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Hydrogen Exchange of Lysozyme Powders. Hydration Dependence of Internal Motions†

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ABSTRACT: The rate of exchange of the labile hydrogens of lysozyme was measured by out-exchange of tritium from the protein in solution and from powder samples of varied hydration level, for pH 2, 3, 5, 7, and 10 at 25 °C. The dependence of exchange of powder samples on the level of hydration was the same for all pHs. Exchange increased strongly with increased hydration until reaching a rate of exchange that is constant above 0.15 g of H_2O/g of protein (120 mol of H_2O/mol of protein). This hydration level corresponds to coverage of less than half the protein surface with a monolayer of water. No additional hydrogen exchange was observed for protein powders with higher water content. Considered in conjunction with other lysozyme hydration data [Rupley, J. A., Gratton, E., & Careri, G. (1983) *Trends Biochem. Sci. (Pers. Ed.) 8*, 18-22], this observation indicates that internal protein dynamics are not strongly coupled to surface properties. The use of powder samples offers control of water activity through regulation of water vapor pressure. The dependence of the exchange rate on water activity was about fourth order. The order was pH independent and was constant from 114 to 8 mol of hydrogen remaining unexchanged/mol of lysozyme. These results indicate that the rate-determining step for protein hydrogen exchange is similar for all backbone amides and involves few water molecules. Powder samples were hydrated either by isopiestic equilibration, with a half-time for hydration of about 1 h, or by addition of solvent to rapidly reach final hydration. Samples hydrated slowly by isopiestic equilibration exhibited more exchange than was observed for samples of the same water content that had been hydrated rapidly by solvent addition. This difference can be explained by salt and pH effects on the nearly dry protein. Such effects would be expected to contribute more strongly during the isopiestic equilibration process. Solution hydrogen exchange measurements made for comparison with the powder measurements are in good agreement with published data. Rank order was proven the same for all pHs by solution pH jump experiments. The effect of ionic strength on hydrogen exchange was examined at pH 2 and pH 5 for protein solutions containing up to 1.0 M added salt. The influence of ionic strength was similar for both pHs and was complex in that the rate increased, but not monotonically, with increased ionic strength.

An understanding of the properties and function of proteins requires knowledge of the protein-water interactions that contribute to these phenomena. Solvent modulates the thermodynamics and dynamics of protein folding, of protein-ligand reactions, and of protein interactions with membranes and other cell organelles. Study of the process of hydrating dry protein to the solution state, because it allows the water concentration to be varied over a wide range, has provided insight into relationships between protein-water interactions and special properties of proteins (Careri et al., 1980; Rupley et al., 1983).

Three stages have been observed for the hydration process for lysozyme: (1) interaction at strong water binding sites, principally charged groups, complete at 0.07 h^1 (grams of

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water per gram of lysozyme); (2) saturation of the remaining polar sites, probably associated with formation of clusters of water molecules, complete at 0.25 *h*; (3) covering of the weakly interacting portions of the surface in a condensation event that leads to full monolayer coverage for lysozyme at 0.38 *h*. At this point, the protein is fully hydrated, in that thermodynamic properties such as volume, heat capacity, enthalpy, etc. have reached their solution values. The thermodynamic properties of the solvent bound to the protein at the point of full hydration differ only slightly from those of the bulk solvent.

The enzymatic activity of lysozyme and the motional properties of a spin probe noncovalently bound at the protein surface exhibit sharp increases at 0.25 *h* (Rupley et al., 1980, 1983). The parallel development of enzymatic activity and spin probe motion is of tenth or greater order in water, consistent with 0.25 *h* being the start of the condensation event that fills the weakly interacting regions of the surface. The mechanism of lysozyme catalysis (Banerjee et al., 1975) includes movement of the substrate at the surface of the enzyme during the rate-determining step, and this may be the explanation of the correlation between spin probe motion and enzymatic activity.

The motional properties of the bound water have been measured as a function of hydration level by dielectric relaxation (Harvey & Hoekstra, 1972; Bone & Pethig, 1982) and NMR methods (Bryant & Shirley, 1980). These observations indicate that water motion increases sharply above 0.2–0.3 *h*, like the changes in spin probe motion. At the point of full hydration, the dynamic properties of the solvent at the interface, like the thermodynamic properties, differ little from those of bulk solvent.

This report describes measurements of the hydrogen exchange of lysozyme powders as a function of hydration level, over the full range of hydration, from dry protein to the solution state. The intent was to compare the hydration dependence of protein internal motions with the hydration dependence of the surface motions, and in particular to determine whether the 0.25 *h* condensation is also a critical event for internal dynamics. The hydrogen exchange measurements reported here show that the motions they monitor are fully established at 0.15 *h*, well below the unfreezing of surface motions that reach their full level only at 0.4 *h* or slightly above. Thus, surface motions and internal motions are relatively uncoupled.

