Individual amide proton exchange rates in thermally unfolded BPTI

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Individual Amide Proton Exchange Rates in Thermally Unfolded Basic Pancreatic Trypsin Inhibitor†

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ABSTRACT: A novel experiment is described for measurements of amide proton exchange rates in proteins with a time resolution of about 1 s. A flow apparatus was used to expose protein solutions in ^2H_2O first to high temperature for a predetermined time period, during which ^1H–^2H exchange proceeded, and then to ice–water. The technique was applied for exchange studies in thermally unfolded, selectively reduced basic pancreatic trypsin inhibitor. Measurements were made by ^1H nuclear magnetic resonance after the exchange was quenched by rapid cooling. Thereby, the sequence-specific resonance assignments for the folded protein could be used, which had been previously obtained. The results of this study indicate that the exchange rates in the thermally unfolded protein are close to those expected for a random chain and that the NH exchange is catalyzed by $^2H^+$ and O^2H^- up to high temperature, with no significant contributions from p^{2}H-independent catalysis. We conclude that the parameters derived by Molday et al. [Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150–158] from measurements with small model peptides can be used to calculate intrinsic exchange rates in unfolded proteins and thus provide a reliable reference for the interpretation of exchange rates measured under native conditions.

Measurements of exchange rates of interior labile protons are a widely used method for investigating internal motility in globular proteins (Linderstrøm-Lang, 1955; Hvidt & Nielsen, 1966; Englander et al., 1972; Wagner & Wüthrich, 1979a). Usually the data are evaluated on the basis of a structural unfolding model:

$$N(H) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O(H) \xrightarrow[^2H_2O]{k_3} O(^2H) \underset{k_1}{\overset{k_2}{\rightleftharpoons}} N(^2H) \qquad (1)$$

N indicates the ensemble of "closed" conformers in which the labile proton considered is not accessible to the deuterated solvent (for example, because of internal hydrogen bonding). O indicates the "open" conformers in which the same proton is in contact with the solvent. k_1 and k_2 describe the rates of interchange between closed and open states of the protein, and k_3 is the intrinsic exchange rate for the solvent-accessible, labile proton. Depending on the ratios of these rate constants, different limiting kinetic situations may arise (Hvidt & Nielsen, 1966). However, the experimental observations presented in the preceding paper (Roder et al., 1985) indicate that for studies of the exchange of interior backbone amide protons in proteins, exchange via an EX_2 mechanism will

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prevail in most instances of practical interest. In this limiting situation, the intrinsic exchange rate, k_3, is small compared to the closing rate, k_2, and the experimentally determined exchange rate, k_{ex}, is

$$k_{ex} = (k_1/k_2)k_3 \quad (2)$$

If the intrinsic exchange rates k_3 are known, information on the equilibria between open and closed states of the protein (eq 1) can be obtained from such experiments.

So far, knowledge of k_3 relies almost exclusively on measurements of the exchange of solvent-exposed amide protons in small model peptides (Berger et al., 1959; Englander & Poulsen, 1969; Molday et al., 1972), and these data have been used frequently to interpret hydrogen exchange measurements in proteins [e.g., see Rosa & Richards (1979), Woodward & Hilton (1979), Wagner & Wüthrich (1982), and Englander & Kallenbach (1984)]. From these model studies, it is known that k_3 depends on the amino acid type and on the nature of the sequentially neighboring residues. In this paper, we describe a novel experimental approach for obtaining individual, intrinsic amide proton exchange rates by ^{1}H nuclear magnetic resonance (NMR)[1] in a thermally unfolded protein. For these measurements of k_3, we selected a modified form of BPTI, which is a small globular protein for which extensive NH exchange data have already been reported (Masson & Wüthrich, 1973; Karplus et al., 1973; Pershina & Hvidt, 1974; Hilton & Woodward, 1978; Richarz et al., 1979; Wagner & Wüthrich, 1982). In the modified protein RCAM-BPTI, the disulfide bond 14–38 had been cleaved (Vincent et al., 1971). This modification lowers the thermal stability of the protein without significantly changing the tertiary structure (Wagner et al., 1979; Stassinopoulou et al., 1984). The reduced stability was needed for the experiments used to measure k_3.

