BIOPOLYMERS VOL. 7, PP. 379–393 (1969)

Hydrogen-Tritium Exchange of the Random Chain Polypeptide

S. W. ENGLANDER and A. POULSEN, *Department of Biochemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Synposis

The hydrogen–tritium exchange character of poly-D,L-alanine was studied in detail as a model for the hydrogen exchange behavior of the unhindered, polymeric peptide group. The random chain nature of poly-D,L-alanine was evident in the uniformity of exchange rate of all its hydrogens and in the similarity between this rate and that of random chain poly-D,L-lysine and other known, unhindered secondary amide groups. An equilibrium isotope effect favoring the binding of tritium over protium to the extent of 21% was measured. Specific acid and base catalysis of the exchange and the absence of detectable general catalysis were demonstrated. Apparent energy of activation is 17 kcal/mole for deprotonation, largely due to dependence of K_w on temperature, and 15 kcal/mole for protonation, which correlates with the extreme apparent pK. The hydrogen–tritium exchange half-time rate of poly-D,L-alanine at any pH and temperature (T; °C) is given by the equation:

$$t_{\frac{1}{2}} \text{ (min)} = 200 \times 10^{0.05T}/[10^{pH-3} + 10^{3-pH}]$$

INTRODUCTION

This paper presents the results of a study of the hydrogen–tritium exchange character of poly-D,L-alanine (PDLA) and poly-D,L-lysine (PDLL). The results show that these polypeptides exist as random chains in solution and, therefore, may serve as models for the hydrogen-exchange (HX) behavior of the peptide group hydrogen when fully exposed to water. Further, the HX picture seen for PDLA may approach as closely as is possible to a "pure" peptide HX behavior, in that it is here unmodified by local charged groups, by bulky hydrophobic side chains, or by potentially catalytic acidic and basic groups.

Other studies of the hydrogen exchange of PDLA have previously appeared. Freeze-drying techniques, which we now know to somewhat distort the data, were used by Berger and Linderstrøm-Lang[1] and by Ikegami and Kono.[2] Bryan and Nielsen[3] and Paiva, et al.[4] reported studies using infrared spectroscopy. The work reported here utilized the more accurate Sephadex methods[5] to extend these studies to the hydrogen-tritium exchange system.

MATERIALS AND METHODS

The several commercial samples of PDLA (DP 29) and the one sample of PDLL (DP 1500) originated at Yeda. Hydrogen exchange techniques used were similar to those discussed in Reference 5. The exchange situation is conveniently discussed in terms of the exchange equation (1), written phenomenologically, without regard to mechanism:

$$\mathrm{P{\cdot}H} + \mathrm{THO} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} \mathrm{P{\cdot}T} + \mathrm{H_2O} \qquad (1)$$

with $K = k_+/k_-$.

The exchangeable hydrogen sites of the polypeptides were initially labeled with tritium by incubating in tritiated, buffered water (THO), so that tritium (T) distributed to equilibrium between water and polymer (P). In the equilibration mixtures, polymer concentration ranged from 3 to 40 mg/ml, and THO levels were 1 to 20 mc/ml.

Sephadex columns were used to separate tritiated polymer (P·T) from free tritium, and the decrease, in time, of bound tritium (exchange-out) kinetics was measured by assaying, in Sephadex column eluant, for polymer concentration and residual, bound tritium. Polymer concentration in Sephadex column eluant was measured by optical density at 220 mμ and tritium that had been carried through the column by the polymer was found by liquid scintillation counting of 0.2 ml of the same samples. Instruments used were a Zeiss PMQII spectrophotometer, calibrated with the hydrogen emission line at 4861 Å, and a Packard Model 3003 counter. The molar extinction coefficient at 220 mμ is 368 l./mole for PDLA and 550 l./mole for PDLL on the basis of peptide nitrogen. In the HX runs, 0.1–0.5 ml samples were passed through G25 Sephadex columns 1 cm wide and 6–8 cm high, in the appropriate buffer. Samples of eluant as small as one drop were collected through the polymer peak region and diluted to 0.3 ml for analysis. It was normally possible to obtain 4 to 6 sequential samples for which the ratio of tritium counts to optical density (C/OD) fell within a total range of less than 5%. Results showing much greater variability than this were discarded. Month to month reproducibility was also of this order.

The ratio C/OD was converted to hydrogen atoms per peptide bond (H) by the formula:

$$H = (110.8\ E/1.21C_0)\ (C/\mathrm{OD}) \qquad (2)$$

C_0 is the initial tritium count level in the equilibration mixture, and 110.8 is the gram-atom concentration of hydrogen in H_2O. E is the molar extinction coefficient for the polymer used. The number 1.21 represents the equilibrium isotope effect for the preferential binding of tritium over protium by the peptide group in water (see text).

Extinction coefficients were determined by using a Kjeldahl technique to measure peptide N concentration in polymer solutions of known absorb-

ance. Peptide N was computed from the Kjeldahl total N by correcting by an exact factor of 2 in the case of PDLL, to account for ϵ-amino N, and by 3.5% in the case of PDLA to account for the amino terminal group (found by pH titration; pK = 7.9) These groups exchange their H too rapidly to be measured by the present techniques. The Kjeldahl technique used was not of the classical kind but utilized sealed tube digestion, transfer of the ammonia into standard HCl by diffusion in Conway dishes, and titration of the unneutralized HCl to a pH endpoint. This technique has, in our hands, checked to within 1% the N concentration of standard serum albumin samples.

