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Hydrogen–Tritium Exchange in Polypeptides. Models of α-Helical and β Conformations†

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ABSTRACT: The rate of hydrogen–tritium exchange of the amide hydrogens for both the α-helical and β-sheet conformation of the same synthetic polypeptide has been measured in aqueous media. A copolymer of L-glutamic acid and L-valine, poly(Glu^{77}Val^{23}), was synthesized whose conformation at pH 4.1 can be varied by heating and cooling. A class of extremely slowly exchanging protons was found which was directly correlated with the amount of β conformation in the copolymer. A greatly reduced rate of exchange was also observed in the soluble aggregate of α-helical poly(Glu), which was directly related to the degree of aggregation. It is suggested that extremely slow hydrogen exchange can be explained by conformation stabilization without restricting such hydrogens to the deep interior of macromolecules. The existence of the previously reported equilibrium isotope effect [Englander, S. W., and Poulsen, A. (1969), *Biopolymers 7*, 379] was observed to be 1.18 for both poly(DL-Lys) and the glutamic acid copolymer.

The exchange rate of protein hydrogens has been proposed as a sensitive technique to provide information about both the conformation and the conformational dynamics of proteins (Berger and Linderstrøm-Lang, 1957). This approach is exemplified in the work of Englander and Staley (1969) on myoglobin where hydrogen exchange studies coupled with the established X-ray structural data have led to valuable insights into the mechanism of hydrogen exchange and the total number of H-bonded peptide groups in this protein.

Recent hydrogen exchange studies of peptides have shown that exchange rates could not be used as a measure of the helical content of proteins as had been originally postulated (*e.g.*, Hvidt, 1964; Blout *et al.*, 1961; Nakanishi *et al.*, 1972). Hydrogen-bonded structures, however, do exchange slowly relative to the intrinsic exchange rate of free (*i.e.*, not hydrogen bonded) amides which has led to the concept that hydrogen-bonded structures themselves could not undergo hydrogen exchange, but that some "opening" of the hydrogen bond must occur first (Linderstrøm-Lang, 1955; Ikegami *et al.*, 1965; Benson *et al.*, 1964; Ikegami and Kono, 1967; Englander and Staley, 1969; Englander *et al.*, 1972; Englander and Mauel, 1972; Englander and Rolfe, 1973). It has been suggested that slowly exchanging protons are representative of hydrogen-bonded structures generally (Englander and Staley, 1969), although steric factors and local environmental effects have been proposed as alternate explanations (Klotz and Mueller, 1969; Leichtling and Klotz, 1966). As the exchange mechanism becomes understood, the use of tritium exchange can lead to information about the dynamic conformations of proteins (*e.g.*, Englander, 1963; Rosenberg and Woodward, 1970; Budzynski and Fraenkel-Conrat, 1970; Hvidt and Corett, 1970; Woodward and Rosenberg, 1970; Englander and Staley, 1969; Englander *et al.*, 1972; Englander and Mauel, 1972; Englander and Rolfe, 1973). Commonly, one observes several classes of exchanging hydrogens as differentiated by their exchange rates, the fastest due to free-amide hydrogens and the slowest due to hindered hydrogen-bonded structures. With these factors in mind, an attempt was made to compare the hydrogen exchange rates of a polypeptide in the β conformation (with its strong hydrophobic stabilization) to that of the same polypeptide in the α-helical form and to analogous peptides in the random conformation. Such data might offer some insight into the origins of the different observed kinetic classes of exchanging protons in proteins.

Materials and Methods

Poly(L-lysine)·HCl (GF-16-69-24). Poly(L-Lys)·HCl was synthesized as previously described (Fasman *et al.*, 1961) and purified as described by Davidson and Fasman (1967) before use. The polymer had an intrinsic viscosity, $[\eta]^{1\ M\ NaCl}_{pH\ 4.3} = 0.76$.

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Poly(L-*glutamic acid* L-*valine*) [*Poly*($Glu^{77}Val^{23}$)] (*GF-18-427-24*). L-Valine-*N*-carboxyanhydride (Bloom *et al.*, 1962) (0.187 g, 1.31×10^{-3} M) dissolved in 1 ml of CH_2Cl_2 and γ-benzyl-L-glutamate-*N*-carboxyanhydride (Blout and Karlson, 1956) (1.37 g, 5.22×10^{-3} M) dissolved in 5 ml of freshly distilled dioxane were added to 140 cm^3 of freshly distilled benzene to give a 1% solution. The polymerization was initiated with $NaOCH_3$ (0.424 cm^3 of 0.385 N) to give anhydride/initiator = 40. After standing 3 days, dry HCl gas was bubbled in until saturation, and then anhydrous HBr was bubbled in for 15 min causing precipitation. On standing overnight, with stirring, a white precipitate formed. The HBr and HCl were removed, as well as the solvent, on a water aspirator. The precipitate was washed three times with ether and then dissolved in 0.01 N NaOH (not all dissolved). The pH was lowered to 7 and the solution was extracted with ether and dialyzed *vs.* H_2O for 4 days, with four changes of water. The solution was then filtered through a sintered glass funnel (M porosity) and the slightly opalescent solution lyophilized; wt = 0.260 g, 32.5% yield; $\eta_{sp/c} = 1.09$ (0.2% in 0.2 M NaCl). The amino acid ratio (Glu:Val) was determined to be 76.8:23.2 by potentiometric titration.

Poly(DL-*glutamic Acid*) (*Sodium Salt*) (*GF-18-431-21*). Dry benzene (200 cm^3) was added to a solution of γ-benzyl-DL-glutamic acid *N*-carboxyanhydride (2.0 g, 7.61×10^{-3} M) dissolved in 5 cm^3 of freshly purified dioxane to give an ≃1% solution. The polymerization was initiated with $NaOCH_3$ (0.0988 cm^3 of 0.385 N) to give anhydride/initiator = 200. After standing 3 days at room temperature, dry HCl gas was bubbled into the slightly viscous clear solution until saturation, and then anhydrous HBr was bubbled in for 20 min causing a white precipitate to form. After standing overnight, nitrogen gas was passed through the solution to remove HCl, HBr, and solvent. The polymer was further precipitated by the addition of 500 cm^3 of ether, and the precipitate was washed with ether after decanting the solvent. The precipitate was further washed with ether and then dissolved in H_2O by the addition of 1 N NaOH to bring the pH to 7. The aqueous solution was extracted twice with ether and the polymer solution was lyophilized to yield a fibrous material. The polymer was dried under vacuum (5 mm) at 80° for 2 hr; wt = 1.06 g; $[\eta]^{0.2\ M\ NaCl}_{pH\ 6.86} = 0.43$.

Poly(L-*glutamic acid*) was purchased from Pilot Chemicals (lot G-99, DP = 550).

Absorption Spectra. Ultraviolet absorption spectra were measured with a Cary 14 spectrophotometer, at 23°, fitted with a light mask for microcuvets. Occasionally, a Zeiss PMQ 11 was used.

pH Measurements. Measurements of hydrogen ion concentration were made routinely with a Radiometer GK2021B combination electrode fitted to a Radiometer Model 25 pH meter. For small volumes a Sargent 4858-L15 miniature combination glass electrode was used, fitted either to a Sargent Model DR or a Corning Model 12 pH meter. The pH meters were standardized with pH 4 buffer prepared from Beckman powdered standard buffers with glass redistilled water according to instructions. Buffer prepared in this manner checked to within 0.01 pH unit with a reference buffer prepared from National Bureau of Standards potassium acid phthalate. At pH 2.0 the instruments were standardized using buffer prepared from Coleman standard buffer tablets. No check was made on the accuracy of this standard buffer.

Ultracentrifugation. The sedimentation-velocity studies were performed in a Beckman-Spinco Model E centrifuge equipped with phase-plate schlieren optics and an electronic speed control. Two-degree 12-mm cells were used for all runs.

