Hydrogen–Deuterium Exchange of Lysozyme. I. Rate Constants and pH Dependence†

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ABSTRACT: The hydrogen exchange of lysozyme in acidic D_2O solutions was studied using the near-infrared region of a Cary 14R spectrophotometer. The near-infrared region apparently has not been used previously to study the exchange of a protein. A computer technique developed to calculate rate constants and class sizes for the exchanging hydrogens is described. Analyses of rate constant data indicate that pH dependence of each class results from specific acid and base catalysis. Furthermore, the pD at the minimum exchange rate, pD_{min}, was nearly the same for all three rate constants. Effects of a net charge on the rates were calculated and extensive modifications of the catalytic constants were predicted. Similarly, pD_{min} showed sizable effects from a net positive charge. The *intrinsic* pD_{min} was found to be 4.7, approximately 1.3 pD units higher than that reported for a small, randomly coiled polypeptide. The difference in pD_{min} was attributed to a change in the properties of the solvent due to the hydrophobic interior of the protein. The number of hydrogens per kinetic class was nearly constant (±5 peptide hydrogens) over the entire pH range investigated for the two most rapidly exchanging groups. That strongly supports previous reports on the stability of lysozyme in acid solution. In addition, such sensitivity should allow hydrogen exchange to detect conformational changes involving as few as 5–10 observable peptide hydrogens. To account for all the peptide hydrogens, it was necessary to include a class that exchanged too rapidly to be detected at 25°. The number in this class agrees well with the number of exposed peptide hydrogens predicted from the X-ray structure of lysozyme.

Hydrogen exchange has been widely used to detect conformational changes in biopolymers (Hvidt and Nielsen, 1966; Englander, 1967; Parker and Bhaskar, 1970). The main advantage for proteins is that hydrogen exchange, in principle, can "see" *all* the amino acid residues. Such sensitivity can be valuable despite making interpretations more difficult. Fortunately, some model-compound studies are available to help us understand exchange in macromolecules. For example, Bryan and Nielsen (1960) and Klotz and Frank (1964) have shown that exchange in simple amides and polypeptides is highly pH dependent and that the minimum exchange rate occurs in the acid region. Although model compounds generally have exhibited only a single rate, proteins show a wide range of exchange rates, presumably corresponding to different portions of the molecule (Segal and Harrington, 1967; Lenz and Bryan, 1969). Therefore, depending on the magnitude of the rate constants, some peptide hydrogens may become unobservable at a given pH, if proteins behave as do model compounds. For example, if the reaction is followed at a pH near the point of minimum exchange for 6–24 hr, only the more rapidly exchanging hydrogens would be observed. On the contrary, at a slightly basic pH most of the hydrogens followed in the acid region should exchange "instantaneously," while those previously too slow to be detected should be observable. Similar arguments probably apply to variations in temperature. Thus, it should be possible to observe all the peptide hydrogens in a protein by knowledgeable selections of pH and temperature. Before such a "mapping" can be accomplished, however, the exact nature of the variations of each rate constant with pH, as well as their magnitude, needs to be determined.

Regarding the nature of the pH dependence expected for proteins, model-compound studies (Klotz and Frank, 1964; Klotz and Mueller, 1969; Kakuda *et al.*, 1971; Klotz and Leichtling, 1966) have shown specific acid and base catalysis to occur in simple amides, polymeric amides, and polypeptides. Regular changes in rate with pH have been observed with several proteins (Hvidt and Nielsen, 1966; Willumsen, 1967) and the pH of minimum exchange has been identified in others (Kägi and Ulmer, 1968; Coleman and Willumsen, 1969). A well-defined pH of minimum exchange based on rate constants, however, has not been established.

Quantitative evaluations of the rate constants associated with hydrogen exchange in native proteins have been reported by Segal and Harrington (1967) and by Lenz and Bryan (1969). In general, they found that two to three rate constants were adequate to describe the time course of the exchange. In neither study, however, did the size of the exchanging groups remain constant over a wide range of pH values.

Lysozyme was used to gain more quantitative information regarding the exchange of peptide hydrogens in proteins. Much evidence had indicated exceptional stability over a wide pH range for lysozyme. For example, reversibility of acid titrations (Tanford and Wagner, 1954), viscosity between pH 3 and 10 (Glaser, 1959), and optical rotatory dispersion studies to pH 2.5 (Ogasahara and Hamaguchi, 1967) have all indicated the absences of any conformational changes. Furthermore, Hvidt (1963) and Hvidt and Kanarek (1963) studied the exchange of lysozyme and found it to vary in a uniform manner with pH. More recently, Praissman and Rupley (1969) have used hydrogen–tritium exchange to show the equivalence of its structure in the dissolved and crystalline states. It is also known that lysozyme exists as a monomer at pH 5 or

† Contribution 128 from the Department of Biochemistry, Kansas Agricultural Experiment Station, Manhattan, Kansas 66502. *Received July 21, 1971.* This material was submitted to Kansas State University, Dec 1970, by P. A. M. as part of a Ph.D. Dissertation. Presented in part at the 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1970.