The pH values of the various buffer solutions used were set by use of a Radiometer PHM4 pH-meter. Commercially available pH standard buffers used for day to day calibration of the pH meter, were themselves calibrated from time to time by using a Harleco blood pH reference buffer solution at pH 7.41 and a buffer at pH 4.01 prepared from National Bureau of Standards phthalic acid according to directions. Buffers, chosen to minimize background optical density at 220 mμ, were simple HCl at appropriate concentration or 0.001M solutions of glycine, formate, or acetate. Ionic strength was maintained at 0.05 for PDLA and 0.2 for PDLL.

RESULTS

Equilibrium Isotope Effect

Analysis of kinetic HX data can yield only two kinds of parameters, numbers of H and their rates of exchange. However, when we use T as a tracer, it is the behavior of tritium, not of hydrogen, that we measure, and we must know how equilibrium and kinetic isotope effects will affect, respectively, the numbers and the rates obtained. In the present system, the equilibrium isotope effect can be assessed in two different ways; by determination of the absolute amount of T bound, and by comparing the T bound in competition with H, in H_2O, with that obtained against D in D_2O.

Direct Determination of Tritium Binding. Polymer was brought to exchange equilibrium in THO. The total amount of tritium bound was found by extrapolating an exchange-out curve to zero time (Fig. 1). It is most informative to recast the data for T binding into terms of apparent H atoms found per peptide bond by use of eq. (2) with the factor 1.21 omitted. The equation in this form assumes the absence of an equlibrium isotope effect since the value $C_0/110.8$, which contains the ratio of tritium counts to gram-atom H concentration in the aqueous equilibration mixture, is used as a direct proportionality constant to convert polymer-bound counts to bound hydrogen. If there were no equilibrium isotope effect, i.e., if at exchange equilibrium T/H on the polymer equaled T/H in the solvent, a binding of 1 H/peptide bond would be found when exchange-out data was calculated by this equation, plotted against time, and back-extrapolated to zero exchange-out time.

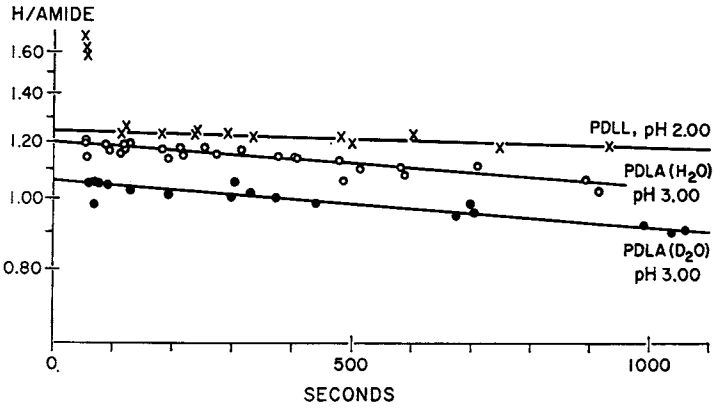

Fig. 1. Equilibrium isotope effect for T vs. H binding to the peptide group in water. The excess binding of T over H is shown both by a direct measurement of T bound per peptide group (upper two curves) and by comparing T vs. H binding with T vs. D binding (lower two curves).

To maximize the accuracy of this extrapolation, the data shown in Figure 1 were obtained under conditions leading to the slowest exchange and data points were taken at early times of exchange-out. Least-squares fitting of the data for PDLA and PDLL, equilibrated with T in H_2O, indicates an intercept value of 1.19 H/peptide bond for PDLA and 1.24⁻ H/peptide bond for PDLL, thus an excess binding of T to the extent of about 21%. (A large part of the apparent difference between the two polymers may arise in a relatively large uncertainty in the extinction coefficient for PDLL).

Comparison of T/H Competition with T/D Competition for Peptide Binding Sites. If T and H compete unequally in binding to the peptide group, then T and D should also compete unequally, and the degree of the inequality in these two cases should differ. If the T/H binding ratio is 1.21, it should also be greater than 1.00 but much less than 1.21 for T/D binding. This kind of comparison may, in many cases, be especially valuable experimentally because the extrapolation to zero time and also the proper scaling of the ordinate in absolute terms are here unnecessary. The effect should be apparent in a simple point to point comparison of the two different exchange-out curves obtained with polymer equilibrated with T in H_2O and in D_2O.

PDLA was incubated to exchange equilibrium in D_2O containing the usual trace of THO. Exchange-out data were obtained by passing samples through Sephadex columns washed with pH 3-buffered H_2O, just as with the other data of Figure 1, so that tritium exchanged out against H_2O as usual. Thus, this experiment differs from the previous only in that equilibration with T occurred in D_2O, so that differences in the data can reflect only the different ability of T to compete with D. Comparative data are shown in Figure 1. The result, that the tritium labeling obtained in D_2O

is less than that in H_2O by about 13%, independently confirms the existence of a T/H equilibrium isotope effect of about the order indicated above.

All data in the remainder of this paper were calculated to indicate H/peptide group using eq. (2) including the factor 1.21 to compensate for the tritium-hydrogen equilibrium isotope effect.

Exchange-In. It might be thought that a third way to identify an equilibrium isotope effect would be to measure the exchange-in rate constant [k_+ of eq. (1)] and the exchange-out rate constant [k_- of eq. (1) as from the slope of Figure 1] and to find K as the directly computed ratio k_+/k_-. In fact, this approach is not possible in the present system.