Circular Dichroism. Polypeptide conformation was determined by circular dichroism[1] measurements using a Cary Model 60 equipped with a Model 6001 circular dichroism attachment, with a slit width programmed to maintain a 15-Å half-bandwidth. Fused-quartz jacketted cells (1 or 2 mm) (Optical Cell Co., Beltsville, Md.) were used. Temperature control was maintained with a Tampson circulating bath. The conformation of the polymers was calculated from measurements of the $[\theta]_{208}$ trough as suggested by Greenfield and Fasman (1969).

$$\% \text{ helix} = \frac{[\theta]_{208} - 4000}{33{,}000 - 4000} \times 100 \qquad (1)$$

Using an analysis of variance, a computer program generously furnished by Dr. Kirk Aune, the relative contributions of each conformation were estimated by comparing the CD measurements of the copolymer to those of poly(L-Lys), taking the latter as the standard reference for each conformational state. More details of the conformational transformations of this copolymer may be found below.

Polypeptide Solutions. Solutions for both CD studies and tritium exchange were prepared as follows: ≃10 mg of copolymer was added to ≃6 cm^3 of solvent (0.01 M sodium acetate–0.01 M NaCl) and 0.1 N NaOH was added to bring the pH to ≃12. The solution was stirred at room temperature for 4–12 hr. The pH was then adjusted slowly to 4.1 by adding 0.1 N HCl with stirring. The solution was clarified by centrifugation at 8000 rpm. Final concentrations used varied between 1 and 10 mg/cm^3.

Tritium Exchange. The exchange experiments were of the exchange-in and exchange-out types previously described (Englander and Poulsen, 1969; Englander and Staley, 1969) with minor operational changes. The slopes and intercepts were determined from least-squares fits to the data. The exchange-in experiments at 25° were conducted as follows: after adjusting the pH, the polymer solution was equilibrated in a 25° thermostat. The exchange-in was initiated by addition of 10–40 mCi of tritium. At the times noted in the Results section, 0.7–0.8-ml aliquots of solution were removed and placed in an ice bath. Aliquots (0.5 ml) were then passed through Sephadex G-25 columns at 0° to remove the free tritium, so that tritium that had exchanged-in to peptide groups of the polymers during the experimental exchange-in time could be measured. The elapsed time between transfer of the solution to 0° and the initiation of the column run was never greater than 1 min. No correction was made for the transit time of the polymer through the column.

The number of hydrogen exchanged atoms per peptide bond, H/A, was determined by eq 2 from the ratio of the tritium counts (C) to the optical density (A) in the fractions

$$H = \frac{110.8EC}{C_0 \quad A} \qquad (2)$$

derived from the column effluent. C_0 is the tritium count rate in the initial equilibrium mixture of polymer and tritiated water and E is the molar residue absorption coefficient of the

[1] Abbreviations used are: H–T, hydrogen–tritium; CD, circular dichroism. The nomenclature and abbreviations for amino acids and synthetic polypeptides (polymerized amino acids) conform to the tentative rules of the IUPAC–IUB Commission on Biochemical Nomenclature as published in *Biochemistry 11*, 942 (1972). All amino acids are L unless otherwise noted.

TABLE I: Equilibrium Isotope Effect for Tritium *vs.* Hydrogen Binding in Polypeptides at 0°.

Polymer	pH	Solvent	Conformation[a]	Ratio T/H[b] ± SE
Poly(L-Lys)	2.00	0.01 M potassium phosphate–0.2 M NaCl	Coil	1.18 ± 0.01
Poly(L-Glu)	5.00	0.01 M sodium acetate–0.2 M KCl	90% helix, 10% coil	1.19 ± 0.01
	5.00	0.01 M sodium acetate–0.1 M NaCl	100% helix	1.12 ± 0.05
	4.50	0.01 M sodium acetate–0.2 M NaCl	100% helix	1.10 ± 0.03
	4.10	0.01 M sodium acetate–0.01 M NaCl	Aggregated[c] 100% helix	1.08 ± 0.06
Poly(DL-Glu)	5.00	0.01 M sodium acetate–0.1 M NaCl	Coil	1.11 ± 0.01
	4.50	Same as above	Coil	1.17 ± 0.03
	4.10	Same as above	Coil	1.18 ± 0.03
	4.10	0.01 M sodium acetate–0.01 M NaCl	Coil	1.20 ± 0.03
Poly(L-Glu^{77}L-Val^{23})-	4.10	0.01 M sodium acetate–0.01 M NaCl	65% helix, 35% β	1.15 ± 0.07
Poly(L-Glu^{77}L-Val^{23})[d]	4.10	Same as above	35% helix, 65% β	1.17 ± 0.04
Poly(DL-Ala)[e]	3.00	μ = 0.05	Coil	1.19
Poly(DL-Lys)[e]	2.00	μ = 0.2	Coil	1.24

[a] As determined by CD studies. [b] See Materials and Methods; SE, standard error. [c] See the results of the CD and sedimentation velocity experiments. [d] After heating the polymer to 80° for 15 min followed by rapid cooling in ice. [e] From Englander and Poulsen (1969).

particular polymer used in the experiment. No correction was made except as noted for the reported equilibrium isotope effect (Englander and Poulsen, 1969) as it was desired to directly detect any change in this parameter under the various experimental conditions.

Since Englander and Poulsen (1969) have shown that the observed exchange-in rate is controlled by the exchange-out rate, we have adopted their method of plotting exchange-in data as the fraction of peptide hydrogens not exchanged (*e.g.*, $1 - [(H/A)/1.13]$). The factor 1.13 (or 1.18) is a correction for the equilibrium isotope effect and was taken from earlier graphical extrapolations of exchange-out data. The equilibrium isotope effects listed in Table I were obtained from a non-linear least-squares computer routine furnished by Dr. Koh of the University of Nevada Computing Center.

The equilibrium isotope effect was measured after the method of Englander and Poulsen (1969) by preequilibrating the polymer with tritiated water, measuring the exchange-out of the tritium label as a function of exchange-out time, and extrapolating to zero time.

Polypeptide Optical Absorption Coefficients. The molar absorption coefficients were determined by the absorbancy of polymer solutions whose concentrations were determined from a micro-Nessler-Kjeldahl nitrogen determination (Lang, 1958). The extinction coefficients obtained in this manner are shown in Table II.

The molar absorption coefficient, A_{220}, for poly(DL-Lys) reported by Englander and Poulsen (1969) is somewhat higher than that determined herein for poly(Lys). Substitution of the latter value into Englander and Poulsen's poly(DL-Lys) data yields nearly identical equilibrium isotope effects for poly(DL-Ala), poly(DL-Lys), and poly(Lys).

Since the total variation of the molar absorption coefficient of poly(L-Glu) from pH 4.9 to 4.1 in 0.01 M sodium acetate (μ = 0.2–0.15) was not more than 1.8%, an average value of 624 was used for all experiments with poly(L-Glu) when these conditions obtained.

In almost every case the reproducibility of independent determinations of the absorption coefficient was better than 2%. The only exception was in the determination of the absorption coefficient of unheated poly(Glu^{77}Val^{23}) where the variability was on the order of about 4%. Values determined after heating aqueous solutions of the copolymer showed much less variability.

TABLE II: Molar Residue Absorption Coefficients, A_{220}, 24°, Used for Hydrogen–Tritium Exchange Studies.

Polymer	pH	Solvent	A_{220}
Poly(L-Lys)	6–7	H_2O	524
Poly(L-Glu)	8.4–8.7	H_2O	576
	4.4–4.8	H_2O	664
	4.94	0.01 M NaOAc–0.2 M KCl	620
	4.40	0.2 M NaOAc–0.2 M KCl	621
	4.48	0.1 M NaOAc–0.2 M NaCl	631
	4.12	0.01 M NaOAc–0.01 M NaCl	626
Poly(L-Glu^{77}L-Val^{23})	5.8–6.7	H_2O	618
	4.11–4.12	0.01 M NaOAc–0.02 M NaCl	641.5
	4.11–4.12	0.01 M NaOAc–0.02 M NaCl	731.5[a]
Poly(DL-Glu)	7	H_2O	575
	4.1	0.01 M NaOAc–0.01 M NaCl	503

[a] After heating to 80° for 15 min and rapid cooling.

TABLE III: Hydrogen–Tritium Exchange Rates of Glutamic Acid Polypeptides at 0°.