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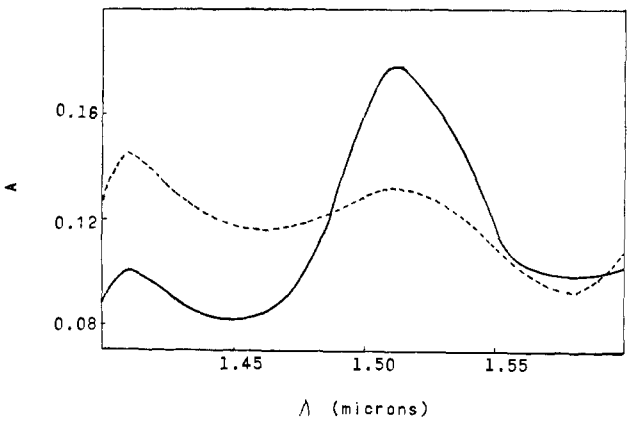

FIGURE 1: Near-infrared spectra of lysozyme in D_2O soon after dissolution (solid line) and after complete exchange (dashed line), 25°.

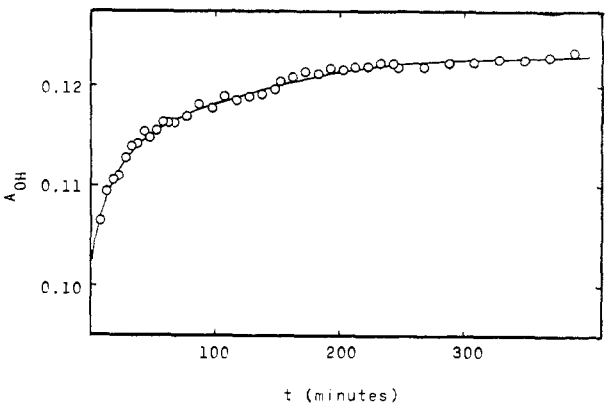

FIGURE 2: HOD absorbance *vs.* time for lysozyme in D_2O, pD 3.83, 25°.

below, even up to 4% solutions (Bruzzesi *et al.*, 1965; Sophianopoulos and van Holde, 1964). Finally, the crystal structure (Phillips, 1967) and the amino acid sequence (Canfield, 1963) have been determined.

Experimental Section

Materials. Hen egg-white lysozyme as a twice-recrystallized, electrophoretically pure soluble powder (lot LY9AA, Worthington Biochemical Corp.), was stored at 5°. D_2O was purchased as 99.8 atom % D from Bio-Rad Laboratories or Merck, Sharp and Dohme. DCl and NaOD (37 and 50% in D_2O, respectively) were from Merck, Sharp and Dohme. Sodium dodecyl sulfate was a Schwarz-Mann product.

Purity of the lysozyme preparation was rechecked with disc gel electrophoresis after 6 months' storage. The procedure was essentially that of Davis (1964) except that the large pore, spacer gel was replaced with G-25 Sephadex in 20% sucrose buffer suspension. Samples (20 μg) of the enzyme in pH 6.8 buffer were layered onto the Sephadex and electrophoresed into 7% polyacrylamide using 2–3 mA/tube and 400 V (dc) for 1.5 hr. The gels were fixed with 20% sulfosalicyclic acid and stained with Coomassie Brilliant Blue R-250. In every case the sample produced a single, narrow band, indicating a homogeneous product.

The stability of lysozyme under conditions similar to those chosen for the exchange studies (0.2 M NaCl and 25°) was inferred from its activity toward *Micrococcus lysodeikticus* suspensions (Shugar, 1952). The activity decreased only 1.25% over 24 hr.

Rate Measurements. Hydrogen–deuterium (H–D) exchange rates were determined using essentially the near-infrared method for model compounds (Scarpa *et al.*, 1967). Briefly, the method consists of adding sufficient DCl or NaOD to a volume of D_2O, containing 0.2 M NaCl, to give the desired final pH after dissolving the lysozyme. The solution was equilibrated to 25° before the lysozyme was dissolved by gentle agitation with a magnetic stirring bar in a sealed flask. Dissolution was complete in 3–5 min and the solution returned to the spectrophotometer cell, which was sealed and placed in the instrument. Generally, 3% solutions were used in 5-cm cells, and spectra were recorded on a Cary 14R spectrophotometer equipped with a 0–0.1 slide-wire.

The solid line in Figure 1 shows a near-infrared spectrum of lysozyme between 1.4 and 1.6 μ soon after it dissolved. The band near 1.41 μ no doubt results from the first overtone of the O–H stretching frequency of HOD and that centered at 1.515 μ from the peptide N–H stretching modes. Although the 16 primary amide groups of lysozyme also absorb in the 1.515-μ region (formamide in dioxane shows overlapping bands with maxima near 1.50 and 1.51 μ), they probably have already exchanged, as discussed later. Furthermore, the band was shifted toward longer wavelengths than those of simple amides. Polypeptides, however, show similar band positions (Hanlon and Klotz, 1965). Thus, it was possible to follow the exchange by measuring either the rate of HOD production at 1.41 μ or the rate of disappearance of N–H at 1.515 μ as a function of time. The values were scaled to readings at 1.185 μ, a region in which no HOD absorbance is detected, to correct for small fluctuations in the spectra at early times.

After following the exchange 6–8 hr, the pH was measured directly in the spectrophotometer cell, using a Beckman Century SS pH meter equipped with a combination electrode. Then immediately afterward, NaOD in D_2O was added with a microburet to give a pH of approximately 8.[1] Exactly the same volume of NaOD solution was added to the reference cell to compensate for any possible HOD in the base solution. At higher pH, exchange is greatly accelerated and absorbance at complete exchange can be obtained after 12–24 hr at room temperature. (Praissman and Rupley (1968) reported that at pH 9.2 in 2% NaCl solution and 0°, 243 of the possible 260 hydrogens exchanged in 24 hr.) The dashed line in Figure 1 is the spectrum after complete exchange, which shows that HOD also has a broad absorption in the 1.5-μ range.