Some data obtained from exchange-in experiments are given in Figure 2. At zero time, exchange-in was initiated by adding a trace of THO to a PDLA solution maintained at pH 3 and 0°C. After various times, the amount of tritium that had exchanged in was found by passing an aliquot of this solution through a Sephadex column to remove free THO, and then measuring PDLA-bound tritium in the eluant. The column runs were controlled to take 1 min and a small correction for the exchange-out expected to occur during the column passage was applied to the values found. The data are plotted in Figure 2 as log (1-hydrogen exchanged in/peptide group) versus exchange-in time so that they may be directly compared with the exchange-out curve obtained under the same conditions and plotted in the usual way.

The exchange-in and exchange-out rates observed by use of a tracer isotope are both controlled by the exchange-out rate constant alone, for reasons developed in the Discussion, and so give no information on the exchange-in rate constant or on the equilibrium isotope effect.

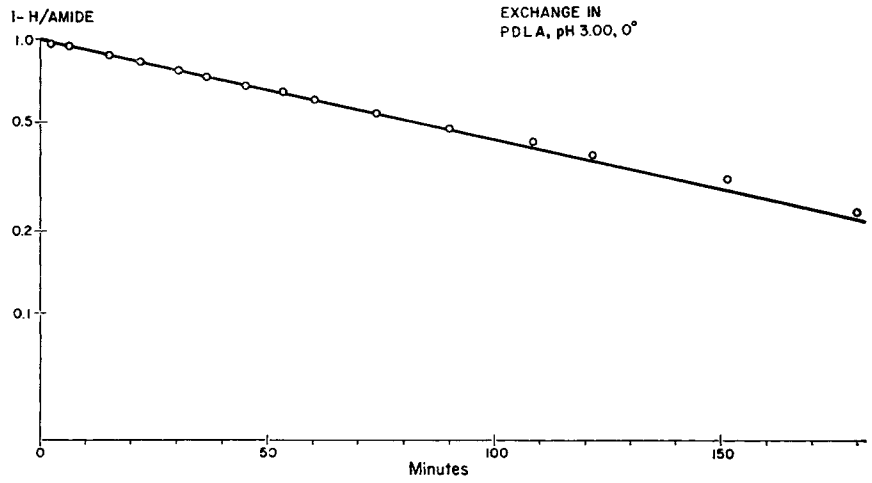

Fig. 2. Comparison of exchange-in and exchange-out rate at pH 3.00, 0°C. The data points from exchange-in experiments match the solid line taken from the exchange-out curve of Fig. 7.

Catalysis of the Exchange Reactions

Specific Acid and Base Catalysis. As shown in Figure 3 for PDLA and in Figure 4 for PDLL, exchange rate decreases with pH to a minimum, then increases again at more acid pH. Measurement of accurate exchange rates was facilitated by carefully determining the zero-time intercept value (Fig. 1), then using this value as an anchor point from which to draw the various pH curves. These results are summarized in Figure 5, wherein the logarithm of the exchange rate is plotted against pH. The solid curve is a theoretical one for simple hydrogen ion and hydroxide ion catalysis, i e., the shape of the curve is fixed and it is moved on the pH and time scales to find the best fit.

The exchange for both polypeptides is simple acid and base catalyzed except near the minimum rate for PDLA at pH 3 where the observed half-time of 84 min is less than the 98 min predicted by the curve. Some "water catalysis" may make a small contribution here.

General Catalysis. The possibility that some of the acidic and basic side chains of proteins might catalyze the exchange of nearby peptide hydrogens has been raised by the work of Klotz and Frank.[6,7] Some data bearing on this point are shown in Figure 6. PDLA equilibrated with THO was passed through a first Sephadex column and the effluent polymer peak was collected, mixed, and dispensed into several test tubes. Within 10 min of the zero time, potential catalyst or control buffer was added to each sample, and the exchange-out was continued for an hour or more in the presence of the catalysts. Tritium still bound to the polymer at the end of this period was determined by passing the samples through a second column and assaying in the usual way. These experiments were carried

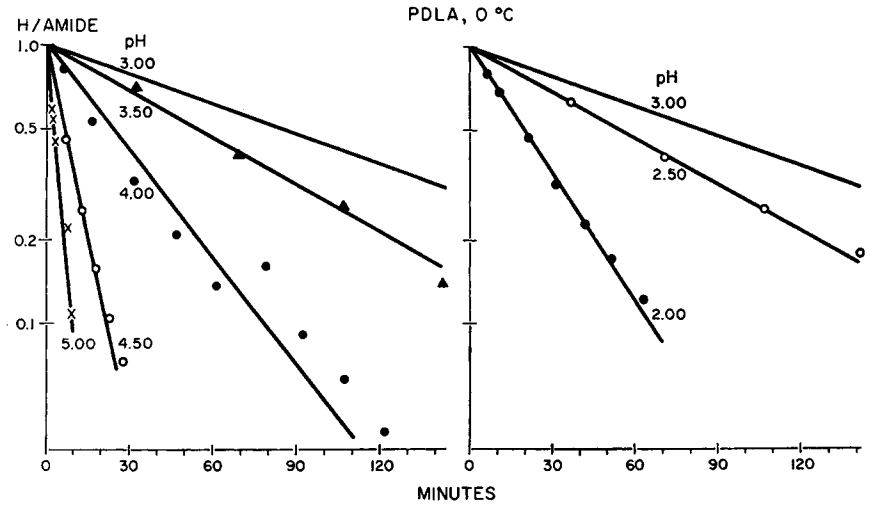

Fig. 3. Hydrogen-exchange rate of PDLA at 0°C as a function of pH. The pH 3.00 curve is taken from Fig. 7.