Materials and Methods

BPTI (Trasylol) obtained from Bayer AG, Leverkusen, West Germany, was used without further purification. Selectively reduced BPTI, with the disulfide bond 14–38 cleaved (RCAM-BPTI), was prepared according to Creighton (1975) using dithiothreitol (Sigma) as a reducing agent and iodoacetamide (Sigma) for blocking the free thiol groups. For proton exchange measurements, 5 mM protein solutions were prepared by dissolving lyophilized RCAM-BPTI in ^2H_2O solutions of glycine-d_5. The p^{2}H of the buffer solutions was adjusted with ^{2}HCl and NaO^{2}H prior to the addition of the protein. Accurate values for p^{2}H were obtained after completion of the exchange measurements by heating the solution to the exchange temperature, for example, 86 °C, and using a combination glass electrode. The pH meter reading was used without correction for isotope effects (Kalinichenko, 1976; Bundi & Wüthrich, 1979; Englander et al., 1979).

The exchange measurements relied on the following procedure. After a ^{1}H NMR spectrum of the freshly prepared ^2H_2O solution of the protein was recorded, the latter was exposed to two consecutive rapid-temperature changes: First, unfolding was initiated by rapid heating to 86 °C, where the solution was kept for a variable time interval between 1.2 and ca. 1000 s. After this exchange time, the reaction was quenched by rapid cooling to 0 °C, which also induced refolding of the protein. The apparatus used is schematically shown in Figure 1. Two thin steel injection needles (0.15-mm inner diameter, 85 mm long) were connected end to end by a short Teflon connector with a 0.2-mm inner diameter. In two separate vessels, the needles were immersed in 86 °C water and in ice–water, respectively. Exposure of the solution to 86 °C for a predetermined time period was achieved by forcing the protein solution through the needles with a constant velocity, v, using a Braun-Melsungen calibrated injection pump. With the known flow velocity, av, the time, t, the solution spends at high temperature is given by

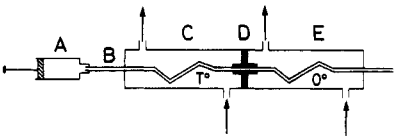

FIGURE 1: Schematic representation of the apparatus used for measurements of amide proton exchange rates in unfolded RCAM-BPTI. From a 5-mL syringe (A), which was driven by an injection pump, the protein solution was forced through a steel needle (B) at a predetermined velocity. In the syringe and the first part of the steel needle, the solution was at ambient temperature, and it passed subsequently through a high-temperature jacket (C), an insulating Teflon connector (D), and an ice–water jacket (E).

$$t = V/av \quad (3)$$

Here, a is the inner cross-section area of the hot part of the apparatus and V the inner volume of the first needle plus the volume of the thermally insulating Teflon connector. The fastest flow velocity used for exchange experiments was 0.5 mL/min, which results in a high-temperature exposure duration of 1.2 s. The time needed to heat the solution from room temperature to 86 °C in the hot needle was estimated by measuring the temperature of the solution with a thermocouple at the exit of the first needle as a function of the flow velocity. An upper limit of 0.15 s for the heating time was found. Due to the strong temperature dependence of the amide proton exchange rates (Englander & Poulsen, 1969), efficient quenching of the reaction in the cold needle was achieved after less than 0.1 s.

^{1}H NMR spectra at 360 MHz were recorded on a Bruker HX-360 spectrometer equipped with an Aspect 2000 computer. The intensity of resolved NH resonances was determined by simulating the spectra in the Aspect 2000 computer using Lorentzian line shapes. Areas were calibrated relative to the two-proton resonance of the nonlabile C$^\epsilon$ protons of Tyr-23. The accuracy of the intensity determination was estimated to about ±5%. Exchange rates for individual NH protons were determined from the resonance intensities as a function of the exchange time (time of exposure at 86 °C in the apparatus described above) by employing exponential regression.

Circular dichroism experiments were performed on a JASCO 500C spectropolarimeter. The ellipticity at 275 nm was recorded as a function of temperature for 1.5×10^{-4} M solutions of RCAM-BPTI (0.1 M NaCl and 0.1 M citrate buffer) in 5-mm quartz cuvettes equipped with a thermostated jacket.