In carrying out the hydrogen exchange measurements, the water activity was varied over a wide range, which allowed determination of the order in water for the exchange process. This was found to be about 4 and the same for both fast- and slow-exchanging protons. Several mechanisms have been proposed to explain the rates of amide hydrogen exchange for proteins (Englander et al., 1972; Wuthrich & Wagner, 1978; Woodward et al., 1979; Richards, 1979). They differ principally in the way in which the protein structure is perturbed to allow formation of a hydrogen-bonded complex between the exchanging amide proton and solvent species (water or hydroxide or hydronium ion). In penetration models, solvent species diffuse through the interior of the protein by propagation of "mobile defects" (Lumry & Rosenberg, 1975) or by motion within transient networks of water molecules connecting the protein surface with interior sites (Richards, 1979). In either case, the opening of a hydrogen bond to allow an amide hydrogen to bond to solvent species is understood as a small and local perturbation. In other models, exposure of amide hydrogens to bulk solvent occurs through local unfolding or domain rocking. The hydration dependence observed for the hydrogen exchange, because it is low order with respect to water and invariant to the extent of shielding,[2] is most simply explained by a penetration model.

Materials and Methods

Materials

Lysozyme, 2 times recrystallized from hen egg white (LY-SF), was purchased from Worthington. The protein was dialyzed for 48 h against three changes of deionized water, lyophilized, and stored refrigerated.

Tritiated water of specific activity 100 mCi/mL was obtained from New England Nuclear, DEAE-Sephadex A-25 from Pharmacia Fine Chemicals, Handifluor complete scintillation cocktail from Mallinckrodt, polyethylene Bantam vials of 6-mL capacity for scintillation counting from Beckman.

Water was deionized with a continental system. All chemicals were reagent grade.

Methods

pH. Values of pH were measured by using a Radiometer PHM 26c meter equipped with a GK2402C glass combination electrode. Standard buffers were of pH 4.01 and 6.5. Additional reference solutions were 0.010 M HCl of pH 2.09 and 0.001 M HCl of pH 3.0. For exchange experiments, pH was determined for 20 mg/mL protein solutions measured after 24 h, the reaction time of most exchange experiments, or after 48 h for jump experiments.

Preparation of Labeled Protein. Stock lysozyme was dissolved in deionized water to a concentration of 50–60 mg/mL and titrated to pH 5. Tritiated water was added to give a specific activity of at least 10 mCi/mL, and the solution was equilibrated at 60 °C for 24 h. Following labeling, the protein solution was titrated to the pH appropriate for exchange-out experiments. The titration steps introduced the only salt present in the preparation, which therefore had different counterion concentrations at each pH. Insoluble material was removed by centrifugation. Two aliquots were withdrawn: one to measure the specific activity as cpm per mole of H and the other to serve as a reserve. The protein solution was frozen in a dry ice–acetone bath and lyophilized overnight in a desiccator containing enough fresh P_2O_5 to adsorb all water. The labeled protein was stored under vacuum in a desiccator containing fresh P_2O_5. To determine the extent of labeling, 10–20 mg of protein powder was dissolved in 10.0 mL of deionized water, and counts were determined in triplicate 0.5-mL aliquots. Incorporation greater than the theoretical value (264 exchangeable hydrogens at pH 2) was observed consistently, reflecting a 1–2% water content and equilibrium isotope effects.

Gel Filtration. Gel filtration was used in analysis of the extent of hydrogen exchange (Englander & Englander, 1972). DEAE-Sephadex A-25, equilibrated with a 0.09 M NaCl–0.01 M sodium acetate solution of pH 4.0, was chosen for analytical separation of tritiated protein from solvent. Lysozyme moves anomalously on a number of chromatographic matrices (Kramer, 1971; Imoto et al., 1972). The pH 4.0 buffer serves to effectively quench the pH-dependent hydrogen exchange

[1] Abbreviations: H_{rem}, labile hydrogens remaining unexchanged at the time of measurement, expressed as moles of H per mole of lysozyme; *h*, hydration level in grams of water per gram of protein; P/P_0, partial pressure of the vapor relative to the vapor pressure of the pure solvent; a_w, activity of water, equal to P/P_0.