Polymer	[NaCl][a] (M)	pH	$t_{1/2}$ (min)	
			Exchange-In	Exchange-Out
Poly(DL-Lys)[e]	0.2	2.00		150
Poly(L-Lys)	0.2	2.00		151
Poly(L-Glu)	0.2	5.00		39
	0.1	5.00	73	80
	0.1	4.75	260	
	0.2	4.50	305	296[d]
	0.1	4.50	384	
	0.1	4.10	98,000	695[d]
Poly(DL-Glu)	0.1	5.00		4.6
	0.1	4.50		6.3
	0.1	4.10		8.2
	0.01	4.10		9.6
Poly(L-Glu[77]L-Val[23])[b]	0.01	4.10	5×10^5[f]	99[d]
Poly(L-Glu[77]L-Val[23])[c]	0.01	4.10	9×10^5[f]	251[d]

[a] Solvent was 0.01 M sodium acetate supplemented with NaCl as indicated. [b] Copolymer unheated, 65% α helix, 35% β structure. [c] Copolymer heated 15 min at 85° and cooled, 35% α helix, 65% β structure. [d] Exchange measured for less than 1 half-life. [e] Englander and Poulsen, 1969.

Results

Equilibrium Isotope Effect. The equilibrium isotope effect of hydrogen–tritium exchange has been carefully determined by Englander and Poulsen (1969) for two random polypeptides, poly(DL-Lys) and poly(DL-Ala). They found values of 1.19 for the alanine polymer and 1.24 for the DL-lysine polymer. A value of 1.18 was found for the random conformation of poly(Lys) in our laboratory (Table I). Essentially, these differences may be ascribed to a 5% lower extinction coefficient obtained herein for poly(Lys) as compared to Englander and Poulsen's value for poly(DL-Lys). The average value for the equilibrium isotope effect of poly(DL-Glu) is close to 1.17.

Although the size of the equilibrium isotope effect for randomly coiled polypeptides has been established with some certainty, it is plausible that this quantity might be different for hydrogen-bonded structures such as the α helix or β structure. The equilibrium isotope effect was therefore determined for poly(Glu) in the pH range of 5.0–4.1 (Table I). There seems to be a slight decrease with decreasing pH; however, the size of the decrease is close to our estimated error of determination. If there truly is a decrease of the value of the equilibrium isotope effect, it is small and it does not result directly from α-helix formation since the values for the α helix, at pH 5, are close to those determined by Englander and Poulsen for the coil conformation.

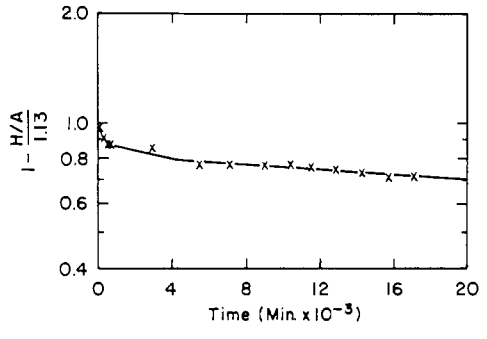

FIGURE 1: Hydrogen exchange rate of poly(Glu) in 0.01 M sodium acetate, pH 4.1, at 0°. The concentration of poly(Glu) was 1.5 mg/ml. The exchange-in method was used. There were no visible signs of aggregation (opalescence or precipitate) throughout the experiment.

The data for heated and unheated poly(Glu[77]Val[23]) indicated little difference in isotope effect between the β structure and α conformation.

Hydrogen–Tritium Exchange of Poly(Glu) and Poly(DL-Glu). The measured exchange rates of the helical conformation of poly(Glu) at different hydrogen ion concentrations together with those of the random copolymer, poly(DL-Glu), under similar conditions, are listed in Table III. Presumably, poly(DL-Glu) is in the random conformation at all measured hydrogen ion concentrations. This assumption is discussed below. The slowing of the exchange rate of the random coil compared to the helix of poly(Glu) is significant. At pH 4.5, for example, the helix exchange rate is $^1/_{61}$th of that of the coil. One very interesting point is the sudden and large slowing of the exchange rate when poly(Glu) is titrated to pH 4.1. Not only does the exchange rate become greatly retarded, but the hydrogens no longer exchange as one class (Figure 1). About 81% of the total number of amide protons exchanged very slowly with a half-life ($t_{1/2}$) of at least 10^5 min. The faster exchanging class had a $t_{1/2}$ = 700 min as measured by the exchange-out technique. It was found that the appearance of the anomalously slow exchange correlated with the aggregation of the poly(Glu) peptide chains. This aggregation has been reported elsewhere (Tomimatsu *et al.*, 1966; Jennings *et al.*, 1965; Schuster, 1965; Zezin *et al.*, 1965) and will be discussed later.

The effect of the degree of ionization on the exchange rate is considerable as can be seen in the case of poly(DL-Glu) (Table III). If specific acid–base catalysis is considered alone, one would have expected an eightfold increase in half-life as the pH was lowered from 5.0 to 4.1 (Englander and Poulsen, 1969), whereas the experimental values show only a 1.7-fold increase. Decreasing the salt concentration tenfold caused about a 20% decrease in the exchange rate at low α (pH 4.1). Possibly the negative carboxyl groups on the polymer electrostatically oppose the attack of hydroxyl ion on the peptide nitrogen, or, equivalently, the microenvironment around the peptide nitrogen is more acidic than the bulk solvent. Re-

TABLE IV: Theoretical Calculation of Poly(DL-glutamic acid) Exchange Rates.[a]

	$t_{1/2}$ (min) at pH		
	5.0	4.5	4.1
Poly(DL-alanine)[b]	2.0	6.3	15.6
Poly(DL-glutamic acid) corrected for neighboring group effect	1.3	3.8	8.8
Poly(DL-glutamic acid) experimental[c]	4.6	6.3	8.2

[a] See text for details of the calculations. [b] Englander and Poulsen, 1969. [c] This work.

ducing the ionic strength tends to increase electrostatic interactions antagonistic to the base-catalyzed exchange.

Recently, Molday *et al.* (1972) showed that the primary structure effects on hydrogen exchange can be considered to be localized (*i.e.*, nearest neighbor). Applying their correction data for the exchange rate of poly(DL-Ala), they were able to accurately predict the exchange profile of oxidized ribonuclease in the pH range 2.0–5.0. This method, applied herein, accurately predicted the exchange rate of poly(DL-Lys) found by Englander and Poulsen (1969) and the exchange rate for random coil poly(Lys) reported herein (Table III). Therefore, calculations were made to see if a similar treatment could be used to predict the poly(DL-Glu) exchange rates determined experimentally. The change of the degree of ionization (α) of poly(DL-Glu) in the pH region investigated was accounted for by use of the rate law (eq 3) (Molday *et al.*, 1972).

$$k_{obsd} = [k_H^{COO^-}(\alpha) + k_H^{COOH}(1 - \alpha)][H^+] + [k_{OH}^{COO^-}(\alpha) + k_{OH}^{COOH}(1 - \alpha)](K_W/[H^+]) \quad (3)$$

Equation 3 gives identical results with eq 4 which formally accounts for the state of ionization on the left- and right-hand sides of the peptide bond. The subscripts denote the acid-(H)

$$k_{obsd} = [k_H^{00}(1 - \alpha)^2 + k_H^{-0}(\alpha)(1 - \alpha) + k_H^{0-}(\alpha)(1 - \alpha) + k_H^{--}(\alpha)^2][H^+] + [k_{OH}^{00}(1 - \alpha)^2 + k_{OH}^{-0}(1 - \alpha)(\alpha) + k_{OH}^{0-}(1 - \alpha)(\alpha) + k_{OH}^{--}(\alpha)^2](K_W/[H^+]) \quad (4)$$

or base-catalyzed (OH) rate constants, the superscripts the location of the un-ionized (0) and ionized (−) side chains, and α denotes the degree of ionization. Hydrogen ion activities were measured potentiometrically and α was determined from the titration data of poly(DL-Glu) in 0.1 M salt reported by Olander and Holtzer (1968). The kinetic constants were derived from those of poly(DL-Ala), modified using the corrections suggested by Molday *et al.* (1972) except for the acid rate constant of the peptide adjacent to an ionized glutamyl side chain. This term was ignored as the base-catalyzed exchange is dominant over the pH range of interest. This method predicts a half-life of 1.3 min at pH 5.0, 3.5 times faster than the experimentally determined half-life of 4.6 min (see Table IV). On the other hand a half-life of 8.8 min is predicted at pH 4.10, which is close to the observed half-life of 8.2 min.