Completeness of exchange under basic conditions was checked by comparing final readings to those obtained in sodium dodecyl sulfate solutions. The dodecyl sulfate was selected because it is a strong denaturant and contains no exchangeable hydrogens. Furthermore, it denatures at relatively low concentrations where there should be no sensible effect on the HOD extinction coefficients. A dodecyl sulfate concentration of 3 g/g of protein was employed, because Reynolds and Tanford (1970) had shown that ratio more than adequate to denature many proteins.

Spectra from the two methods were quite similar in the N–H region when matched at 1.41 μ. Small corrections (usually less than 5% of the total change in absorbance) for the slightly higher absorbance at 1.515 μ with the NaOD method were applied to each final reading.

[1] A small amount of precipitate (<1% of the sample) occasionally formed in this step.

TABLE I: Variations in Root-Mean-Square Deviations with Number of Parameters RMSD × 10^4.

	Parameters	
pD	4	6
3.62	3.39	3.41
3.83	5.95	4.29
4.48	7.02	4.48
4.88	6.58	6.72

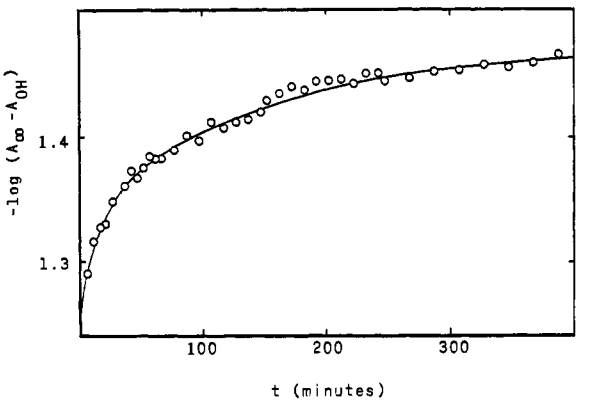

FIGURE 3: First-order plot of HOD absorbance for lysozyme in D_2O, pD 3.83. The solid line is calculated, 25°.

To convert changes in absorbance to numbers of peptide hydrogens, an extinction coefficient for HOD was determined at 1.41 μ by adding H_2O with a microburet to a weighed quantity of D_2O in a spectrophotometer cell equilibrated at 25.0°. HOD absorbance obeyed Beer's law over the range 0.2–1 M.

Samples of lysozyme were dried at 110° for extended periods and 5–6% water was found.

Calculations. Figure 2 shows the change in absorbance at 1.41 μ with time for a typical run. The substantial background absorbance at zero time is due to rapidly exchanging, side-chain hydrogens and residual water, as verified below. Figure 3 shows the same data on a log ΔA *vs.* t graph. Clearly, the exchange process cannot be described by a single first-order process. However, as Linderstrom-Lang (1955) has shown, hydrogen exchange in proteins can be described by a sum of pseudo-first-order terms, which for the present purposes can be written as

$$(A_\infty - A_{OH}) = \sum_i A_i e^{-k_i t} \quad (1)$$

where A_{OH} is the 1.41-μ absorbance reading at any time, t; A_∞ the reading upon complete exchange; A_i the change in absorbance associated with each group i; and k_i is the first-order rate constant for that group. Generally, 20–30 points were taken over a 6- to 8-hr period for each computational analysis. In all the runs with lysozyme, two or three rate constants were sufficient to fit the data within the precision of the measurements. For example, the solid line in Figure 3 was calculated from the equation

$$(A_\infty - A_{OH}) = 0.0095e^{-0.077t} + 0.0121e^{-0.0082t} + 0.0341e^{-0.000006t} \quad (2)$$

A Fortran program was written for the computational analysis. Basically, it was a combination of a linear and nonlinear least-squares routines. Initial estimates of the rate constants were obtained from a plot of log $(A_\infty - A_{OH})$ *vs.* t and used in eq 1 to find the initial values of A_i by a linear least-squares analysis. In turn, those values of A_i were used in the nonlinear subroutine to find the best values of k_i. That procedure was repeated until neither set of constants was further refined. Convergence in the nonlinear portion was greatly accelerated by recognizing that the error surface is an elliptical paraboloid (Sillen, 1964) in the vicinity of the minimum. Thus, each k_i *vs.* error cross section is parabolic and the properties of a parabola can be used to estimate the value of k_i, which will minimize the error. With this method increments assigned to move k_i toward its minimum are automatically damped and serious overshoots in the optimization process are virtually eliminated. Another advantage is that there is no requirement for equally spaced time intervals.

The program was checked by fitting 60 points generated from eq 3, which had been obtained from hydrogen–tritium-exchange data on myosin (Segal and Harrington, 1967).

$$H(t) = 1190e^{-0.0798t} + 1176e^{-0.0105t} + 1029e^{-0.00171t} \quad (3)$$

These values were generated by our program: $H(t) = 1190e^{-0.0803t} + 1185e^{-0.0105t} + 1023e^{-0.00169t}$. Clearly, all six constants were reproduced within 1%.

The criteria applied to determine the number of parameters needed to adequately fit a set of data were based on a comparison between the overall root-mean-square deviation, RMSD, and the precision of the absorbance measurements, ±0.0004 unit. RMSD was calculated according to eq 4

$$\text{RMSD} = \left[\frac{\sum_i^n (\Delta A_i - \Delta A_{calcd})^2}{n - p}\right]^{1/2} \quad (4)$$

where $\Delta A_i = A_\infty - A_{OH}$ are the experimental values at any time, t, ΔA_{calcd} the corresponding theoretical changes, n the number of data, and p the number of parameters. Some comparisons are given in Table I. The pD 3.83 and 4.48 data clearly indicate the need for an additional set, A_i and k_i, to satisfy experimental precision. On the other hand, the pD 3.62 and 4.88 sets appear to be adequately reproduced with only two sets of parameters.