[2] By shielding, we mean the set of mechanisms that operate to reduce the rate of amide hydrogen exchange. Thus, there need be no relationship between extent of shielding and distance from the surface.

reaction because pH 4 is the reported pH_{min} for lysozyme exchange (McBride-Warren & Mueller, 1972; Kakuda & Mueller, 1975). The columns were 1.0 × 6.0 cm, with a 5-cm eluent head. Freshly equilibrated columns were used for each data point. Eluent was pumped with a Technicon peristaltic pump and manifold tubing, giving a flow rate of at least 3.5 mL/min. Eluate was monitored for protein by using a Chromatronix UV system. The void volume was collected as one fraction. Fractions through the protein peak were collected at 10 drops each. Later fractions, analyzed for tritium in order to calculate material balances and the specific activity of water bound to powder samples, were generally of greater volume. Fraction volumes, when measured, were based on weight, assuming a density of 1.0 g/mL. The total time from sample dissolution through collecting the protein peak fractions was 1.5–2.5 min. All gel filtration was performed at room temperature, usually 24–25 °C. Recovery was 100% for protein and 95% for tritium.

Analysis of Gel Filtration Fractions and Calculation of Labile Hydrogens Remaining Unexchanged. Gel filtration fractions through the protein peak were diluted by adding 2.5 mL of eluent. Protein absorbance was measured at 280 nm with a Zeiss PMQII spectrophotometer. Protein concentrations were calculated by using $E^{1\%}_{1cm} = 26.25$ and a molecular weight of 14306. Tritium counting was done in duplicate with a Beckman LS7000 microprocessor controlled scintillation counter, on samples of 0.5 mL of a diluted fraction added to 3.5 mL of cocktail. Counting time was 5 min. Background counts per minute were calculated as the average of the void volume fraction counts within an experiment. Counts per minute used for calculation of hydrogen exchange were the average of the duplicate analyses minus background cpm. This value, expressed as cpm per milliliter, was converted to moles of H per milliliter by use of the specific activity measured during labeled protein preparation. Dividing by the protein concentration yields H_{rem}, the moles of $H_{unexchanged}$ per mole of lysozyme. Reported H_{rem} values are averages through the protein peak.

Solution Hydrogen Exchange. To start an experiment, labeled stock lysozyme was dissolved to give a concentration of 20 mg/mL in 0.010 M HCl solution (for pH 2), 0.001 M HCl (for pH 3), deionized water (for pH 5), deionized water (for pH 7), or 0.010–0.020 M sodium carbonate buffer solution (for pH 10). Equilibration was at 25 °C. At preselected times after the start of the experiment (time zero), 100-μL aliquots were withdrawn for hydrogen exchange analysis by gel filtration.

Powder Hydrogen Exchange. Labeled lysozyme (at least 40 mg) was weighed into a 75-mL tared equilibration bottle by use of a Mettler B6 semimicro analytical balance. An empty 10-mL glass beaker was set inside. The bottle was capped and stored in a desiccator containing fresh P_2O_5. To start an experiment, 5 mL of a solution of the appropriate volume percent H_2SO_4 (for pH 2–7) or weight percent NaOH (for pH 10) was dispensed into the beaker to control the vapor pressure for isopiestic equilibration. In order to maximize the exposed surface of the protein powder, it was spread thinly over the bottom of the bottle. Of the arrangements tested, this geometry gave the most rapid equilibration between protein and the solvent sink. The bottle was tightly capped; a strip of parafilm served as an additional seal. The sealed bottle was placed in a 25 °C water bath. After being equilibrated for 24 h, the bottle was dried, and the contents (minus the beaker) were reweighed to determine the extent of hydration. The protein was dissolved with gel column eluent to give a concentration of 20 mg/mL, and a 100-μL aliquot was taken for analysis of the extent of hydrogen exchange by gel filtration.

Jump Hydrogen Exchange. Two types of experiments were performed: solution pH jump and hydrated powder to solution jump.

For solution pH jumps, the extent of exchange after 24 h at the initial pH was determined by duplicate analysis. After jump to the final pH of the sample, aliquots taken at preselected times were analyzed for exchange.

Powder to solution jumps required three powder samples hydrated in parallel. After 24 h, two samples were analyzed for hydration level and hydrogen exchange to characterize the powder and to establish the value for H_{rem} for the protein in solution at the start of the jump. The remaining sample was jumped by dissolving in solvent to a concentration of 20 mg/mL. Aliquots were withdrawn at preselected times for hydrogen exchange analysis.