The work of Molday *et al.* (1972) predicts that the exchange rate of poly(DL-Glu) ought to be consistently faster than that of poly(DL-Ala), but, in fact, the glutamic acid polymer exchanges appreciably slower than poly(DL-Ala) as the degree of ionization increases (see Figure 6 or Table IV).

It should be pointed out that the rate shift for protonated glutamyl residues was derived for model di- and tripeptides.

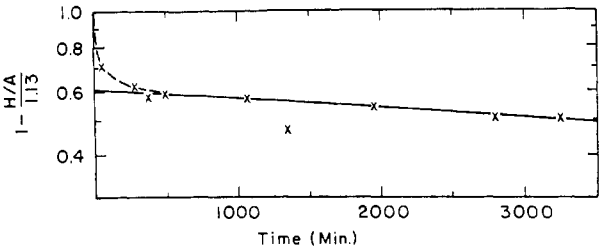

FIGURE 2: Hydrogen exchange rate of poly(Glu) in 0.01 M sodium acetate–0.01 M NaCl, pH 4.1, at 25°. The polymer concentration was 2 mg/ml. No visible signs of aggregation were observed during the experiment. The exchange-in method was employed.

If the conformation of the polypeptide varies from an extended structure at high degrees of ionization to a collapsed structure at low ionization, the variation of exchange rate with pH may contain electrostatic interactions peculiar to the dimensions of the polymer. Whatever the reasons, the neighboring group correction factors, while good at low ionization, do not quite account for all of the rate behavior of poly(DL-Glu) as the degree of ionization increases, indicating a necessity to consider the effect of the more remote side chains as the charge density becomes large.

These higher order effects are apparently strongly inhibitory on exchange rates as the poly(Glu) exchange rate at pH 5 is slower than that of poly(DL-Ala). It is difficult to devise an explanation of this phenomenon which is consistent with the exact rate prediction obtained by Molday *et al.* (1972) for fully ionized poly(Lys).

From the foregoing it is apparent that it is difficult to compare the exchange rates of poly(Glu) and poly(DL-Glu). One reason, at least, is that the degree of ionization at the same pH is quite different for the two polymers. Since the quantitative effect of carboxyl ionization on the exchange rate of the glutamate polymers is not known, only a roundabout approximation can be made. The corresponding pH was determined for an identical degree of ionization on the two polymers from the potentiometric titration data published by Olander and Holtzer (1968). Plots of half-life *vs.* α (degree of ionization) are linear for poly(DL-Glu) and the half-life at any α may be estimated. With this information comparison of the exchange rates of the two polymers at identical α, but at different pH, can be made. The data may be compared at identical pH and α, if one assumes the pH dependency, due to specific acid–base catalysis, such as observed by Englander and Poulsen (1969), operates at all degrees of ionization. Using this approach, one may estimate the exchange slowing due to helix formation. Comparison of poly(Glu) at pH 5.00 (100% helix) to poly(DL-Glu) at pH 5.00 (0% helix) calculated at an identical degree of ionization indicates only a 21-fold increase in half-life; this slowing factor is 83 if the comparison is made in the same manner at a nominal pH 4.5.

Leichtling and Klotz (1966) working with a mixed solvent system (dioxane–water, 1:1) report a slowing factor of 10^3 due to helix formation for poly(Glu), although they are not specific about the model and conditions used to determine the rate of exchange of the coil. The exchange rates which they observed for poly(Glu) are considerably slower than those reported here. Using their rate constants and Arrhenius activation energy, an exchange half-life of 5.3×10^5 min was calculated at pH 4.1, 0° or about five times slower than observed here for the aggregated helix.

Effect of Temperature on the Tritium–Hydrogen Exchange of Aggregated Poly(Glu). The hydrogen exchange of poly(Glu), at 25° in 0.01 M NaCl, is seen in Figure 2. This plot is multi-

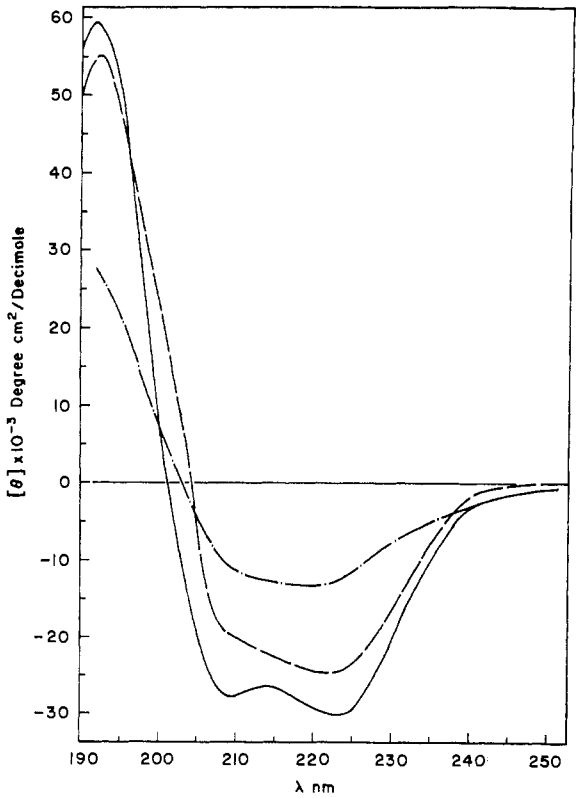

FIGURE 3: Circular dichroism spectra of poly(Glu^{77}Val^{23}). The spectra were obtained at (a) 20° (—); (b) 80° (– ·); (c) at 0° after cooling from 80° (– –). The per cent helix, β structure, and random coil from a computer fit of the data are: (a) 20°, % helix = 65, % β structure = 35, % random coil = 0; (b) 80°, % helix = 16, % β structure = 62, % random coil = 22; (c) 0° after heating to 80°, % helix = 35, % β structure = 65, random coil = 0. The polymer (1 mg/ml) was dissolved in 0.01 M sodium acetate–0.01 M NaCl (pH 4.10) at 0°; no aggregation was observed.

phasic, as it is at 0°, the slow component exchanging with a half-life of about 1.1×10^4 min. It is interesting to compare the exchange rate of the slow component at 25° to that observed at 0°. From Englander's work (Englander and Poulsen, 1969) one would predict that the half-life should decrease by a factor of 1/17.7 for such a temperature rise; however, the observed factor was only 1/8. The size of the slowly exchanging class of protons was reduced from 81% of all amide protons at 0° to 61% at 25°.

This discrepancy is probably more apparent than real. Because of the time limit of the exchange reaction, only a fraction of the total number of slowly exchanging protons is measured. This includes those protons at the ends of the helices, which are expected to be somewhat more labile to exchange than those toward the interior due to the lower stability at the helix termini (Nakanishi *et al.*, 1972; Wee and Miller, 1973). Likewise, in proteins the length of any helical section will also affect the helical unit lifetime and consequently the exchange rate (Miller, 1973). Therefore, one might expect the "slow" class of protons to be different at the two temperatures. That this may be the case is suggested by Figures 1 and 2 where the size of the "slow" class is significantly reduced at 25°.

Sedimentation Velocity of Poly(Glu). Because of the distinct differences between the slow exchanging protons of poly(Glu) at pH 4.1 and the faster exchanging protons at higher pH, the state of aggregation of the polymer was investigated by sedimentation velocity at 25°. At low polymer concentration (2 mg/ml) the sedimentation coefficient (s_{20}) in 0.1 M NaCl was 6.8 S and in 0.01 M NaCl, 6.6 S. At this low concentration there appeared to be a new boundary forming at the meniscus which could not be resolved further. Increasing the polymer concentration to 10 mg/ml revealed the presence of a slow sedimenting material with a sedimentation velocity of 1.8 S in 0.01 M salt and 1.9 S in 0.1 M salt as well as a fast sedimenting component with s values of 5.6 and 7.4 S in 0.01 and 0.1 M salt, respectively. The low salt schlieren patterns showed hypersharpening. The fast sedimenting material accounted for 57% of the sedimenting material at low salt and 61% at high salt. At low salt the per cent aggregated material correlates well with the class size of the slowly exchanging protons. However, the decrease in the class size of the slow exchanging protons at high salt concentration cannot be explained by a dissociation of the slow sedimenting material. From the sedimentation velocity, assuming a rod-like shape for the unaggregated and aggregated material alike and assuming a hydration of 0.85 g of H_2O/g of protein, one can estimate a mol wt of 275,000 daltons for the aggregated species and 70,000 for the unassociated polymer compared to 81,000 calculated from the degree of polymerization.