Results

In order to study the exchange from the unfolded form of the protein, it was first necessary to find conditions of temperature and pH where RCAM-BPTI could be reversibly unfolded. To monitor the equilibrium between folded and unfolded protein, the aromatic bands in the circular dichroism spectrum were used as a probe for the native protein structure.

[1] Abbreviations: BPTI, basic pancreatic trypsin inhibitor; RCAM-BPTI, chemical modification of BPTI obtained by reduction of the disulfide bond 14–38 and protection of the free sulfhydryls by carboxamidomethylation; CD, circular dichroism; NMR, nuclear magnetic resonance.

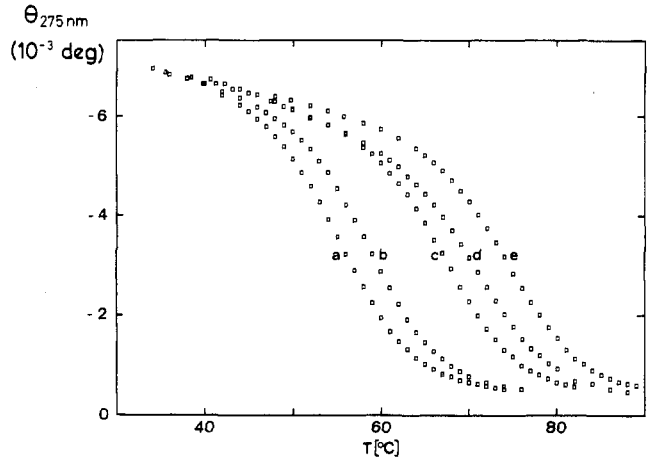

FIGURE 2: CD studies of the thermal denaturation of RCAM-BPTI at five different pH values. The ellipticity at 275 nm is plotted vs. temperature for 1.5×10^{-4} M solutions of RCAM-BPTI containing 0.1 M NaCl and 0.1 M sodium citrate at pH 2.1 (a), 2.8 (b), 3.8 (c), 4.3 (d), and 4.9 (e).

Figure 2 shows the temperature dependence of the ellipticity at 275 nm at five pH values in buffered solutions of RCAM-BPTI. Since BPTI contains no tryptophan, the negative CD band at 275 nm is due to the four tyrosine residues. When the tyrosine rings are released from the asymmetric environment within the protein, these bands disappear almost completely. Figure 2 indicates that at pH below 4.3 RCAM-BPTI is fully unfolded at temperatures above 85 °C. When the solutions were cooled down from 90 °C, essentially identical transition curves were obtained as on heating, indicating nearly complete reversibility of the unfolding transition.

Exchange experiments were performed for eight individual amide protons at 86 °C in the p^2H range between 1.5 and 4.0. After a reference spectrum of the freshly prepared sample was recorded, the solution was exposed to denaturing conditions at 86 °C for an appropriate time interval, as described under Materials and Methods. The exchange reaction was then quenched by rapid cooling, and a second spectrum was recorded to determine the remaining NH intensity. All ^1H NMR spectra were recorded at 25 °C, where the exchange of the amide protons studied is of the order of days (Richarz et al., 1979). The heating–cooling–recording cycle was repeated for the same protein sample at least five times, choosing successively longer exchange times. All resolved NH resonances showed exponential decay of the intensity as a function of the totally accumulated exchange time. Figure 3 shows logarithmic plots of the exchange rates, calculated by exponential regression, vs. p^2H for eight individually assigned NH protons.