Comparison of jump data with previously determined values for solution exchange (denoted the control data) required two adjustment steps. First, because the control data and jump data were for slightly different pHs, the control data were rescaled in time to adjust them to the pH of the jump data, by use of the relationship

$$\log t_2 = \log t_1 + \alpha(pH_1 - pH_2) \quad (1)$$

where α is the measured dependence of exchange rate on pH ($\Delta k_{obsd}/\Delta pH$), t_1 and t_2 are the observed and adjusted exchange times of the control data, respectively, pH_1 is the pH for the control data, and pH_2 is the pH for the jump data. This adjustment of the control data is justified by the pH invariance of the rank order of exchange, which is proven below. Second, the jump data were transposed graphically along the time axis until the time-zero point met the control curve. The time dependence of the exchange-out after the jump was then compared with the control.

Degree of Hydration. Hydration levels were determined by weight for each sample of a hydration experiment. The determination consisted of two parts. First, a weighed amount of the unhydrated stock lysozyme powder was heated at 102–104 °C. After 24 h, it was equilibrated to room temperature in a desiccator containing fresh P_2O_5 and then reweighed to determine protein dry weight. The difference between the initial weight and the dry weight gave the percent water in the stock lysozyme powder. This value was used to correct each hydration sample's initial weight to obtain dry weight. Second, each protein powder sample, hydrated by 24 h of isopiestic equilibration, was reweighed prior to being dissolved for gel filtration. The weight difference between dry and hydrated protein is the hydration water. Dividing by sample dry weight gives the hydration, expressed as grams of H_2O per gram of lysozyme (denoted h).

Control Experiments. As a check on the stock lysozyme preparations, two control measurements were routinely made in conjunction with each powder hydrogen exchange experiment.

The value C_0 is the maximum observable H_{rem} for the conditions of sample preparation and analysis. It was measured by gel filtration analysis of an aliquot of stock labeled protein dissolved in eluent and immediately analyzed. It represents a measurement of the changes that occurred within the dead time of the technique. It is a sensitive monitor for loss of tritium from labeled stock protein. The value of C_0 depends on the pH of the solution from which the stock protein was obtained by lyophilization. Typical values are given in Table I for pH 2–10. Because the pH after dissolution in the

Table I: H_{rem} Values for Solution Hydrogen Exchange at pH 2–10 in the Absence of Added Salt[a]

time (min)	pH[b] 2	3	5	7	10
C_0[c]	133	134	132.9	92.6	46.1
1.0	138	131	105.5	71.8	29.6
2.0	128	122	103.3	62.7	26.0
3.0	130	118	96.0	59.4	ND[e]
4.0	128	115	94.0	58.2	23.2
6.0	130	114	89.8	55.1	ND
8.0	123	111	85.3	51.5	20.3
10.0	124	109	85.8	51.6	20.1
20	122	105	77.3	46.1	17.3
40	109	96	73.4	40.7	15.1
80	106	89	66.4	36.6	12.1
160	96	85	62.0	33.1	9.4
320	90	79	56.8	29.0	5.8[d]
640	79	74	50.0	24.4	4.2
1440	69	68	43.2	21.5	2.1

[a] All solutions equilibrated at 20 mg/mL and 25 °C. Solvents: pH 2, 0.010 M HCl; pH 3, 0.001 M HCl; pH 5, titrated deionized water; pH 7, deionized water; pH 10, 0.020 M carbonate. [b] Measured pH after equilibration: 1.86, 2.94, 5.08, 6.88, and 10.03. [c] C_0 is the maximum observable H_{rem} at a given pH; it is a dead-time measurement for the gel filtration procedure. Mean values over all preparations for each pH are given for C_0. [d] Aliquot taken at 390 min. [e] ND, not determined.

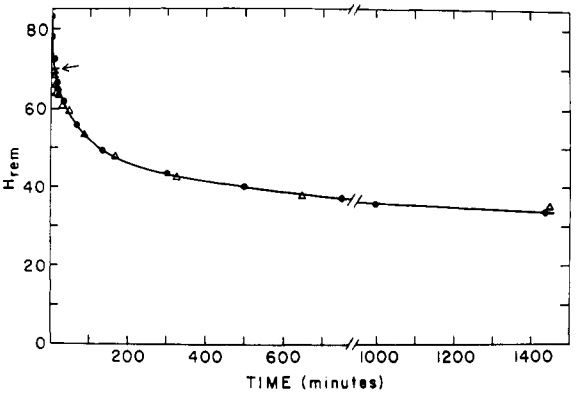


FIGURE 1: pH 3 to pH 5 jump in solution. Data from an unbuffered pH 2.92 to pH 5.86 solution pH jump experiment (△) were compared to pH 5.11 solution control data adjusted to pH 5.86 (●). In the jump experiment, fully labeled lyophilized pH 3 lysozyme was dissolved to a concentration of 20 mg/mL. After 24 h of equilibration at 25 °C, two 100-μL aliquots were analyzed to establish time-zero H_{rem} (←) for the jump, and then the solution was jumped by addition of an equal volume of 0.100 M phosphate, pH 6.03. The sample continued to equilibrate at 25 °C for another 24 h, with aliquots taken for analysis at times from 1.0 min to 24 h. For this comparison, the time-zero H_{rem} value of the jumped sample was located on the control curve at the corresponding H_{rem} point.

pH 4 quench buffer is the same for powder samples of different pHs, the different values of C_0 must represent transient control by the protein of the local environment during dissolution.