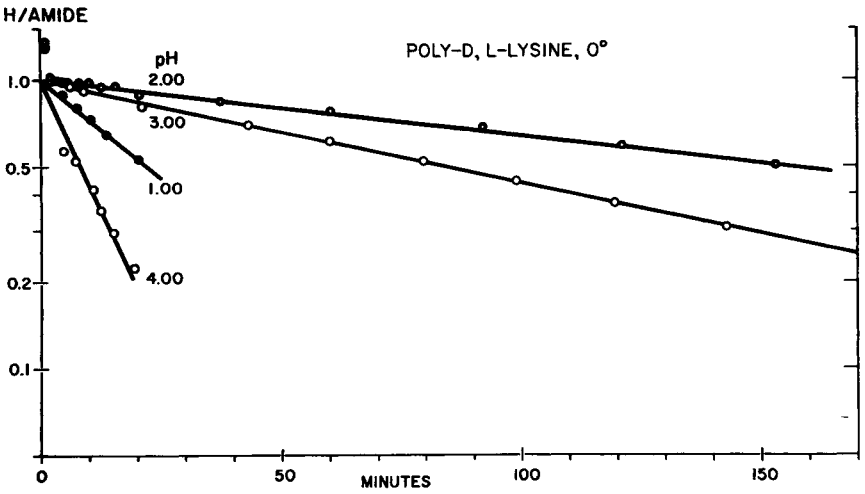

Fig. 4. Hydrogen-exchange rate of PDLL at 0°C as a function of pH.

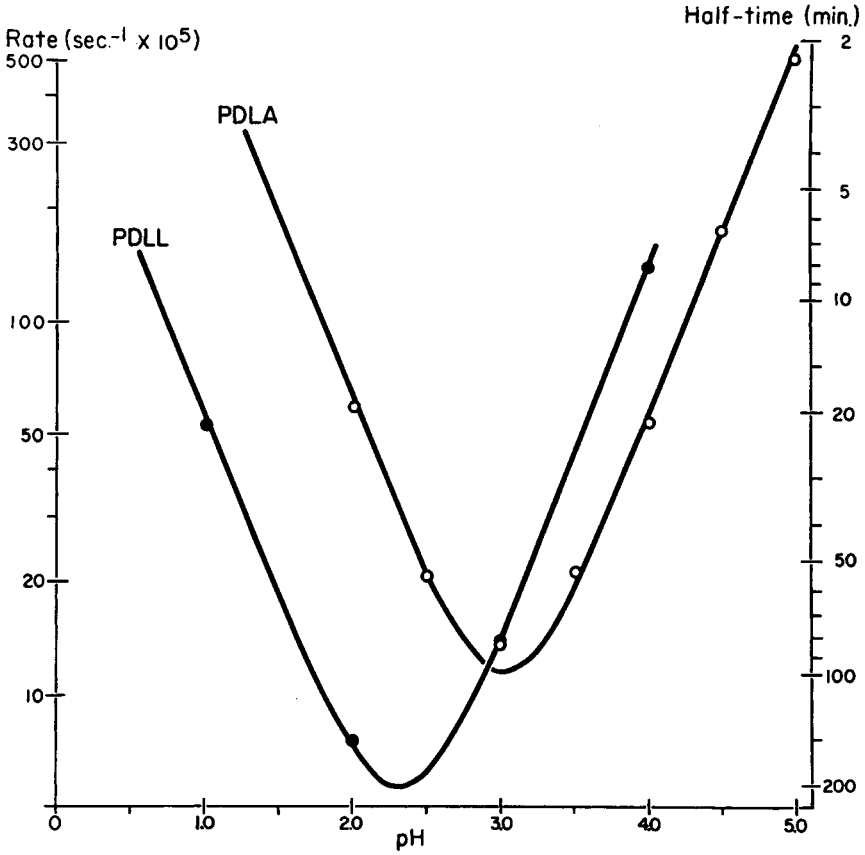

Fig. 5. Log rate vs. pH. The rate data of Figs. 3 and 4 are fitted with a theoretical curve for specific acid and base catalysis.

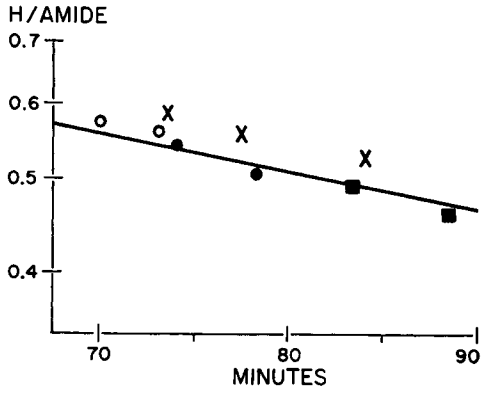

Fig. 6. HX in the presence of some possible general catalysts; PDLA exchanged-out for over 1 hr at pH 3.00, 0°C, in the presence of (●) 0.5*M* arginine; (■) 0.25*M* histidine; (○) 0.5*M* Tris; (×) 0.5*M* trichloroacetate. The solid line is from Fig. 7.

out at pH 3 where specific catalysis is at a minimum so that the additive contribution due to general catalysis might more readily be measured. The data of Figure 6 show that no effect was detected. Exchange-out in the presence of 0.5*M* catalysts proceeded at the same rate as in their absence.