Material, at low concentration, was stored at room temperature for 3 days, and the sedimentation velocity was redetermined. No significant change was found indicating that the aggregated polymer is very stable, and does not form higher aggregates on standing. Polymer stored at 10 mg/ml at room temperature showed no visible signs of aggregation.

Hydrogen Exchange Rate for the β Conformation. One of the main obstacles in the interpretation of tritium exchange in conformational studies has been that only the rates of exchange for the α-helical and random conformation are known. The rate of exchange of the β structure has never been reported. In this paper the relative rates of exchange of the three conformations commonly found in proteins, α, β, and random coil, are reported.

Because it is well known that the environmental effects near and about the exchangeable hydrogens greatly affect rates of exchange (see review by Englander *et al.*, 1972) the ideal model would be one that could be transformed from one conformation to another. Such a model system has been synthesized and studied herein. The model system is a synthetic random copolypeptide of L-glutamic acid and L-valine, poly(Glu^{77}Val^{23}). Poly(Glu^{77}Val^{23}) is an interesting copolymer in that under the proper conditions it will assume the α-helix, β-pleated sheet, or random coil conformations. Under certain conditions, it will assume various proportions of all three conformations simultaneously. Furthermore, this copolymer of valine and glutamic acid could assume the β conformation at low pH without leading to nonspecific aggregation. The circular dichroism (CD) spectra of this copolymer at pH 4.1 (0.01 M sodium acetate) are shown in Figure 3. The polymer contains 35% β structure, and 65% α helix, as estimated by the procedure of Greenfield and Fasman (1969). Heating this polypeptide solution to 80° for 15 min and then cooling to 24° cause an increase in the β content to 65% and a corresponding decrease in the helical content. The amount of β formed is somewhat concentration dependent. The heated polymer solution is stable for several days if the polymer concentration is not above 2 mg/ml; higher polymer concentrations lead to nonspecific aggregation and eventual precipitation. The unheated polymer is stable indefinitely at all polymer concentrations investigated (1–10 mg/ml).

The results of hydrogen–tritium exchange experiments on poly(Glu^{77}Val^{23}) are shown in Figure 4. The most important feature to be noted is that, for both the heated and unheated

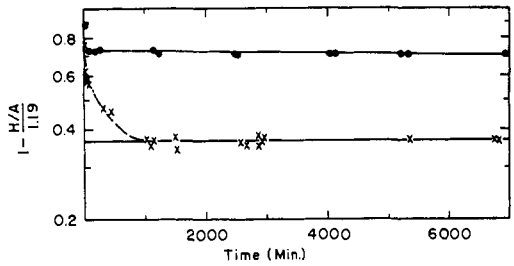

FIGURE 4: Hydrogen exchange rate of poly(Glu^{77}Val^{23}) at 25°: (a) copolymer exchange before heating (×); (b) after heating for 15 min in an 80° water bath followed by cooling in a 25° thermostat (●). Polymer solution: 0.5 mg/ml of polymer solution in 0.01 M sodium acetate–0.01 M NaCl (pH 4.1). No opalescence was observed. In both cases the exchange-in method was used. (Data shown are taken from three experiments.)

polymers, the size of the slowly exchanging class correlates well with the amount of β structure shown to be present by CD, *i.e.*, compare 35% to 65%. The slow class of exchanging protons seems to have an extremely long half-life under the conditions of the investigation.

Calculations from Figure 4 indicate a half-life of 5×10^5 min for the slow exchanging protons of the unheated copolymer and 9×10^5 for the heated polymer. Because of the short fraction of the half-life measured, these numbers should not be considered as significantly different. These rates are at least one order of magnitude slower than the slow exchange of the aggregated α helix (see below), indicating a difference between the dynamic states of these two newly studied aggregated conformations and the α helix.

Sedimentation Velocity of Poly(Glu^{77}Val^{23}). The sedimentation coefficient (s_{20}) of poly(Glu^{77}Val^{23}) was measured in 0.01 M sodium acetate–0.01 M NaCl at 25°. Two peaks (s_{20} = 4.7, 2.2 S) were observed at pH 4.1, whereas only one peak (s_{20} = 2.1 S) was found at pH 4.5. Circular dichroism studies at pH 4.5 indicate no β structure and that the conformation of the polymer is 100% α helical. Calculations based on these sedimentation rates (Martin, 1964) indicate that the molecular weight of a single copolymer chain is about 90,000 and three–four of these chains aggregate to form the β structure.

Is Poly(DL-glutamic acid) a True Random Polymer? The question of the randomness of poly(DL-Glu) arises from the consideration of the polymerization kinetics where two situations are possible yielding either a true random sequence of D- and L-glutamic acid residues, or a polymer composed of short sequences of nearly pure D and nearly pure L monomer. In this latter case it would be possible for the polymer to exist as short left- and right-handed helices. Such a situation would not be detectable by the CD technique used in this study because such polymers would be internally compensating and the respective CD signals cancel. Although intuitively one might expect the existence of large amounts of nonrandom structure in poly(DL-Glu) to be rather remote, experiments by Olander and Holtzer (1968) measuring the ultraviolet hypochromicity (a measure of all ordered structure) of poly(DL-Glu) at 200 nm indicated that the polymer might be 62% helical at $\alpha = 0$. It is important to establish the presence of any helical structure because comparison of all the measured hydrogen exchange rates to the "random coil" is made to determine the relative rates of ordered conformations. If the experimental standard is not truly random, serious underestimation of the slowing, due to the formation of an ordered structure, is possible.

Hydrogen–tritium exchange rates are influenced by the presence of an all-ordered structure. Therefore, studies of the hydrogen exchange of poly(DL-Glu) at pH 4.1 in 0.1 M

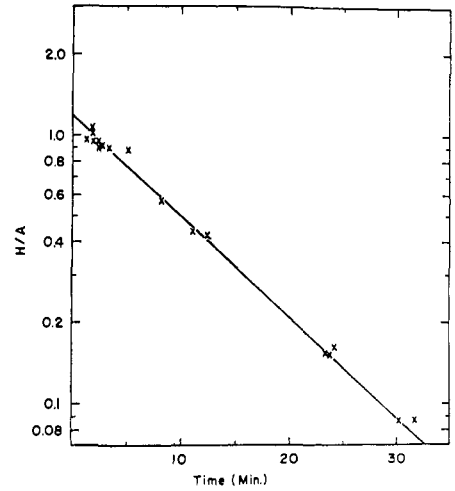

FIGURE 5: Hydrogen exchange-out rate of poly(DL-Glu). The polymer concentration was 2 mg/ml in 0.01 M sodium acetate–0.1 M NaCl (pH 4.1). The single column exchange-out method was used.

NaCl were undertaken. From the data of Olander and Holtzer (1968), the degree of ionization, under these conditions, was estimated to be $\alpha = 0.19$. From their hypochromicity data it was estimated that the helical content of the racemic polymer was at least 43%. If the polymer is partially helical, one can envision two possible situations. (1) The polymer is composed of stable helical sections (right and left handed) and coil sections connecting these. In this case one would observe two distinct classes of exchanging protons, the size of the classes being directly proportional to the relative amounts of helix and coil present in the polymer. The data of Figure 5 show only one single class of exchanging protons even after 91% of the amide protons had exchanged. (2) There are no definite regions of helix or coil but rather the helical regions of either sense fluctuate throughout the polypeptide chain. This could arise if the D and L monomers were so arranged that all sections of the polypeptide chain had equal probability of forming a transitory unstable helix. The latter possibility is highly unlikely.

The second possibility is difficult to test because one would expect to see only a drop in rate which corresponded to the amount of helix formed. This can be estimated as [1 − (fraction helix)] × the coil-exchange rate. If the hypochromicity data do predict the correct helical content of the racemic polymer, an exchange rate approximately half as great as that of a randomly coiled polymer is expected.