The magnitude of the rate constant, under a given set of conditions, largely determines the information that can be derived from a method. Figure 4 shows the log of the absorbance change over 6 hr for each of the first-order reactions given in eq 2. $\Delta A'$, $\Delta A''$, and $\Delta A'''$ were calculated from eq 5, 6, and 7. The solid lines in Figure 4 were evaluated from the

$$\Delta A' \equiv (A_\infty - A_{OH}) - A_2e^{-k_2t} - A_3e^{-k_3t} \quad (5)$$

$$\Delta A'' \equiv (A_\infty - A_{OH}) - A_1e^{-k_1t} - A_3e^{-k_3t} \quad (6)$$

$$\Delta A''' \equiv (A_\infty - A_{OH}) - A_1e^{-kt} - A_2e^{-k_2t} \quad (7)$$

terms $A_1e^{-k_1t}$, $A_2e^{-k_2t}$, and $A_3e^{-k_3t}$ for $\Delta A'$, $\Delta A''$, and $\Delta A'''$, respectively. The reaction characterized by k_1 was detected for only a short time, because the limit of measurable change

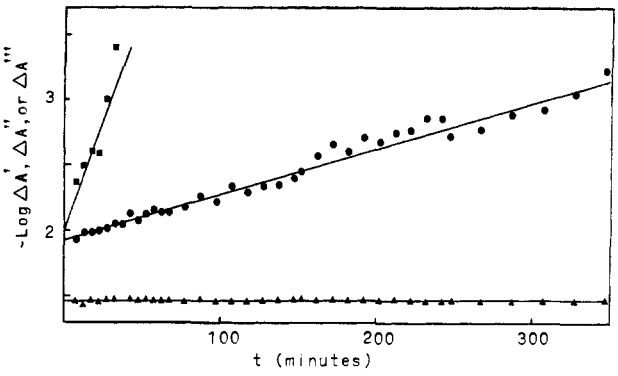

FIGURE 4: First-order plot of the HOD absorbance change for each class of exchangeable hydrogens in lysozyme, pD 3.83: (■) log $\Delta A'$; (●) log $\Delta A''$, and (▲) log $\Delta A'''$, 25°.

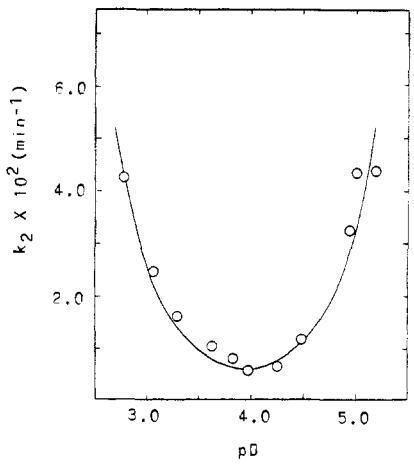

FIGURE 5: Rate–pD profile for k_2. Solid line is calculated, 25°.

TABLE II: Effect of A_∞ on the Reaction Parameters.

	A_∞		
	0.1514 (Actual)	0.1337 (24 hr)	% Change
k_1 (min^{-1})	0.077	0.091	15
k_2 (min^{-1})	0.0082	0.0091	10
$k_3 \times 10^4$ (min^{-1})	0.06	0.59	90
RMSD $\times 10^4$	4.29	4.27	
A_1	0.0095	0.0096	
A_2	0.0121	0.0123	<2
A_3	0.0277	0.0104	62

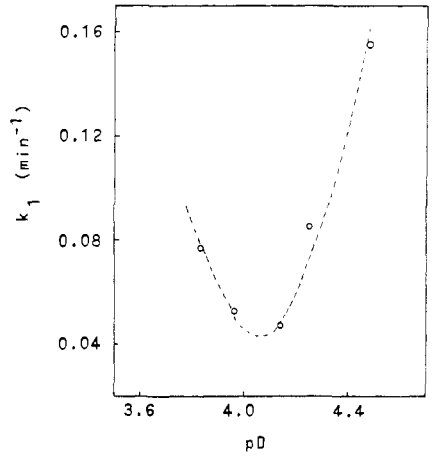

FIGURE 6: Rate–pD profile for k_1. Dashed line is estimated, 25°.

corresponds to log $\Delta A = -3.4$. Therefore, beyond approximately 30 min, that reaction no longer contributes to the observed change. However, the reaction governed by k_2 was successfully followed over the entire time period. The slowest reaction contributed little to the total change during 6 hr. Under present conditions, therefore, k_2 probably will be the most reliably determined rate constant, k_1 slightly less reliably, and k_3 considerably less.

Effects on rate constants from variations in the complete exchange reading are another aspect of quantitative hydrogen–deuterium exchange that needs to be investigated. Values of $A_\infty - A_0$, the total change in absorbance, averaged 0.053 from pD 3.83 to 4.48 with a RMSD of ±0.003. Most of the scatter appeared to lie in the A_∞ reading. Segal and Harrington (1967) reported that variations in A_∞ had little effect on k_1, A_1 and k_2, A_2 pairs, but a large effect on the k_3, A_3 set. Table II shows the change in reaction parameters with a drastic reduction in A_∞. Therefore, variations of the order of RMSD (0.003) should lead to 2, 1, and 15% errors in k_1, k_2, and k_3, respectively. Similarly, 0.2, 0.3, and 11% variations are predicted for A_1, A_2, and A_3, respectively. Thus, again k_1, A_1 and k_2, A_2 results are seen as considerably more reliable than those for the k_3, A_3 pair.

