work was provided by a grant from the National Institutes of Health (PHS 5R01 AM 27526). High-field NMR spectra (360 MHz) were obtained on an instrument in the National Science Foundation Regional Instrumentation Facility for NMR at the University of Illinois (NSF CHE 83-13703).
* Address correspondence to this author at the School of Chemical Sciences, University of Illinois.

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