These data incidentally demonstrate the insensitivity of PDLA exchange rate to ionic strength, which was at 0.05 in the absence of added trial catalysts and ranged to over 1.0 in these solutions.

Conformation of PDLA in Solution

Comparison with Poly-D,L-lysine. If the peptide group hydrogens of PDLA were involved in hydrogen bonding, we would expect their exchange with water to be greatly slowed. In Figure 5, HX rates of PDLA and PDLL are compared. The high charge density of PDLL keeps it from folding into any kind of condensed structure so that its peptide hydrogens are surely not involved in hydrogen bonding. With respect to the rates found for PDLA, the pH–rate curve for PDLL is shifted on the pH axis by −0.75 pH units, and on the rate axis by a factor 2.0 to *slower* rates.

Uniformity of PDLA Conformation. The data for PDLA in Figure 1 rule out the existence of any significant number of sites exchanging at rates moderately faster than the majority. Any possible faster class of H would have to be much faster indeed to be completely missed here. In this respect, it may be noted (Fig. 1, uppermost points) that we were able to detect the tail end of the exchange of the ε-amino groups of PDLL, which are exchanging here with a half-time of about 20 sec.

The data of Figure 7, which depicts over 90% of the exchange-out reaction, indicate the essential uniformity of exchange rate of all the PDLA H, though there is a suggestion that a fraction of the hydrogens exchange at a rate slightly slower than that found for the majority.

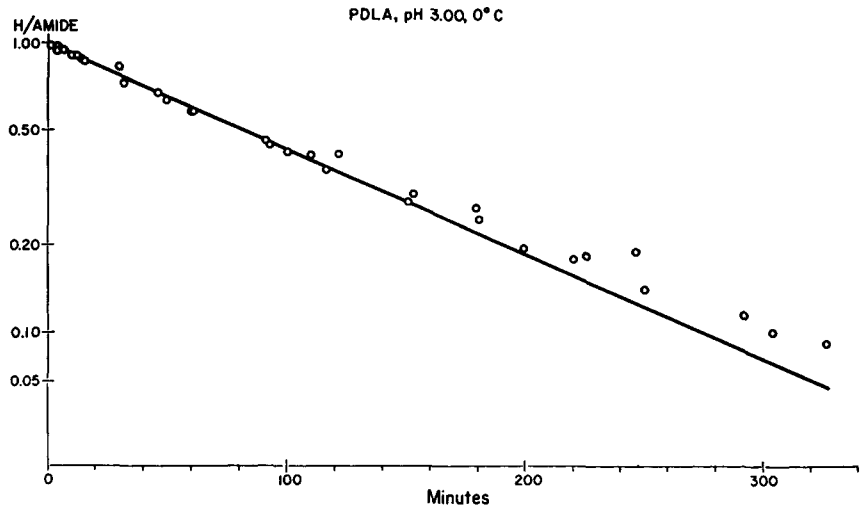

Fig. 7. Long-time exchange-out data for PDLA at pH 3.00, 0°C.

Temperature Dependence of Peptide Hydrogen Exchange

Figure 8 shows the effect of temperature on exchange rate of PDLA at pH 4 and pH 2 where the exchange is, respectively, 99% base-catalyzed and 99% acid-catalyzed. Van't Hoff plots of these data (Fig. 9) show the energy of activation for the hydroxide-catalyzed reaction to be 17 kcal/mole and for the acid-catalyzed reaction to be 15 kcal/mole.

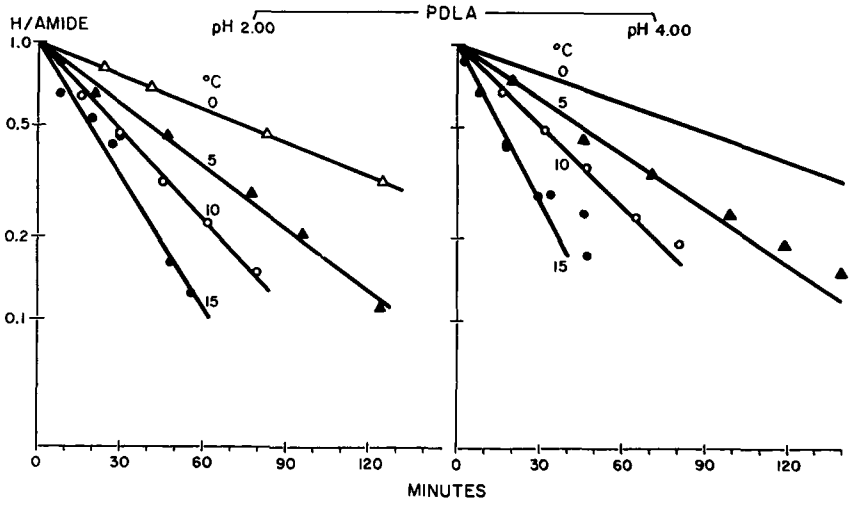

Fig. 8. Temperature dependence of PDLA hydrogen-exchange rate when 99% acid-catalyzed (pH 2) and 99% base-catalyzed (pH 4). The curve at pH 4.00, 0°C, is taken from Fig. 3.