Results

pH Dependence. Equation 8 describes the rate constant in terms of specific acid, k_D, and base, k_{OD}, catalytic constants

$$k = k_0 + k_D(D^+) + k_{OD}(OD^-) \quad (8)$$

and a spontaneous rate constant k_0. (D^+) and (OD^-) are the activities of the deuterium and deuteriooxide ions, respectively. For computational purposes eq 8 may be rewritten as

$$k = k_0 + k_D(D^+) + \frac{k_{OD}K_{D_2O}}{(D^+)} \quad (9)$$

where K_{D_2O} is the autoionization constant of the solvent.

The dependence of k_2 on pD is shown in Figure 5. The solid line through the points was calculated from eq 9 with $k_0 = 0$ using a nonlinear, least-squares program (Kakuda *et al.*, 1971).[2] Clearly, variation of k_2 with pD can be adequately described by eq 8 with k_0 taken as zero. Interestingly, k_1 and k_3 also show the parabolic rate *vs.* pD profiles characteristic of acid and base catalysis. The data for k_1 rapidly became too fast to follow on either side of the minimum, as shown in Figure 6. A calculated fit to eq 9 was not possible for k_1 as too few points were available. Table III summarizes the cat-

[2] Catalytic constants are usually determined from the slopes of k *vs.* (H^+) and (OH^-) plots. The observed rate constants, however, are significantly base or acid catalyzed even at 1 pH unit above or below the minimum, respectively. Thus, only a few points at the extremes of the profile are useful. To the contrary, a nonlinear least-squares technique weighs all the points equally and the data near the minimum can be included.

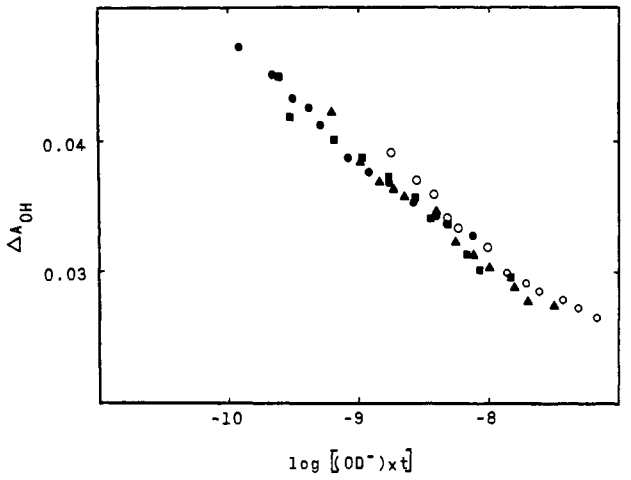

FIGURE 7: ΔA_{HOD} *vs.* log [$(OD^-) \times t$]: (○) pD 5.18, (▲) pD 4.88, (■) pD 4.48, and (●) pD 4.25.

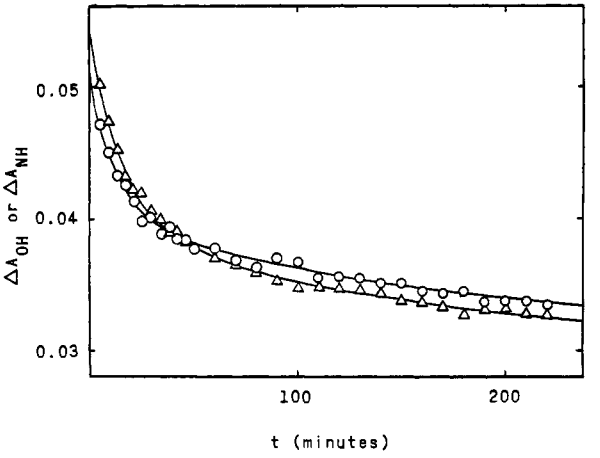

FIGURE 8: ΔA_{OH} (⊙) and ΔA_{NH} (△) *vs.* time for lysozyme in D_2O, pD 4.25, 25°. Solid lines are calculated and the circle size indicates the estimated precision of ±0.0004 absorbance unit.

TABLE III: Catalytic Constants, k_{min} and pD_{min}.

	k_D (M^{-1} min^{-1})	$k_{OD}K_{D_2O}$ (min^{-1})	k_{min} × (min^{-1})	pD_{min}
k_2 (min^{-1})	26 ± 2	(3.5 ± 0.2) × 10^{-7}	6.0	3.94
k_3 (min^{-1})	0.4 ± 0.2	(5 ± 1) × 10^{-6}	0.09	3.95

alytic constants found for k_2 and k_3, as well as the calculated values of pD_{min} and k_{min}, the value of the observed rate constant at pD_{min}.

Base catalysis of exchange for widely varying rates can be demonstrated also by superposition of ΔA *vs.* log [$(OD^-) \times t$] as a function of pD (Willumsen, 1967). However, superposition will occur only after correction for charge effects and when all the classes of hydrogens have the same pD_{min}. The analysis presented below indicates that all the peptide hydrogens observed under the present conditions do satisfy these criteria. Accordingly, Figure 7 shows the results obtained on the basic side of pD_{min} plotted in this manner. Nearly all data fall along a single line again indicating base catalysis.