A comparison of the observed exchange rates of poly(DL-Glu) and poly(DL-Ala) and the expected exchange rate of poly(DL-Glu) based on the reported neighboring group effect of Molday *et al.* (1972) is seen in Table IV. If the racemic polymer is 43% helical at pH 4.1 the expected half-life would be 15.5 min [= 8.8 min/(1 − 0.43]. While the experimental exchange rates are slower than the calculated rates at pH 5.0 and 4.5, the experimental rate is very close to the theoretical at pH 4.1 and about twice as fast as poly(DL-Ala). This would strongly argue against the formation of appreciable amounts of helix at low polymer ionization.

A comparison of the half-lives of poly(DL-Glu) with those of poly(DL-Lys) and poly(DL-Ala) as a function of pH is seen in Figure 6. The data for poly(DL-Lys) and poly(DL-Ala) are those of Englander and Poulsen (1969). Note that the exchange rates of the lysine and alanine polymers are quite close to the values reported herein for poly(DL-Glu) over the pH range 5.0–4.1. The rates are identical for poly(DL-Ala) and poly(DL-Glu) at pH 4.5 and are very close for poly(DL-Lys) and poly-

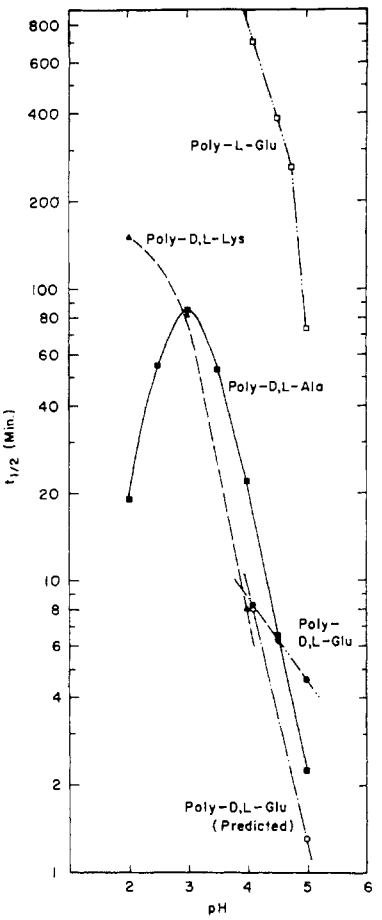

FIGURE 6: Log $t_{1/2}$ *vs.* pH. Comparison of the dependence of exchange rate, $t_{1/2}$, upon hydrogen ion concentration for glutamic acid polymers. The data were obtained at 0° in 0.01 M sodium acetate–0.1 M NaCl except for poly(Glu) at pH 4.1 where the NaCl concentration was 0.01 M. The data for poly(DL-Lys) and poly(DL-Ala) are taken from Englander and Staley (1969).

(DL-Glu) near pH 4. The comparison is complicated by the small negative slope of the DL-glutamic acid polymer. Normally, this slope (log half-life *vs.* pH) would be one showing the direct concentration dependence of exchange rate with the concentration of a specific catalyst; however, steric and electrostatic factors have altered the exchange behavior in this pH region. Olander and Holtzer's (1968) hypochromicity data showing increasing helical content with increasing hydrogen ion concentration lead one to expect that the slope of the poly(DL-Glu) line in Figure 6 ought to have a negative value greater than one. In fact, the slope has a negative value much less than one. However, it is possible to argue that the electrostatic factors are so great they completely mask the relative slowing of exchange caused by helix formation.

Taken altogether, the hydrogen exchange data do not seem to provide any basis for believing that poly(DL-Glu) is in a helical conformation at pH 4.1. The infrared measurements of the amide I and amide II bands of poly(DL-Glu) in dioxane–D_2O at pH 3.5 by Nakanishi *et al.* (1972) support this conclusion.

Discussion

An equilibrium isotope effect, *i.e.* a selective binding of one of the isotopes of hydrogen to sites on the polypeptide, has clearly been demonstrated in this study (Table I). This value must be known to convert bound tritium counts to bound hydrogen to evaluate the kinetics of exchange.

Equilibrium Isotope Effect. The magnitude of the observed equilibrium isotope effect with poly(DL-Glu) was 1.17 (Table I), quite close to that determined by Englander and Poulsen (1969) for poly(DL-Ala). The value of 1.18 for poly(Lys) in the random conformation was lower than that observed by Englander and Poulsen (1969) for poly(DL-Lys). As explained above, this calculated difference is due to the lower extinction coefficient used herein for poly(Lys). It is suggested that the earlier reported isotope effect of the lysine polymer was an overestimate; Englander and Poulsen (1969) had stated their value was uncertain. An average value of 1.16 was observed for poly($Glu^{77}Val^{23}$) in various conformations.

It is interesting that helical poly(Glu) gave a consistently lower observed equilibrium isotope effect of 1.11. The magnitude is similar to the excess tritium binding reported by Tashian (1970). However, this value is probably an underestimate, but it is impossible to get zero time extrapolation from the published data.

Several authors have reported finding no isotope effect at all (Byrne and Bryan, 1970; Lees and von Hippel, 1968; Ikegami and Kono, 1967; Emery, 1967; Woodward and Rosenberg, 1971). However, the results of the work herein and of Englander's group unquestionably establish the equilibrium isotope effect with a value of 1.18.

Slowly Exchanging Protons. Both the aggregated α helices [poly(Glu)] and the β structure [poly($Glu^{77}Val^{23}$)] exchange very slowly (at pH 4.1 normalized to 0°, $t_{1/2} = 9.8 \times 10^4$ and 7.8×10^6 min, respectively). The slowly exchanging classes of protons in proteins are reported to exchange at comparable rates. In Table V, the exchange rates of several proteins are compared after extrapolation to pH 4.1, 0°, assuming that the exchange rate varied with pH and that the temperature effect on exchange is the same as that observed by Englander and Poulsen (1969) for synthetic polypeptides. This table is not to be construed as a prediction of the actual exchange rates of these proteins under these conditions but is simply a device to provide approximate comparisons of exchange rates in native proteins with those observed in polypeptides with specific conformations.

The rate of the slowly exchanging protons of poly(Glu), listed in Table V, is, of course, the fastest possible rate of this general class. There may be still slower exchanging protons present but our experiments were not continued over a sufficiently long period of time to reveal them. Nonetheless, the comparison of rates found in Table V is interesting. Few protons exchange more slowly than those of the copolymer in the β structure and some proteins show protons with exchange rates of the same order of magnitude as the aggregated α helices of poly(Glu).

Where measured, classes of protons exchanging at a rate slower than that measured for the copolymer β structure accounted for a relatively small percentage of the total exchanging protons. For instance, the slowest exchanging class of protons observed for light meromyosin (Segal and Harrington, 1967) accounted for, at most, 2% of the total amide protons.

Both myoglobin and light meromyosin are proteins of high helical content. Light meromyosin contains a significant number of protons ($\simeq$25%) with exchange rates slower than those observed in the aggregated α helix. The slowest class of amide protons measured by Englander and Staley (1969) in myoglobin exchanges with a rate much slower than those of the aggregated α helices.

The exchange rates observed for poly(Glu) in dioxane–D_2O (1:1) are compared to those obtained in H_2O in Table V. The observed aggregation of poly(Glu) in water suggests that

aggregation may have occurred in dioxane–D_2O and may be responsible for the slow rate of exchange observed by Nakanishi *et al.* (1972).

If aggregation is not occurring in the dioxane–D_2O solvent, it may be that this solvent is more favorable to helix formation than H_2O and possibly approximates the microenvironment of the aggregated α helices. This is suggested because the slowing of hydrogen exchange due to helix formation in dioxane–D_2O is 800-fold at pH 4 and 500-fold at pH 4.5. This compares to the slowing factors observed in the work reported herein of 72-fold at pH 4.1 and 61-fold at pH 4.5 for poly(Glu) (the above numbers are not corrected for differences in carboxyl ionization). This difference corresponds to approximately 1.3 kcal of additional stabilization of the closed (helical) conformation of the polypeptide, assuming the slowing factor can be interpreted as an equilibrium constant between the open and closed forms of the helix. Dioxane also depresses the [OH^-] in aqueous solutions due to the decrease in K_w and would accordingly account for a slowing of ≃100 times on the chemical rate alone (Englander *et al.*, 1972). Other work has also shown that the organic water solvent systems favor helix formation relative to water alone (Fasman *et al.*, 1964; Iizuka and Yang, 1965; Cassim and Taylor, 1965; Fasman, 1967; Matsumoto *et al.*, 1968; Conio and Patrone, 1969; Dubin, 1973).