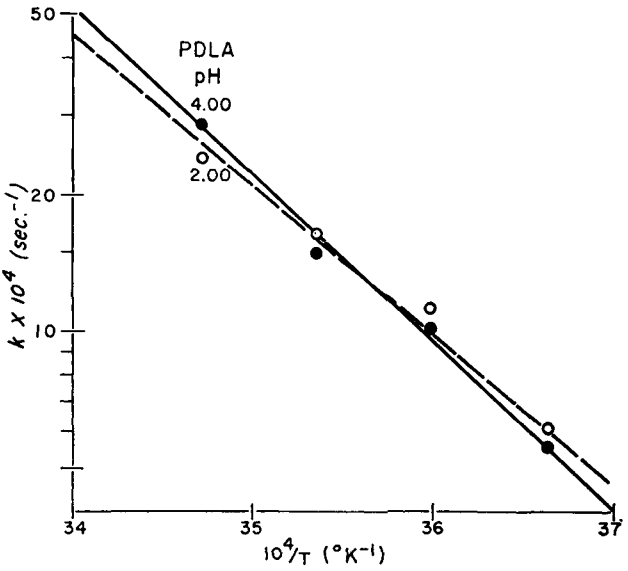

Fig. 9. Rate constant for PDLA hydrogen exchange in the acid and the base regions as a function of reciprocal temperature.

DISCUSSION

Conformation of PDLA

The motivation for the present study of PDLA hydrogen exchange was the belief that this molecule might afford a relatively ideal model for the polymeric, unhindered peptide group. However, in view of the considerable uncertainty that has existed concerning the solution conformation of this polymer, its acceptance as a random chain model requires justification.

Previous Results. Infrared spectroscopic observations were early interpreted to indicate the alpha-fold in PDLA but later optical rotatory dispersion results in the same laboratory favored the random conformation.[8,9] Berger and Linderstrøm-Lang[1] found rather slow HX ates for PDLA, and, since the freeze-drying techniques they used had always measured free peptide H as exchanging in less than 2 min, they considered PDLA to be largely hydrogen-bonded. This conclusion was first challenged by Bryan and Nielsen following a restudy of the hydrogen exchange of PDLA[3] and some di- and tripeptides[10] by an infrared spectroscopic technique. Their data showed that small peptides actually exchanged rather slowly, and that PDLA exchanged only about five times slower. In support of the random chain nature of PDLA, these authors also stressed the first-order nature of the exchange kinetics and the relatively small effect on exchange rates that they and Berger and Linderstrøm-Lang[1] had found some denaturants to exert. These observations have been balanced off against the later discovery by Gratzer and Doty[11] of the remarkable resistance of the

poly-L-alanine helix to denaturation by detergent, concentrated urea and guanidine, and high temperature. More directly, these latter authors reported a far-ultraviolet hypochromicity for PDLA reminiscent of that seen for known helical polypeptides. We have repeated this observation.

Free Peptide Rate. In the present work, hydrogen exchange of PDLA has been compared with that of poly-D,L-lysine (Fig. 5), which is clearly not hydrogen-bonded in the low pH range studied. The curve of rate versus pH for positively-charged PDLL is, as expected, moved to lower pH, but overall rates seen for this open chain polymer are even a little slower than for PDLA. The positive charge on PDLL should shift, by the same amount, the pK's for both protonation (H_3O^+ catalyzed reaction) and deprotonation (OH^- catalyzed reaction) of the peptide group, so that the pH–rate curve would only be displaced to lower pH.[12] Apparently superimposed on this effect is a slowing, by a factor of 2, of both forward rates, perhaps owing to the rather long hydrocarbon side chains[13] of lysine. Thus peptide hydrogen exchange in PDLA is even a bit less hindered than in PDLL. This strongly suggests that PDLA peptide groups are not involved in internal hydrogen bonding.

The PDLA rates might also be compared with rates reported in the literature for a number of small molecules containing the peptide group. Exact comparisons are generally not possible because of characteristics of these molecules (vicinal charges, inductive effects) or of the exchange system (incompletely aqueous solvent, H–D exchange) which may be difficult to correct for. Insofar as comparisons can be made, the small molecule and the PDLA rates agree to well within an order of magnitude.

The constancy of peptide HX rates within the PDLA molecule (Figs. 1 and 7) also argues for an essentially random chain conformation for PDLA. Even a uniform α-helix has four unbonded NH groups near its amino terminus. Such free groups would account for over 10% of the 29-mer used here. Further, Ingwell et al.[14] have shown the unusual stability of poly-L-alanine helix to depend on the folding over of helical lengths so that sidechain contacts may be made. Even one folding over of a supposed PDLA helix would then leave 25% of the peptide hydrogens unbonded and exchanging more rapidly. The data in this paper rule out the possibility that any significant number of peptide hydrogens exchange at some free-peptide characteristic rate that would be rather more rapid than the major-class rate actually observed.

Implicit in this argument is the assumption that involvement in a hydrogen bond would seriously slow the hydrogen-exchange rate. This position is supported both by theory and experiment. For a proton transfer reaction to occur, it is thought necessary to first form a hydrogen bond between the proton donor (here the peptide NH) and the acceptor (e.g., the OH^-). If the peptide hydrogen is internally linearly hydrogen-bonded, hydrogen-bond formation to OH^- is not possible unless the pre-existing bond is first greatly distorted or broken. Thus the observed exchange rate would be reduced, and the relative reduction in rate may approximate the

fraction of time the bond is open. Reduction in exchange rate by a factor of 10^1 to 10^5 has been seen for some small molecules that hydrogen-bond internally[15,16] and for poly(glutamic acid) following a coil-to-helix transition.[12,17]

A fraction of PDLA hydrogens do exchange with slightly slowed rates. This effect may stem from the occurrence in some PDLA molecules of blocks of L- and D-residues arranged in such a way as to allow transitory helices to form. Again, in this rather small polymer, the more centrally located residues may simply experience a higher time average apolar surround and, on that account, exchange more slowly. The same kind of effect might account for the general two-fold slowing seen in the much larger polymer, PDLL.