Another quantity of interest related to pH dependence is the pD at the point of minimum exchange, pD_{min}. Leichtling and Klotz (1966) derived eq 10 from 9. Evaluations with

$$(D^+)_{min} = \left[\frac{k_{OD}K_{D_2O}}{k_D}\right]^{1/2} \quad (10)$$

eq 10 indicated that pD_{min} was virtually identical for the exchange characterized by either k_2 or k_3 (Table III). In addition, Figure 6 shows that k_1 probably has a very similar pD of minimum exchange.

Information regarding the nature of the exchanging group was inferred from rates of exchange calculated from HOD production (1.41 μ) and N–H diminution (1.515 μ). Figure 8 compares ΔA_{OH} and ΔA_{NH} for a typical experiment followed for over 5 hr. Although the net extinction coefficient for >N–H at 1.515 μ is close to that for HOD at 1.410 μ, they are not identical and exact superposition cannot be expected. The time course of the exchange, however, is very comparable, as reflected in the calculated rate constants shown in Table IV.

TABLE IV: Comparison of First-Order Rate Constants (min^{-1}) from OH Increase and N–H Diminution.

	1.41 μ		1.515 μ	
pD	k_1	k_2	k_1	k_2
2.77		0.043		0.046
4.25	0.086	0.0067	0.068	0.0091
4.48	0.16	0.012	0.13	0.015
5.01		0.044		0.044

Overall, the values for k_1 usually agreed within 25, and those for k_2, within 15%. If labile hydrogens other than peptide and amide N–H contributed to the production of HOD, rates calculated from 1.41-μ data should be consistently higher than those from 1.515 μ. No such systematic differences were apparent; instead the variations undoubtedly reflect errors inherent in the technique. Furthermore, the rates are the same whether 1.50-, 1.53-, or 1.515-μ data are used in the N–H region. However, RMSD increases rapidly as one moves away from the peak absorbance at 1.515 μ.

The preexponential factors, A_i, represent absorbance changes associated with peptide hydrogens exchanging with rate constants k_i. Values of A_i were converted to numbers of peptide hydrogens using an extinction coefficient of 53.5 cm^2/mole for HOD at 1.41 μ and 25°. The total change in absorbance, ΔA_{OH}, expected for the 128 peptide hudrogens can be readily calculated from the concentration of lysozyme.[3] In turn, eq 11 gave the number of amino acis in each ex-

$$n_i = \frac{A_i}{\Delta A_{OH}} 128 \quad (11)$$

changing class. The results are shown in Table V.

Interestingly, n_1 and n_2 seem to show only a slight variation about their means, not a trend with pD. The number of hydrogens in the n_3 class varied much more, but again with no

[3] Lysozyme concentrations were corrected for the 5–6% water in the commercial preparation.

TABLE V: Exchangeable Hydrogens in Lysozyme (Peptide H/Molecule).

pD	n_1	n_2	n_3	n_t	n_0
2.77		17	98		
3.07		20	99		
3.28		(23)	*a*		
3.62		25	59		
3.83	18	23	64	105	23
3.96	14	15	*b*		
4.10	18	14	*b*		
4.25	20	17	66	103	25
4.48	20	22	56	98	30
4.88		22	87		
5.01		23	22		
5.18		23	60		
Average	18	20	68	102	26

[a] No complete exchange reading. An average value was used for the calculations of n_2. [b] No N–H data available.

apparent trend. Greater scatter in the n_3 class reflects both the difficulty in following slow rates and errors inherent in the complete exchange readings. The difference between 128 and $n_1 + n_2 + n_3 \equiv n_t$ has been designated n_0 and is intended to represent those peptide hydrogens that exchange too rapidly to be detected under present conditions.

Apparently, the number of peptide hydrogens in a class can be measured to within ± 5 provided their rates can be brought into a readily observable range ($10 \leq t_{1/2} \leq 1000$ min). Comparison to the predicted sensitivity of ± 0.0004 absorbance unit shows that the theoretical limit would be about ± 2 hydrogens (ΔA for 102 hydrogens was 0.053). A variation of ± 5, therefore, is two to three times the theoretical limit.

Discussion

The near-infrared region on a Cary 14 is convenient and gives precise hydrogen-exchange data on proteins. One advantage is that HOD production and >N–H decrease can be followed on the same sample. Besides giving a double set of data on one experiment, the Cary 14 permits one to infer the nature of the exchanging group by comparing rate constants calculated from the two spectral regions. Such comparisons (Table IV) have shown that only peptide or primary amide hydrogen exchange is detectable after 5–10 min. However, if the data on *N*-methylacetamide (Klotz and Feidelseit, 1966) can be applied to a primary amide, a rate constant of about 0.5 min^{-1} at the pD_{min} of 5.42 and 25° would be predicted. Rates of the primary amides in a protein could be slowed (as the rates for peptide hydrogens are) below rates for simple molecules by being buried in the interior of the molecule, but X-ray diffraction data of Phillips (1967) show that only Gln-57 is shielded. Thus, at pD values of 5 or less, anticipated rates for Asn- and Gln-NH_2 exchange would be 0.62 min^{-1}, or larger. Even the 32 primary amide hydrogens in lysozyme, when exchanging at a half-life time of 1 min or less, would make a negligible contribution to the absorbance change after 5 min. Hence, for all practical purposes only peptide hydrogen exchange was observed.