The presently used procedure can provide reliable measurements of the exchange from the unfolded protein only if (i) the unfolding reaction after the heating jump is fast compared to the exchange reaction and (ii) refolding after cooling is fast compared to the intrinsic NH exchange at 0 °C. That (i) holds was confirmed by showing that the degree of exchange is independent of the number of unfolding–refolding cycles: *Within experimental error, the same percentage of the* protons were exchanged independent of whether the exchange was initiated by several heating–cooling steps with short exchange periods or by a single cycle with a long exchange period. Direct measurements of unfolding and refolding rates (Roder, 1981) further confirmed that the exchange from the unfolded state is the rate-limiting process. That refolding is fast compared to the exchange at 0 °C was verified by the observation that after a temperature jump from 80 to 0 °C

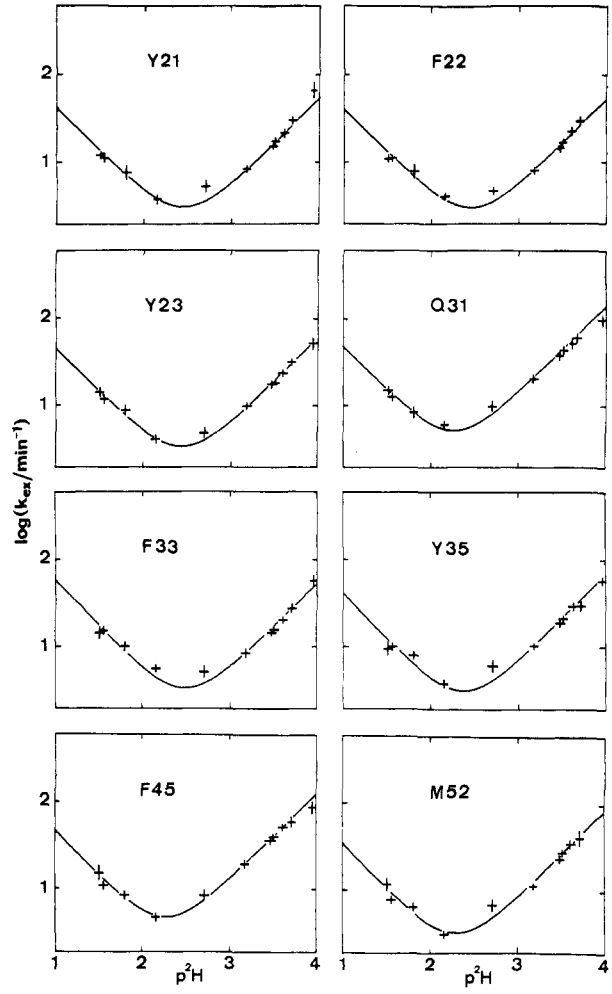

FIGURE 3: Logarithmic plots of the exchange rates as a function of p^2H for eight individually assigned amide protons in RCAM-BPTI at 86 °C in ^2H_2O containing 0.1 M glycine-d_5. The experimental points are indicated by crosses, where the vertical bars indicate the standard deviation of the exponential regression used to obtain k_{ex} from the measured resonance intensities, and the horizontal bars indicate the estimated undertainty of the p^2H measurements (0.1 p^2H unit). The curves are fits of eq 4 to the data, from which the values for p^2H_{min} and k_{min} in Table I were extracted.

at p^2H 2.5, the resonances of native RCAM-BPTI appeared in less than a minute, whereas the exchange from small random-coil peptides at 0 °C is of the order of 100 min at pH 3 (Englander & Poulsen, 1969).

The p^2H dependence of the exchange rates (Figure 3) corresponds to the theoretically expected shape for an acid- and base-catalyzed process. On the left and right sides of the p^2H minimum, the rates are proportional to the $^2H_3O^+$ and O^2H^- concentrations, respectively. Near the p^2H minimum, both processes contribute to the kinetics. The data in Figure 3 were fitted by curves of the type

$$k_{ex} = (k_{min}/2)(10^{p^2H-p^2H_{min}} + 10^{p^2H_{min}-p^2H}) \qquad (4)$$

The parameters obtained from this fit, k_{min} and pH_{min}, are listed in Table I.