H-T Exchange Character of PDLA

Equilibrium Isotope Effect. The results of Figure 1 demonstrate an equilibrium isotope effect of about 21% favoring the binding of T over H by the free peptide group in water. The effect was shown both by direct measurement of T binding for two independent polypeptides and by the relative difference found in T versus H and T versus D competition for binding to PDLA. In interpreting T-H exchange experiments with proteins, it will be necessary to compensate for this effect, as in eq. (2). However, as pointed out by Hvidt and Nielson,[18] the size of the effect may be modified for groups in hydrogen bonds. A hydrogen-bonded site will tend to accumulate that isotope which least strains the bond. If this modification is sizable, then the 13% difference between T versus H and T versus D binding found here will also change, but in a completely unpredictable way. For example, a site might then bind D more avidly than either H or T.

In recent work[2] on the H-T exchange of some polypeptides including PDLA no equilibrium isotope effect was found. Apparently this discrepancy lies in the calibration of the PDLA concentration measurement. These authors report an extinction coefficient at 190 mμ of 7200 l./mole whereas we find for this value 5800 l./mole in agreement with Gratzer and Doty.[11] The difference, just over 20%, probably accounts for the difference in conclusions concerning the size of the equilibrium isotope effect. A previous report from this laboratory of no isotope effect[5,22] was based largely on data for oxidized ribonuclease. Further work with this polypeptide shows its HX curve to slope upward at early exchange times to values consistent with the present effect.

The equilibrium isotope effect found here refers specifically to the distribution of T between the peptide group and liquid water. Other T-H isotope effects in the literature might be cited, but their relationship to the present result is generally not straightforward since they refer to somewhat different systems. Other work in this laboratory shows the primary amide group to have a somewhat smaller isotope effect (1.12) than found here and the amino groups of nucleic acids to have no isotope effect.

Kinetic Isotope Effect. It is of interest to compare T–H exchange rates found here with D–H exchange rates for PDLA reported in the literature.[1,3,4] When the temperatures used in the different investigations are taken into account (see below), we find T–H exchange to be slower by a factor of about 2 or 3 than rates found for exchange-out of hydrogen in D_2O. About the same factor is found in a comparison with the peptide analog, N-methylacetamide[6,7,19,20] when inductive effects on the pH profile are taken into account.

In using T–H or D–H exchange methods to study proteins, etc., the kind of systematic kinetic isotope effect seen here is not a problem. It is only necessary to relate observed rates to the pertinently calibrated system.

Acid and Base Catalysis. From the data of Figure 5 for PDLA, we calculate a H_3O^+ catalytic constant of $5.9 \times 10^{-2} M^{-1}$ sec^{-1} and a OH^- catalytic constant of $5.9 \times 10^{7} M^{-1}$ sec^{-1}, using 15 for the pK_w of water at 0°C. If these reactions proceeded at diffusion-controlled rates, so that every collision with H_3O^+ or OH^- resulted in exchange, these rate constants would be about $2 \times 10^{10} M^{-1}$ sec^{-1}.[15] Thus the base-catalyzed reaction is slowed by a factor of $10^{2.5}$ and the acid reaction by $10^{11.5}$.

The OH^- catalyzed reaction presumably consists in a direct attack by OH^- on the peptide NH,[19] in which H^+ distributes between OH^- and N^-. The distribution reached in the encounter complex is determined by the relative basicities of these two competing moieties In the simple case, successful proton transfer occurs only once in $10^{2.5}$ collisions because the peptide pK is higher than that of HOH (16.7 at 0°C) by 2.5 units. Thus we may estimate the pK for deprotonation of the peptide group at about 19. Berger et al.[19] have suggested the rate-limiting H^+-catalyzed step to be a protonation by H_3O^+ at the peptide N. If the above approach is applied here, the observed slowing factor of $10^{11.5}$ taken together with the pK for deprotonating H_3O^+ (-1.7) leads to a pK for protonating the peptide N of -13. (Re-examination of the mechanism for H^+ catalysis may be indicated).

Klotz and his co-workers[6,7,12] in studies of H–D exchange of some peptide analogs in mixed dioxane–water solvent, have observed general catalysis by a number of analogs of protein side chain groups. The results of Figure 9 demonstrate the absence of catalysis by groups including analogs of all the acidic and basic groups of proteins. Thus we may expect peptide groups to show no general catalysis when exchanging in fully aqueous solvent. However, the observations of Klotz and his co-workers may have serious implications for the exchange of protein–surface groups having neighboring apolar side chains and for the HX of groups normally buried and exposed to exchange by a transitory "opening" of their local, apolar structure.

Temperature Dependence. The data of Figures 8 and 9 lead to energies of activation of 17 kcal/mole for the OH^- reaction and 15 kcal/mole for the H^+ reaction. These values may not have high accuracy since only a 15°C temperature range was covered.