The extent of exchange at 1440 min (24 h) in solution was measured in duplicate, also as a control for the exchange behavior of the stock protein. Generally, a set of measurements on powder samples of different hydration level but of a single pH were done together, and a solution 24-h control sample was run in parallel. Typical values are given in Table I.

Error. Values of H_{rem} are given as averages with standard deviations for measurements of the protein peak fractions. The error in H_{rem} consists of contributions from determinations of protein absorbance, volume, sample cpm, and cpm per mole of H. Estimated uncertainties of 0.5–1.5% for each of the above operations give a total estimated uncertainty of 2%. This estimate agrees with the observed error.

The estimated uncertainty in hydration level, reflecting the six weighings in its determination, is 2.5% for 0.2 *h*, assuming 0.1-mg uncertainty in each weighing.

RESULTS

Solution Hydrogen Exchange. The two groups of experiments described below were necessary to establish the solution exchange behavior of lysozyme for use in comparison with measurements on partially hydrated powder. First, hydrogen exchange was measured for solutions of pH 2–10. The results are summarized in Table I. At pH 2 and 3 and short times of exchange, the least shielded of the 128 amide hydrogens of lysozyme are observed in the 24-h time window. At pH 10, there is nearly complete accessibility of the amide hydrogens to exchange, and only the most shielded hydrogens are observed.

In order to use the above data to generate a complete solution exchange profile, it was necessary to demonstrate that the rank order of exchange is preserved as the pH is changed. This was done through pH jump experiments that spanned the range of pH 3–10. Figure 1 gives results for the jump of an unbuffered pH 3 solution to pH 5 by addition of an equal volume of 0.1 M phosphate buffer of pH 6.03. The jump data were compared to solution controls as described under Methods. The close agreement between the two exchange curve segments shows that rank order of exchange is maintained between pH 3 and pH 5. Similar results were obtained for jumps from pH 5–7 and pH 7–10, confirming the report of Knox & Rosenberg (1980) that the rank order of exchange is maintained for lysozyme under change in pH.

The complete solution hydrogen exchange profile was then constructed by transposing semilogarithmic plots of the solution H_{rem} data to achieve the best overlap, as described under Methods. The results are displayed as the solid line of Figure 9. At least 8 orders of magnitude in hydrogen exchange rate can be observed through selection of the pH of measurement. The transpositions in time needed to obtain the overlaps required to construct the solution exchange profile derive from the effect of the change in pH on the exchange rate. The overlap factors, $\Delta(\log t)/\Delta pH$, used to construct Figure 9 are the following: pH 2–3, 0.53; pH 3–5, 0.54; pH 5–7, 0.98; pH 7–10, 0.75.

Ionic Strength Effects. The influence of ionic strength on solution exchange was measured in order to interpret results of isopiestically equilibrated powder samples, in which ionic strength is expected to vary markedly in the early stage of hydration. The effect of added salt on 24-h solution hydrogen exchange was measured for pH 2 and pH 5. The data are given in Table II. At pH 5, the addition of salt increased the pH, as expected for an ionic strength effect on the p*K* of groups of a positively charged protein. Table II gives, in parentheses, H_{rem} values corrected to the pH of the salt-free solution. The ionic strength effect does not change monotonically as salt is added. At both pH 2 and pH 5, H_{rem} at 24 h decreases by four to six protons with ionic strength increase from 0 to 0.1. H_{rem} then increases slightly between ionic strength 0.1 and 0.5 and decreases again with increase in ionic strength above 0.5.

The similarity in the dependence of the exchange on salt concentration for the two pHs is notable, since the net and total charges are different at the two pHs. Net charge is +18 and +12, and total charge is 20 and 26, for pH 2 and 5, respectively.