The following additional observations indicate that intermolecular aggregation in the unfolded state, which could significantly affect the exchange rates of certain amide protons, is unlikely. First, the reversibility of the unfolding transition observed with CD implies that irreversible aggregation is minimal. In addition, no changes in the native ^1H NMR spectrum were observed even after several heating–cooling cycles. Reversible aggregation of unfolded protein appears

Table I: Intrinsic Amide Proton Exchange Rates in RCAM-BPTI at 86 °C[a]

condition	exchange parameters	amide proton							
		Tyr-21	Phe-22	Tyr-23	Gln-31	Phe-33	Tyr-35	Phe-45	Met-52
A	k_{min} (min^{-1})	3.1	2.9	3.3	5.0	3.4	3.2	4.7	3.4
	p^2H_{min}	2.4	2.4	2.4	2.2	2.5	2.4	2.3	2.3
B	k_{min} (min^{-1})	4.2	3.7	3.7	5.3	4.0	4.7	7.5	5.6
	p^2H_{min}	2.5	2.7	2.6	2.3	2.5	2.8	2.6	2.5

[a] The p^2H profiles for eight amide protons (Figure 3) are characterized by the minimal exchange rate, k_{min} (min^{-1} ±10%), and the corresponding p^2H minimum, p^2H_{min} (±0.15) (eq 4). Condition A is for exchange measured in thermally unfolded RCAM-BPTI at 86 °C. Condition B is for exchange parameters for the same eight amide protons obtained by extrapolation of low-temperature measurements with model peptides to 86 °C (see text) (Englander & Poulsen, 1969; Molday et al., 1972; Covington et al., 1966).

unlikely since ^1H NMR spectra of heat-unfolded RCAM-BPTI show no line broadening. Evidence against aggregation of the heat-unfolded protein was also obtained from the following experiment with native BPTI: After adding 3 M guanidinium chloride, which is known to dissociate protein aggregates (Tanford, 1968), the exchange rates at 90 °C were slowed down by a factor of 1.5–2, in accordance with the slowing effect of the denaturant on the intrinsic exchange in model peptides (Woodward et al., 1975). If aggregates were present, one would expect, in contrast, the solvent effect to be compensated by acceleration of the exchange due to increased solvent accessibility.

Discussion

The presently described quench method for measurements of proton exchange rates up to ca. 100 min^{-1} bridges a gap between direct, "real-time" NMR measurements of exchange in ^2H_2O solutions at the exchange temperature, which are limited to rates slower than ca. 0.1 min^{-1}, and measurements based on NMR line-shape analysis and saturation transfer in H_2O solutions, which cover rates from ca. 60 to 60 000 min^{-1}. In the application to exchange studies in thermally unfolded RCAM-BPTI, the individual resonance assignments for the native protein can be used. Even though no resonance assignments are available for the unfolded protein, the exchange rates can thus be attributed to specific sites in the amino acid sequence. While proton exchange from denaturated proteins was previously measured with different methods (Molday et al., 1972; Woodward & Rosenberg, 1970), Figure 3 presents the first exchange data which can be attributed to particular amino acid residues in an unfolded polypeptide chain.

The data in Figure 3 confirm that the individual amide proton exchange rates in thermally unfolded RCAM-BPTI are governed by acid catalysis at low p^2H and by base catalysis at higher p^2H. The p^2H dependence of the rates could be closely fitted with eq 4 which can, for the sake of improved clarity, be rewritten as

$$k_{ex} = k_{^2H^+}[^2H^+] + k_{O^2H^-}[O^2H^-] \quad (5)$$

where $k_{^2H^+}$ and $k_{O^2H^-}$ are the rates for acid- and base-catalyzed exchange. p^2H-independent exchange due to general ^2H_2O catalysis is generally considered to be negligible (Englander et al., 1972). Gregory et al. (1983), however, concluded from their recent experiments with poly(DL-alanine) that p^2H-independent "water catalysis" should make a significant contribution to the exchange behavior at elevated temperature. If their activation parameters are used to extrapolate the model peptide exchange rates to 86 °C, it is found that near p^2H 3 the p^2H-independent term would be dominant relative to $^2H^+$ and O^2H^- catalysis by a factor of 4 and the p^2H profile would be nearly flat over 2 p^2H units. In contrast, the present exchange data, measured at 86 °C, exhibit linear dependence on $[^2H^+]$ and $[O^2H^-]$, respectively (Figure 3), coinciding with the observations made previously for unstructured model peptides at 0 °C (Molday et al., 1972). There is thus no indication of p^2H-independent catalysis in our data. The deviations from ideal acid and base catalysis, such as a slope $\neq 1$ in a log k_m vs. p^2H plot or the position of p^2H_{min} (Richarz et al., 1979; Wagner & Wüthrich, 1979a; Hilton & Woodward, 1978), can thus not be due to nonideal behavior of the intrinsic exchange step. They must be attributed to p^2H-dependent conformation changes, which have previously been correlated with the deprotonation of ionizable groups in BPTI (Wagner & Wüthrich, 1979b). The apparent discrepancy between the reports of p^2H-independent catalysis at low temperature (Englander et al., 1979; Gregory et al., 1983) and our measurements at high temperature would be resolved if the activation enthalpy of "^2H_2O catalysis" were lower than that of the $^2H^+$ and O^2H^- mechanisms (Hvidt et al., 1983). Deviations from the simple acid/base catalysis would then be more pronounced at low temperature.