Analyses of the hydrogen-exchange data, as described in the section on calculations, indicated that the data could be adequately reproduced with either two or three rate constant and class size parameters. Actually, such an analysis probably does not mean that there are, for example, exactly 18 hydrogens exchanging with a single rate constant k_1. On the contrary, k_1 may represent the mean of some distribution of rate constants, as suggested by Laiken and Printz (1970). However, if the distribution were not fairly narrow, abrupt shifts in parameters, as those given in Table I, probably could not be observed. Further, in the absence of *a priori* knowledge on the type of distribution expected in a protein, the present type of analysis seems sufficient, especially because any number of distributions can be expected to adequately describe hydrogen-exchange data (Laiken and Printz, 1970). Thus, descriptions of hydrogen exchange in terms of distributions of rate constants may not have any more physical validity than discrete k_i analysis. In any event, conformational changes should be just as detectable with either computational method.

Traditionally, hydrogen-exchange data on biopolymers are interpreted in terms of the motility concept as envisioned by Linderstrom-Lang and coworkers (Hvidt and Nielsen, 1966). In their mechanism

$$N \underset{k_r}{\overset{k_f}{\rightleftharpoons}} I \xrightarrow{k_x} \text{exchange}$$

where N is the native form of the macromolecule and I represents an instantaneous state in which a normally buried hydrogen is exposed to solvent and becomes exchangeable, k_f and k_r are the forward and reverse rate constants for the transition, respectively, and k_x is the rate constant for the exchange step. Under steady-state conditions the pseudo-first-order rate constant for a hydrogen in the *i*th class becomes

$$k_i = \frac{k_f k_x}{k_r + k_x} \tag{12}$$

If $k_r \gg k_x$ then $k_i = k_f k_x / k_r$. In that case, the exchange will show the pH dependence of k_x, and be slowed by the appropriate factor k_f/k_r for each class of hydrogens.

It has been shown that parabolic rate–pD profiles for all observable classes of hydrogens in lysozyme result from specific acid and base catalysis, as do those for polypeptides and for simple and polymeric amides. However, that does not necessarily substantiate the motility mechanism, because other factors as steric inhibition could produce slow exchange. What the data do show is that under conditions where conformational changes are absent, specific acid and base catalysis can be used to predict the pH dependence on the rate of exchange. Alternately, deviations from the anticipated behavior could be taken as evidence for a conformational change.

To correctly predict changes in exchange rates with pH, however, the effect of a net charge must be included. Berger *et al.* (1959) proposed that exchange in simple amides passes through charged intermediates.

acid catalysis

$$>N\text{–}H + D_3O^+ \longrightarrow >\underset{\underset{D}{|}}{N^+}\text{–}H + D_2O \xrightarrow{\text{fast}} >N\text{–}D$$

base catalysis

$$>N\text{–}H + OD^- \longrightarrow >N^- + HOD \xrightarrow{\text{fast}} >ND$$

Therefore a net positive charge on a biopolymer should slow D^+ catalysis and accelerate OD^- catalysis. Coleman and Willumsen (1969) found that exchange data for insulin followed the OD^- catalysis expected from model compound behavior only after correction for charge effects as measured from titration studies.

Recently, the direct effect of a net positive charge on the exchange of a polymeric amide group was reported by Kakuda *et al.* (1971). Although a Linderström-Lang type of analysis was found to qualitatively account for the decrease in the acid region, w, the electrostatic interaction factor, was about twice that predicted on the same basis from titration of the carboxyl side chains. The authors also proposed that under conditions where a biopolymer carries a net charge, eq 9 should be rewritten as

$$k = k_0 + k_D^0 \times 10^{-wz}(D^+) + \frac{k_{OD}^0 \times 10^{wz} K_{D_2O}}{(D^+)} \quad (13)$$

where z, a function of pH, is the net charge on the macromolecule, and k_D^0 and k_{OD}^0 are the *intrinsic* acid and base catalytic constants, respectively.

Titrations of lysozyme under conditions similar to those for exchange gave a w of about 0.04 for the 10 groups protonated between pH 5.3 and 2.5. The net charge on lysozyme, however, cannot be calculated directly from the titration data, because Carr (1953) found that Cl^- ions bind in the acid region and the amount bound increases with an increase in net positive charge. A combination of the Cl^- and H^+ binding data indicates that z increases by only $+1$ over the pH range 5.0–2.8 despite binding some 8 protons. If the average z is taken as $+9$ over the range and the results from polymeric amide studies can be extended to proteins, then w would be 0.08 (2×0.04), and the intrinsic catalytic constants k_D^0 and k_{DO}^0 for k_2 become: $k_D^0 = k_D \times 10^{0.72} = 136\ M^{-1}\ min^{-1}$ and $k_{OD}^0 K_{D_2O} = k_{OD} K_{D_2O} \times 10^{-0.72} = 6.7 \times 10^{-8}\ min^{-1}$. Comparisons to the results presented in Table III show that charge effects indeed sizably alter observed catalytic constants. However, to a good approximation, a net charge will not affect k_{min}, the observed rate constant at pD_{min}.

The value of pD_{min} also will show a charge effect and the magnitude of the shift can be predicted from eq 14 (Kakuda *et al.*, 1971)

$$\Delta pD_{min} = pD_{min} - pD_{min}^0 = -2wz \quad (14)$$

where w is that obtained for a carboxyl group and pD_{min}^0 is the *intrinsic* pD_{min}. Using the values of z and w given above and the observed pD_{min} of 3.95, pD_{min}^0 is found to equal 4.7. Thus the effect of a net positive charge is to *reduce* pD_{min} by 0.72 unit.

Table VI compares pD_{min}^0 from several studies. In general, simple and polymeric amides have minima near 5.4. On the other hand, small peptides and polypeptides show greatly reduced pD_{min}^0, due to inductive effects along the peptide chain (Leichtling and Klotz, 1966). Therefore, a minimum rate would be expected in the vicinity of pD 3.4 for a protein based on backbone inductive effects alone. However, pD_{min}^0 of lysozyme is 1.3 units higher.