An ionic strength jump experiment at pH 2 was performed to test for preservation of rank order with change in salt concentration. Initial and final ionic strengths (0.017 and 0.500) were chosen for equal 24-h extents of exchange (Table

Table II: Effect of Added Salt on Solution Hydrogen Exchange at 24 h[a]

ionic strength[b]	pH 2		pH 5	
	H_{rem}[c]	pH	H_{rem}[c]	pH
0.0	71.3 ± 1.2	1.87	43.1 ± 0.9	5.10
0.010	69.0 ● 1.8	1.91	42.3 ± 1.2 (44.6)	5.24
0.017	68.4 ± 2.2	1.89	41.9 ± 1.5 (43.9)	5.22
0.025	67.6 ± 1.4	1.90	40.6 ● 0.8 (43.2)	5.26
0.050	66.2 ± 1.8	1.91	39.8 ± 0.6 (43.1)	5.32
0.075	65.9 ± 1.6	1.92	39.6 ± 0.8 (43.7)	5.36
0.100	65.4 ± 3.0	1.92	39.3 ± 1.6 (43.4)	5.38
0.250	65.4 ± 1.9	1.91	40.1 ± 1.3 (46.0)	5.48
0.500	68.2 ± 2.3	1.89	40.7 ± 1.4 (47.6)	5.52
0.750	67.1[d]	1.86	39.9 ± 1.7 (46.4)	5.52
1.000	65.8 ± 0.9	1.81	38.9 ± 0.4 (45.3)	5.52

[a] All samples dissolved and equilibrated at 20 mg/mL and 25 °C. [b] To obtain the solvent, NaCl was dissolved in 0.010 M HCl for pH 2 and in titrated deionized water for pH 5. The ionic strength listed is the NaCl molarity. [c] H_{rem} values are single determinations except at zero ionic strength, for which the mean of two measurements is given. Values of H_{rem} in parentheses are the measured values adjusted to pH 5.10. [d] H_{rem} value for peak fraction of gel analysis.

Table III: Ionic Strength Jump at pH 2[a]

sample[b]	H_{rem}	pH
0.017	60.6 ± 1.0	1.90
0.500	58.6 ± 0.6	1.88
jump	59.8 ● 0.8	1.90
	58.6 ● 0.8	

[a] All samples dissolved and equilibrated at 20 mg/mL and 25 °C for 48 h. Jump sample was equilibrated at 0.017 ionic strength for 24 h and then jumped to 0.500 ionic strength for another 24 h. [b] Molarity of NaCl dissolved in 0.010 M HCl.

II). Thus, the jump should result in no change in H_{rem} if rank order is preserved, and this behavior was found (Table III).

The solution measurements made here can be compared with published hydrogen exchange measurements on lysozyme (Hvidt & Kanarek, 1963; Hvidt, 1963; Knox & Rosenberg, 1980). The normalized exchange curve of Figure 9 differs from the similar profile given by Knox & Rosenberg (1980) by being nine protons lower, on average, between −1.0 and 3.5 log time and is in close agreement with the Knox and Rosenberg data between 3.5 and 5.5 log time. The transposition of data for various pHs required to generate the profile gives a measure of the dependence of the exchange rate on the specific base catalyst concentration. The average value of d log k_{obsd}/d pH = 0.83 determined from data spanning the entire range from pH 5 to pH 10 is the same as the value for pH 7–8 given by Knox & Rosenberg (1980). However, the value for any given pH interval can differ markedly from the average, as seen above.

These results provide new information on the ionic strength dependence of solution exchange for lysozyme. (The Knox and Rosenberg results are for ionic strength 0.15.) The measurements reported here show that the ionic strength dependence is complex over the range 0–1.0 (Table II) and that ionic strength effects on the pK of protein groups have to be taken into consideration.

Furthermore, the effect of ionic strength on the exchange of lysozyme in solution is different from that for polypeptide models. The specific acid- and base-catalyzed exchange of small amide compounds (Englander et al., 1972) and of uncharged polypeptides (Kim & Baldwin, 1982) is insensitive to change in ionic strength. As expected from consideration of electrostatic interactions, positively charged polypeptides show a slight increase in rate of acid-catalyzed exchange (3-fold) and a strong decrease in rate of base-catalyzed exchange

Table IV: Time Course of Lysozyme Hydration[a]

time (h)	0.95 P/P_0		0.82 P/P_0	
	h	fractional h	h	fractional h
0.0	0.014	0.04	0.030	0.21
0.5	0.121	0.31	0.091	0.62
1.0	0.142	0.36	0.113	0.77
2.0	0.190	0.48	0.134	0.92
4.0	0.246	0.62	0.155	1.06
8.0	0.284	0.72	0.147	1.01
24.0	0.371	0.94	0.144	0.99
72.0	0.394	1.00	0.146	1.00
half-time	2.0[b]		0.5[b]	

[a] Samples of pH 7 protein were equilibrated against 5% or 15% (v/v) H_2SO_4, corresponding to 0.95 and 0.82 P/P_0. Each datum is for a separate sample that had equilibrated the indicated time. [b] Half-time in hours.