These two values, though similar in magnitude, arise from entirely different components. If pH is held constant as in these experiments, OH^- concentration changes in parallel with K_W, which shows a $\Delta H°$ of 15 kcal/mole, and this largely accounts for the apparent E_a of the OH^--catalyzed reaction. (The ΔpK between water and peptide group probably changes little with temperature.) The largest part of the apparent E_a for the H^+ reaction actually derives from the enthalpic part of the $\Delta F°$ for distributing H^+ between H_2O and the peptide group in the encounter complex. The OH^- reaction has a similar, though much smaller, component.

Exchange-in. We wish to justify an earlier statement that both exchange-in and exchange-out kinetics in the present system are determined by the exchange-out rate constant alone. From eq. (2), the general expression for change in polymer-bound T during an exchange-in experiment is:

$$d[P\cdot T]/dt = k_+[P\cdot H]\,[THO] - k_-[P\cdot T]\,[H_2O] \qquad (3)$$

(These rate constants do not correspond to the catalytic constants evaluated above but this difference is irrelevant to the point being made here). Because T is present only in tracer amounts ($T/H < 10^{-5}$), the quantity [P·H] is constant throughout these experiments. The quantity [THO] is also a constant. This is true in exchange-in experiments because [P·H]/[H_2O] is small ($<10^{-4}$) so that no significant amount of [THO] is ever lost. The converse holds in exchange-out experiments, wherein [THO] is reduced to zero at zero time, and subsequently no kinetically significant amount is formed. Thus the time-dependent part of eq. (3) involves only the exchange-out constant k_-. If k_+ and k_- are unequal, the equilibrium isotope effect will not be unity. In fact, at equilibrium, $[P\cdot T]/[P\cdot H] = (k_+/k_-)$ ([THO]/[HOH]). But the first-order kinetics of the approach to (exchange-in) or the fall from (exchange-out) this equilibrium level is determined by k_- alone. The equivalence of exchange-in and exchange-out kinetics is experimentally demonstrated in Figure 2.

This fact can be useful in H–T exchange studies of structured macromolecules. In the study of proteins, the more rapidly exchanging hydrogen can best be observed by equilibrating with THO only briefly so that the faster hydrogens are preferentially labeled. The knowledge that exchange-in proceeds precisely down the total exchange-out curve allows meaningful comparisons to be made between experiments on the totally labeled and the partially labeled protein. This kind of comparison has been used to distinguish and measure intrinsically faster H (non-hydrogen-bonded peptides) and intrinsically slow hydrogen in myoglobin.[21]

It is a pleasure to acknowledge the many helpful discussions we have had with Dr. Roland G. Kallen. This work was supported by a research grant (AM–11,295) and a general research support grant (FR–5415) from the National Institutes of Health, U. S. Public Health Service.

References

1. A. Berger and K. Linderstrøm-Lang, *Arch. Biochem. Biophys.*, **69**, 106 (1957).
2. A. Ikegami and N. Kono, *J. Mol. Biol.*, **29**, 251 (1967).
3. W. P. Bryan and S. O. Nielson, *Biochem. Biophys. Acta*, **42**, 552 (1960).
4. T. B. Paiva, A. C. M. Paiva, and H. A. Scheraga, *Biochemistry*, **2**, 1327 (1963).
5. S. W. Englander, *Biochemistry*, **2**, 798 (1963).
6. I. M. Klotz and B. H. Frank, *Science*, **138**, 830 (1962).
7. I. M. Klotz and B. H. Frank, *J. Amer. Chem. Soc.*, **87**, 2721 (1965).
8. A. R. Downie, A. Elliott, W. E. Hanby, and B. R. Malcolm, *Proc. Roy. Soc.*, (London), **A242**, 325 (1967).
9. A. Elliott, in *Polyamino Acids, Polypeptides and Proteins*, M. A. Stahmann, Ed., Univ. Wiscon. Press, Madison, Wisc., 1962, p. 119.
10. S. O. Nielson, W. P. Bryan, and K. Mikkelsen, *Biochem. Biophys. Acta*, **42**, 550 (1960).
11. W. B. Gratzer and P. Doty, *J. Amer. Chem. Soc.*, **85**, 1193 (1963).
12. B. H. Leichtling and I. M. Klotz, *Biochemistry*, **5**, 4026 (1966).
13. J. S. Scarpa, D. D. Mueller, and I. M. Kotz, *J. Amer. Chem. Soc.*, **89**, 6024 (1967).
14. R. T. Ingwall, H. A. Scheraga, N. Lotan, A. Berger, and E. Katchalski, *Biopolymers*, **6**, 331 (1968).
15. M. Eigen, *Angew. Chem. Intern. Ed.*, **3**, 1, 1964.
16. E. M. Eyring and D. L. Cole, in *Fast Reactions and Primary Processes in Chemical Kinetics*, (Nobel Symposium 5), S. Claesson, Ed., Interscience, New York, 1968, p. 255.
17. A. Ikegami, S. Yamamoto, and F. Oosawa, *Biopolymers*, **3**, 555 (1965).
18. A. Hvidt and S. O. Nielsen, *Adv. Protein Chem.*, **21**, 287 (1966).
19. A. Berger, A. Loewenstein, and S. Meiboom, *J. Amer. Chem. Soc.*, **81**, 62 (1959).
20. S. O. Nielsen, *Biochim. Biophys. Acta*, **37**, 146 (1960).
21. S. W. Englander and R. Stayle, in preparation.
22. S. W. Englander in *Poly-α-Amino Acids*, G. Fasman, Ed., Marcel Dekker, Inc., N. Y., 1967, p. 346.

Received July 1, 1968
Revised September 8, 1968