The pD_{min} of a peptide group also can be altered in other ways. Equation 10 shows that a change in k_{OD}/k_D, or in K_{D_2O} will shift the value. The ratio of intrinsic catalytic constants depends on inductive effects among the peptide groups. As a reasonable approximation, however, the inductive effects should be about the same in a protein as in a polypeptide. In that case, a shift in pD_{min} would result from a change in K_{D_2O}.

TABLE VI: Intrinsic pD_{min} for Model Compounds and for Lysozyme.

Compound	pD_{min}^0
N-Methylacetamide[a]	5.4
Poly(*N*-isopropylacrylamide)[b]	5.0
Poly(ε-aminomethacrylyl-L-lysine)[c]	5.5
Poly-DL-alanine[d]	3.4
Lysozyme	4.7[e]

[a] Klotz and Frank (1964). [b] Scarpa *et al.* (1967). [c] Kakuda *et al.* (1971). [d] Englander and Poulsen (1969). [e] Calculated value. See text for details.

Klotz and Frank (1964) have shown that pD_{min} is increased by approximately 1.5 units in passing from pure D_2O to a 50:50 mixture of D_2O–dioxane, which has a dielectric constant of about 40. Furthermore, in a 50:50 mixture of H_2O–dioxane, K_w is about 10^{-17} at 25° (Harned and Owen, 1958). Equation 10 indicates that an increase of 1.3 units would require a decrease in K_{D_2O} of 2.5×10^{-3}. There is abundant evidence that hydrophobic residues can alter the structure of water, so such a decrease could occur in the apolar interior of lysozyme. Thus, at the moment of exchange the environment of the peptide >N–H group appears much more apolar than that for a small, randomly coiled polypeptide.

Estimates of pD_{min} based on the number of hydrogens exchanged at various times have been reported. Kägi and Ulmer (1968) found pD_{min} to lie between 4 and 6 for the number of hydrogens exchanged at 10 min, 1 hr, and 24 hr in cytochrome *c* (pI ~10). Coleman and Willumsen (1969) reported a pD_{min} of 2.5 for all the peptide hydrogens observable in zinc insulin (60%) at times between 2 min and 24 hr. Interestingly, that pD_{min} is below the 3.4 expected from inductive effects alone. Insulin, however, has a net positive charge of 6 and a w of 0.2 (corrected for association) at this pD. Therefore, by the arguments presented earlier, ΔpD_{min} would be -2.4 and pD_{min}^0 4.9, which is very near that for lysozyme.

Thus, there appear to be two opposing effects on the pD_{min} of proteins. First, the effect of a net positive charge is to *lower* pD_{min} from that expected from backbone inductive effects alone. Second, the apolar interior of a globular protein tends to raise pD_{min}. In the case of lysozyme, the apolar factor outweighed the first and the observed pD_{min} was above 3.4. With insulin the opposite effect is seen in that the charge factor dominated and the observed pD_{min} was less than 3.4. The intrinsic pD_{min}, however, was nearly the same for both proteins. It appears, therefore, that pD_{min}^0 may fall near 4.8 for the exchange of interior peptide groups in most proteins.

An additional item related to the value of pD_{min} is of interest. First, Hvidt (1963) reported that the number of hydrogens exchanged at pH 3.0 and 3.2 (pD 3.4 and 3.6) were nearly identical, while considerably more exchanged in the same time period at pH 4.2. Figure 5 shows that pD 3.6 and 4.6 lie on either side of the minimum and that their rates should be about the same. A possible explanation for this discrepancy may be that their pH measurements were made in

H_2O solutions before lyophilization. We have found that pH can rise slightly after lyophilization of unbuffered acidic solutions. In addition, Hvidt reported that 55 hydrogens remained unexchanged after 24 hr at pH 3.2 and 38°. Extrapolation of the pD 3.83 and 4.25 data to 24 hr gave 49 and 58 hydrogens unexchanged, respectively. In general, the agreement between the two techniques appears satisfactory.

Regarding class-size analysis, data in Table VI indicate that n_1 and n_2 can be determined within ±5 peptide units. Such a near constancy of class size strongly supports earlier observations that lysozyme maintains structural integrity in acid solution. The third class, n_3, varied much more, apparently due to scatter in the complete exchange readings. An average value of 68, nonetheless, shows that there are many very slowly exchanging hydrogens in lysozyme. The slowest class, however, accounts for all the hydrogens between n_2 and complete exchange. It undoubtedly is comprised of additional classes that will become detectable only at a different pH or temperature.

Finally, the differences between 128 and n_t indicated some 26 hydrogens in lysozyme exchange with a $t_{1/2}$ of <10 min at 25°. The n_0 class depends on the A_∞ reading representing exactly 100% exchange. The magnitude of the n_0 class could be in error if the exchange is not really complete. Furthermore, pD_{min} may be different for these exposed hydrogens.

Interestingly, Praissman and Rupley (1968) reported that a space-filling, scale model of lysozyme prepared from X-ray diffraction coordinates indicated 80% of the peptide hydrogens were buried. The 20% fully exposed to solvent corresponds to 26 hydrogens, the same as n_0. However, such close agreement is probably fortuitous.

In general, it should be possible to determine most of the class sizes of exchanging peptide hydrogens to within ±5 groups by selecting proper temperature and pH. Therefore, it appears that H–D exchange in the near-infrared region can be used to detect conformational changes involving as few as 5–10 peptide residues.

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