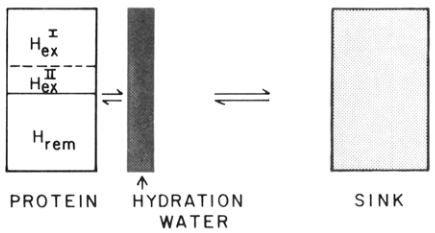


FIGURE 2: Scheme for hydrogen exchange of a protein powder.

(50-fold) for an increase from 0 to 1 M added salt (Kim & Baldwin, 1982). For lysozyme, however, change from 0 to 1 M added salt produces at pH 2 a 4-fold increase and at pH 5 a 3-fold increase in the rate of exchange of lysozyme (calculated from transpositions along the time axis in Figure 9 of the data of Table II). Thus, for the hydrogens of rank order in H_{rem} near 70 and near 40, the ionic strength effects are complex and different from that found for model compounds, as judged by the shape, sign, and magnitude of effects at high ionic strength.

Lysozyme Powder Hydrogen Exchange. Isopiestic Equilibration Half-Time. The time course of the hydration process is given in Table IV for two vapor pressures, P/P_0 = 0.82 and 0.95, corresponding to final hydration levels of 0.15 and 0.4 h, respectively. The half-time for hydration is 4 times greater for the higher vapor pressure (2.0 vs. 0.5 h). The point to be made is that the equilibration half-time is short (0.5 h or less) for the hydration levels below 0.2 h that are of importance for the following discussion. The long half-time for hydration to higher levels probably reflects the large mass of weakly bound water that must be transported to and bound at the protein surface. However, when hydration was performed by mixing a lyophilized powder sample directly with the appropriate volume of water to give a desired final hydration level, a high level of hydration could be established in effect instantaneously

Values of the hydration level for lysozyme measured as a function of vapor pressure at pH 7 and 25 °C agreed well with the sorption isotherm data for lysozyme at pH 6.8–7 and 27 °C, measured by Hnojewyj & Reyerson (1961).

Picture of Powder Hydrogen Exchange. Hydrogen exchange from protein powders is represented in Figure 2. At any time of exchange, the labile hydrogens of the protein can be viewed as divided among three classes. H_{ex}^{I} protons exchange rapidly during the analysis and are unobservable; i.e., they do not contribute to C_0 exchange values. H_{ex}^{II} protons exchange-out during the experiment and do not exchange during analysis. H_{rem} protons are not exchanged during the experiment and subsequent analysis on gel columns. For

simplicity, it is assumed that the tritium-labeled labile hydrogens of the protein exchange only with the hydration water bound at the surface of the protein and that the hydration water exchanges through the vapor phase with the solvent sink of zero specific tritrium activity. If exchange between hydration water and sink is slow compared to exchange between protein and hydration water, then tritium can accumulate in the hydration water. In this case, the specific activity of the protons H_{ex}^{I} and H_{ex}^{II} after these have exchanged in the protein is greater than the specific activity of the sink and is equal to the specific activity of the protons of the hydration water. Consequently, experimental exchange measurements from powders give H_{obsd}, which is H_{rem} plus a contribution from H_{ex}^{II} that is dependent upon the specific activity of the hydration water. It is necessary to correct for the contribution of H_{ex}^{II}.

Measurements on the eluate from gel filtration analyses of exchanged powders allow one to determine the total tritium counts in the water that elutes from the gel column after the protein. This value, the counts free, has contributions from the residual tritium in the hydration water and the protons of class H_{ex}^{I} that exchange with the eluant during column analysis and that are of the same specific activity as the hydration water. From the counts free, one can calculate the specific activity of the hydration water, in counts per mole of water protons:

$$\text{s.a.}(H_2O) = (\text{counts free})(1590h + H_{ex}^{I})^{-1} \quad (2)$$

where h is the hydration level (in grams of H_2O per gram of lysozyme), 1590 is twice the ratio of the molecular weight of lysozyme to that of water, and counts free is expressed per mole of lysozyme. H_{ex}^{I}, expressed as moles of H per mole of lysozyme, is determined from the specific activity of the protein preparation and the solution zero-time measurement, C_0. As shown by Table I, H_{ex}^{I} varies with pH.

The value of H_{rem} is obtained by correcting H_{obsd} by use of the above value for the specific activity of the hydration water:

$$H_{rem} = H_{obsd}\{\text{s.a.(start)}/[\text{s.a.(start)} - \text{s.a.}(H_2O)]\} - C_0\{\text{s.a.}(H_2O)/[\text{s.a.(start)} - \text{s.a.}(H_2O)]\} \quad (3)$$

where s.a.(start) is the specific activity of the tritiated water used in labeling the protein.