The exchange parameters obtained from the measurements in unfolded RCAM-BPTI (Table IA) were compared with corresponding data in small model peptides (Table IB) as follows. Since the exchange in such peptides cannot be quenched by "trapping" internal amide protons in a folded form, the technique used here for the unfolded protein could not be applied. Therefore, using the rules on the sequence dependence of intrinsic exchange rates derived by Molday et al. (1972) from systematic studies of model peptides, we computed intrinsic exchange rates for the amide protons in RCAM-BPTI at 0 °C, and these data were then extrapolated to 86 °C (Table IB). For this estimation, the rate constants for acid and base catalysis were extrapolated from $k_{^2H^+}(0\ °C) = 3.468\ min^{-1}\ M^{-1}$ and $k_{O^2H^-}(0\ °C) = 2.599 \times 10^{10}\ min^{-1}\ M^{-1}$ to 86 °C with activation enthalpies $\Delta H^{\ddagger}_{^2H^+} = 15$ kcal M^{-1} and $\Delta H^{\ddagger}_{O^2H^-} = 2.6$ kcal M^{-1}, respectively (Englander & Poulsen, 1969). The temperature dependence of the water dissociation constant, $K_{^2H_2O}$, was calculated according to Covington et al. (1966), taking account for the temperature dependence of the enthalpy of ^2H_2O ionization by

$$pK_{^2H_2O} = 4913T^{-1} - 7.60 + 0.02009T \quad (6)$$

This yields $pK_{^2H_2O}(86\ °C) = 13.29$ and $pK_{^2H_2O}(0\ °C) = 15.875$. Since this value for $pK_{^2H_2O}(0\ °C)$ deviates from the value of 15.0 used by Englander & Poulsen (1969), we adopted a modified value for $k_{O^2H^-}(0\ °C) = 2.599\ min^{-1}\ M^{-1}$ to match the model peptide data at 0 °C. Table I shows that there is close coincidence between the values for k_{min} and p^2H_{min} obtained in the unfolded protein and by the model peptide approach. For all protons studied, the rates measured in the unfolded protein are slightly smaller (up to 40%) than the extrapolated model peptide rates, but in view of the uncertainty in the activation enthalpies used for the high-temperature extrapolation, which are based on measurements of the temperature dependence in poly(DL-alanine) between 0 and 15 °C (Englander & Poulsen, 1969), the agreement is surprisingly good. Note that in both data sets the highest values for k_{min}

prevail for Gln-31 and Phe-45. This result argues against the presence of hydrogen-bonded residual structure in the denatured protein which would lead to slower exchange rates. Further evidence against significant residual spatial structure effects in the heat-denatured protein comes from the aforementioned observation that addition of 3 M guanidinium chloride did not increase the NH exchange rates.

In conclusion, the present measurements of individual amide proton exchange rates in unfolded RCAM-BPTI show that the intrinsic exchange reaction in a natural polypeptide chain is qualitatively closely similar to the behavior anticipated from the data available from small model peptides. After the well-known electrostatic effects due to side chains and neighboring peptide groups (Molday et al., 1972) are taken into account, no unexpected further sequence-dependent effects are observed. Thus, the procedures of Molday et al. (1972) can be used to calculate intrinsic exchange rates in unfolded proteins and in this way represent a useful reference for the interpretation of individual amide proton exchange rates measured under native conditions.

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Registry No. Basic pancreatic trypsin inhibitor, 9087-70-1; H_2, 1333-74-0.

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