Rationale for Rate Decrease. Which factors are responsible for the very slow proton exchange of poly(Glu) in the aggregated state? Generally, it is assumed that the intrapolypeptide hydrogen bond must be broken before exchange may occur (Berger and Linderstrøm-Lang, 1957; Hvidt and Nielsen, 1966). By this model the rate of hydrogen exchange would be determined by the intrinsic exchange rate of the peptide hydrogen, the rate of opening and closing of intrapeptide hydrogen bonds present, and the accessibility of the solvent to the open hydrogen bond. The slow exchange of the aggregated poly(Glu) helices, therefore, might be explained by assuming that the amide hydrogens in the aggregated polymer are not available to the bulk solvent. From measurements of the sedimentation velocity, it was estimated that four polypeptides associated to form the aggregate. If it is assumed that the conformation is that of individual helices aligned side by side (and there is some support for this model; see Jennings *et al.*, 1965; Schuster, 1965) then geometrically it can be estimated that about 76% of the amide protons would be as available to the solvent in the aggregated state as in the isolated helix. The results show that, at 25°, the per cent of slowly exchanging protons is the same as the per cent of fast sedimenting material, *i.e.*, essentially all of the protons in the aggregated species exchange at the slower rate. This would seem to eliminate solvent accessibility as an argument. The foregoing is essentially not altered in principle if there is some variation in the number of polypeptide chains in the aggregate or the aggregate has a somewhat different conformation (*e.g.*, superhelices). Since these experiments involve only changes in the number of associating polypeptide chains, and assuming there is no major change in the primary and secondary structures, local environmental effects would also seem to be eliminated as a possible rationalization of the slow exchange. The most plausible explanation remaining is that the hydrogen bond remains relatively more closed in the aggregated structure than in the corresponding free helix. The thermodynamic factors involved in aggregation also stabilize the closed peptide hydrogen bond.

The factors discussed above may also be given as reasons for the difference between the exchange rate of the β form of the copolymer and the aggregated poly(Glu). In the generally accepted model of a β-pleated sheet, several chains of a polypeptide associate side by side, through hydrogen bonding and the interaction of hydrophobic side chains, so that solvent accessibility may also be a factor. This may also occur by folding of the same polypeptide back on itself. This latter situation probably pertains herein as the size of the β aggregate was small (three–four chains), which is similar to that found for the aggregated poly(Glu). Model building would suggest

TABLE V: Conversion of Exchange Rates of Proteins Extrapolated to pH 4.1, 0°.[a]

	Conformation	$t_{1/2}$ (min)
Poly(L-Glu)[b]	α Helix	6.95×10^2, exchange-out
	Aggregated α helices	9.80×10^4, exchange-in
Poly(L-Glu,L-Val)[c]	β conformation	4.9×10^6 [k]
Poly(L-Glu)[d] in dioxane–H_2O	α Helix	4.12×10^2 7.25×10^3
Human erythrocyte carbonic anhydrase[e]		1.9×10^9
Metmyoglobin[f]		2.4×10^6
Metmyoglobin[g]		1.0×10^{10}
Lysozyme[h]		2.2×10^4
Lysozyme[i]		5.7×10
		4.3×10^2
		6.6×10^4
Light meromyosin[j]		7.1×10^5
		1.4×10^6
		4.0×10^7
Heavy meromyosin[j]		9.0×10^5
		4.4×10^8
Myosin[j]		1.58×10^6

[a] In general, exchange rates observed for slowly exchanging protons were extrapolated to pH 4.1, 0°, assuming direct dependence on hydroxide ion concentration and a ΔH of 17 kcal/mol. The source of the published data and the experimental pH are indicated in the footnotes. Where more than one $t_{1/2}$ is listed for a polypeptide, they represent different rate classes, or data were obtained under different conditions. [b] pH 4.1, 0.01 M NaCl from Table III. [c] This paper, pH 4.1, 0.01 M NaCl. [d] Nakanishi *et al.* (1972), pH 4.0, in dioxane–water (1:1), 0.2 M NaCl, 25°. Solvent and temperature effects were corrected by multiplying by a factor derived by comparing the exchange rate of poly(DL-Glu) in water (Table III) to that observed in mixed solvent at corresponding pH and pD. [e] Tashian (1970), data collected over a wide range of hydrogen ion concentrations. Data represent a range of slowly exchanging protons. [f] Benson (1959), pH 7.0. [g] Englander and Staley (1969), pH 5.0, 0°. [h] Hvidt (1963), pH 3.9. [i] McBride-Warren and Mueller (1972), pH 4.25, rate classes are those of the authors. Rates were normalized for solvent and temperature using a factor relating the intrinsic exchange rates described in footnote *c*. [j] Hartshorne and Stracher (1965) at 25°. The data are derived from the slowest measured class at a variety of hydrogen ion concentrations. These are, in order of increasing half-life, light meromyosin, pH 6.05, 6.3, 8.2; heavy meromyosin, pH 5.96, 8.2; myosin, pH 6.3. [k] This is an average value of the slowly exchanging protons from both heated and unheated polymers, extrapolated from 25 to 0°.

TABLE VI: Equilibrium Constants of the Transconformational Reaction.

Material	k_2/k_1	$t_{1/2}$ (min)[a]
Poly(L-Glu)[b] (helix)	66	6.95×10^2
Myosin (class B)[c]	95×10^2 [d]	1.5×10^5
Light meromyosin (class C)[c]	160×10^2 [d]	2.55×10^5
Poly(L-Glu-L-Ala) 11[b]	50[e]	5.00×10^2

[a] The half-life of each sample was normalized to pH 4.1, 0°, by assuming k_1/k_2 remained constant, and that the exchange rate was directly proportional to hydrogen ion concentration. The rate was considered proportion to $10^{0.0466\Delta T}$ where ΔT is the temperature change in degrees. The factor 0.0466 was derived from the measured Arrhenius activation energy of 17,000 cal/mol of the base-catalyzed exchange and includes the enthalpy of the self-dissociation of water (Englander and Poulsen, 1969). [b] The intrinsic rate of exchange (k_3) was calculated by the method described in Molday *et al.* (1972), allowing for changes in amino acid composition using the formula: $k_3 = X\{k_{OH}^{COO^-}(\alpha) + k_{OH}^{COOH}(1 - \alpha)\}(K_w/[H]) + Y\{k_{OH}^{poly\text{-}(DL\text{-}Ala)}\}(k_w/[H])$ (where the second-order rate constants are derived from Molday *et al.* (1972)). The k_{11} terms are negligible. The symbols X and Y represent respectively the fraction of glutamic acid and alanine residues in the copolymers. The values of X, Y, and α for poly(L-Glu-L-Ala) are given in Ikegami and Kono (1967). [c] Classification of Segal and Harrington (1967). [d] Results of Segal and Harrington (1967). [e] Results of Ikegami and Kono (1967).

that the intrapeptide hydrogen bonds are more shielded from the bulk solvent in the β conformation than in the α conformation. It is suggested that the strong hydrophobic interactions, known to play a role in the stabilization of the β structure (Fasman, 1967), may also further stabilize the closed form of the hydrogen bond. Because of the similarity of the amino acid composition of poly(Glu) and poly($Glu^{77}Val^{23}$), changes in the intrinsic rate of exchange will be relatively small and can be predicted from the work of Molday *et al.* (1972). It is interesting to note that a slowly exchanging class of protons was observed by Ikegami and Kono (1967) in a copolymer of glutamic acid and alanine.

The above may be restated in terms of the Linderstrom-Lang model where a hydrogen on the polypeptide, through local transconformational changes, is alternately completely shielded from any exchange with the solvent or in an open conformation and exchangeable. The nature of the shielded conformer is not defined. The equilibrium between the shielded and open forms, k_1/k_2, controls the apparent rate of exchange as

$$\text{shielded} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{open} \xrightarrow{k_3} \text{exchange} \qquad (5)$$

where k_3 is the intrinsic rate of exchange of a peptide hydrogen. [For additional comments on the validity and interpretation of this type of mechanism see Woodward and Rosenberg (1971)]. If solvent accessibility was more important than the transconformation reaction, it would be expected that proteins of high molecular weight (or greater molecular diameter) ought to exchange, in general, more slowly than those of lower molecular weight or with a smaller radius. Willumson (1971), however, found no such trend in his review of several different proteins of widely varying exchange rates. If it is assumed that the exchange reaction does not disturb the equilibrium between the open and closed forms of the peptide hydrogens, then the exchange rate of an arbitrary peptide hydrogen (m), in a polypeptide, is given by the following expression (Berger and Linderstrøm-Lang, 1957; Hvidt and Nielsen, 1966)

$$k_m = \frac{k_1 k_3}{k_1 + k_2 + k_3} \qquad (6)$$

If those protons are considered which spend little of their time in the open conformation (*i.e.*, $k_2 \geq 10k_1$), then the rate constant of the mth peptide reduces to

$$k_m = \frac{k_1 k_3}{k_2 + k_3} \qquad (7)$$

From a plot of $1/k_m$ as a function of $1/k_3$, it is possible to estimate the values of k_1 and k_2 from the slope (k_2/k_1) and the intercept ($1/k_1$) (Segal and Harrington, 1967). Segal and Harrington (1967) divided the exchanging protons of myosin and light meromyosin into discrete exchanging classes of protons (for a critical discussion of this type of class analysis, see Willumson, 1971). The rates of these classes were determined as a function of hydrogen ion concentration and taken as k_m in the above expressions. The intrinsic rate of exchange (k_3) was taken from poly(DL-Ala) data. While kinetic class analysis is open to criticism, transconformational alterations cause far greater changes in observed exchange rates than those due to primary structure effects, and, therefore, it may be reasonable to group protons into broad exchanging classes. However, this criticism does not pertain to homopolymers. Therefore, a plot of the reciprocal of the observed exchange rate ($1/k_m$) of poly(Glu) in the α-helical conformation as a function of $1/k_3$ was made. The intrinsic rate of exchange was determined from calculations of the expected exchange rate of random poly(Glu) using the method of Molday *et al.* (1972). The calculated rate was used rather than the poly(DL-Glu) data, as the hydrogen ion concentration rate dependency of poly(Glu) in this region more closely paralleled the calculated data and this procedure accounts for differences in ionization between the L and DL polymers.

While the double reciprocal plot of Segal and Harrington (1967) will formally lead to the determination of the opening and closing rate constants, the extrapolations to infinite k_3 to yield k_1 will be seriously in error if $1/k_1$ is much smaller than k_2/k_1k_3, as eq 7 will reduce to $1/k_m = (k_2/k_1)(1/k_3)$. The validity of such a treatment is only assured if the experiment can be conducted into a region where the exchange rate becomes independent of hydrogen ion concentration. If we reject the pH 5.0 point because the rate of exchange is anomalous (see Figure 6), a plot of $1/k_m$ *vs.* $1/k_3$ for poly(Glu) has a slope of 66 while k_3 varies from 0.08 to 0.3 min^{-1}. The value of k_1 from the Y intercept is 0.0053 min^{-1} compared to 0.019 and 0.033 min^{-1} determined for myosin and light meromyosin by Segal and Harrington (1967). In Table VI the ratios of k_2/k_1 of poly(GluAla) and some muscle proteins, which have large helical contents, are compared. As expected the exchange rate correlates with the ratio of k_2/k_1. When $k_3 < k_2$ eq 5 reduces to

$$k_m = k_1 k_3 / k_2 \qquad (8)$$

where k_1/k_2 is simply an equilibrium constant and equivalent to the slowing factor described in the Results section.

The free-energy change of helix–coil transitions can be calculated by the statistical theories of helix–coil transitions (Zimm and Bragg, 1959; Zimm and Rice, 1960). Olander and Holtzer (1968) utilizing such free energies calculated that the

fraction of helix in the un-ionized poly(Glu) was 0.987, and therefore suggested that even the un-ionized polymer may not be 100% helical. This has an important implication for hydrogen exchange. Referring back to the Linderstrøm-Lang model of the exchange mechanism, where the closed hydrogen-bonded helical form would be nonexchangeable and the coil form freely exchangeable, the helix fraction calculated by Olander and Holtzer would predict that the helix should exchange with a rate 1/77th of that of the coil. This is close to the slowing factor calculated from k_2/k_1 above (Table VI) and very close to the slowing factor (1/83) calculated from the ratio of the exchange rates of poly(Glu) and poly(DL-Glu) at pH 4.5, after correction for the differences in carboxyl ionization. Despite the uncertainties of such a comparison, this result supports the Linderstrøm-Lang model.

The results with the glutamic acid polymers have shown that electrostatic and conformational factors can be extremely important in determining exchange rates. Attempts to account for the deviation of poly(DL-Glu) from the expected dependency on the hydrogen ion concentration by incorporation of electrostatic work factors such as those employed by Willumson (1971) have thus far not been successful, leading to the hypothesis that the collapse of the extended structure of this polymer, as the degree of ionization is lowered, also exerts a powerful influence on the exchange rate. The deviation from the expected dependence on the specific catalyst concentration is also evident for poly(Glu) (Figure 6). The slope of the line (log half-life *vs.* pH) has a smaller negative slope than that expected over the pH range 4.75–4.1. This may be due to the reduction of the charge on the helix surface with increasing ion concentration. The slope becomes much steeper between pH 4.75 and 5.0 and the reason for this is not readily apparent. CD measurements indicated that poly(Glu) is completely helical under the conditions of the exchange experiment. It is possible that the optical measurements overestimated the helical content by a small amount thus leading to the observed discrepancy.

Summary

The results presented in this paper would suggest that extremely slow hydrogen exchange can occur in polypeptides where solvent accessibility or local environmental effects cannot provide a reasonable explanation. This fact may have important consequences in the evaluation of protein hydrogen exchange where the classes of such slowly exchanging protons are modified by pH and the binding of small molecules such as substrates or metal ions.

While hydrogen exchange can be a sensitive probe of relative conformational changes in proteins, the assignment of exchange rates to specific types of secondary or tertiary structure has remained speculative. Englander (Englander and Poulsen, 1969; Englander and Staley, 1969; Englander and Mauel, 1972; Englander and Rolfe, 1973) has attempted to correlate the progress curve of exchanging myoglobin with the known X-ray structure. While this work revealed interesting relationships, one still cannot proceed with confidence to assign specific structural characteristics on the basis of hydrogen-exchange data alone.

A conspicuous feature of protein hydrogen exchange has been the existence of a class of extremely slowly exchanging protons. This feature has often been ascribed to solvent accessibility, that is, these protons exchanged slowly because the exposure to solvent was severely limited by the steric effect of surrounding amino acid residues. However, Willumson (1971) did not find a correlation with the bulk of the protein and the slowly exchanging protons. This argues against any simple form of steric hindrance of solvent penetration into the interior of the protein. The present paper offers significant insight into the understanding of proton exchange relative to the conformation of polypeptides and proteins. The data herein show that the extremely slowly exchanging amide protons can be correlated with one of the well-known secondary structures, the β-pleated sheet. Similarly, helices, which show intermediate exchange rates (half-life in the hundreds of minutes), are transposed to species with *very* slow exchange rates when interhelical association occurs, a condition found frequently in many biological structures, *e.g.*, muscle proteins. In this case it was shown that solvent accessibility could not account, in any way, for the observed shift to slower rates.

The above information will be useful when considering the exchange curves of native proteins. While one cannot disregard steric restraints on solvent penetration into the interior of proteins, it is much more fruitful to consider the exchange rates as a measure of the relative rigidity of the hydrogen-bonded structures of the protein without reference to the inner or outer topography. The work presented herein indicates that such rigid structures can equally well be on the protein surface as deep in the interior. Since hydrogen exchange measures the relative fraction of time the peptide unit spends in an open, non-hydrogen-bonded phase of a "breathing" reaction, it is possible that on the protein surface some areas will show much more "rigidity" (a greater relative amount of time in a closed, hydrogen-bonded conformation) than others within the interior. These rigid areas may be very important matrices for determining the properties of proteins, *e.g.*, the nature of ligand and substrate binding sites.

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