Time Course of Powder Exchange and Hydration. Time courses of exchange for lysozyme powders were measured at pH 7 for two vapor pressures. The data are given in Figure 3. At high relative vapor pressure (triangles; $P/P_0 = 0.95$), powder exchange closely follows solution exchange (solid line; data from Table I). At low relative vapor pressure (circles; $P/P_0 = 0.34$), powder exchange is slow, with an apparent exchange half-time of 2 h. It is important to note that the half-time for hydration at the low vapor pressure is less than 0.5 h (Table IV).

From consideration of the time courses for hydration and exchange of the powder, it was decided to measure exchange at 24 h at varied levels of powder hydration. The use of single time points for measurement is justified as follows. Because of the exponential character of amide hydrogen exchange and the dispersion of the rate constants for a native protein, there is relatively little change in H_{rem} between 24-h exchange and several times this period of reaction. At 24-h equilibration, hydration is more than 90% complete (Table IV). Thus, measurements at 24 h are significant, experimentally convenient, and give results not markedly different from those for several days longer reaction times. Short times of reaction (1 min to 1 h), during which there is a large drop in H_{rem} in solution (e.g., from 71.8 to 36.6 at pH 7; Table I), are not appropriate for powder measurements, because the half-time

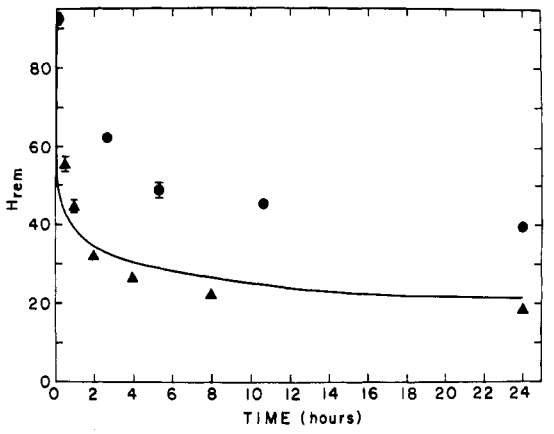


FIGURE 3: Time dependence of powder hydrogen exchange. Individual samples of pH 7 fully labeled, lyophilized lysozyme were isopiestically equilibrated at 25 °C and at relative vapor pressures of 0.34 (●) or 0.95 (▲), using 36% or 5% (v/v) H_2SO_4 to control vapor pressure. At the times shown, samples were dissolved to a concentration of 20 mg/mL, and 100-μL aliquots were analyzed by gel filtration. The solid line represents solution exchange at pH 7. The zero-time value of H_{rem} (●) is the C_0 value.

for hydration is ca. 0.5 h or longer. The relatively small changes in H_{rem} between 4–10 h of exchange (the least time needed for substantial completion of hydration) and 24 h make progress curves for exchange from the powder relatively uninteresting.

It is important to note that quantitative information on the rate of amide hydrogen exchange can be extracted from a single time point, even though there is a dispersion in the distribution of protein exchange rates over many orders of magnitude. At any particular time of exchange, most exchangeable sites on a protein will be either fully exchanged or not exchanged at all. Therefore, the time of exchange is equal to the weighted average characteristic time, which defines the average first-order exchange rate constant for the small subset of hydrogens that are partially exchanged. As long as the rank order of exchange is invariant to change in reaction conditions (such as pH, ionic strength, etc.), it follows that (1) at a given H_{rem} value, the same set of hydrogens is being observed whatever the conditions, and their average exchange rate constant, as defined by the time of reaction required to reach a fixed H_{rem} value, can be compared for varied conditions and (2) changes in the value of H_{rem} at a fixed time of reaction can be used as a qualitative measure of the effect of varying conditions on the protein amide exchange rates, in that a lower H_{rem} value reflects faster exchange.

Lysozyme Powder Exchange at pH 5. Table V gives the hydration dependence of 24-h hydrogen exchange from lysozyme powder at pH 5. These data are typical of those obtained at other pHs. The data illustrate the appreciable specific activity of tritium in the hydration water (column 5) for powders of hydration level below about 0.05 h. For such samples, the half-time for hydration is short, about 0.5 h, in contrast with the near 5-h half-time for exchange of the hydration water with the solvent sink (estimated from Table V, column 5). The greatly different half-times for hydration and exchange of the surface water reflect the comparable difference in the on and off rates for water at the protein surface at low hydration. The observation of slow equilibrium between hydration water and sink at low hydration is in accord with the picture presented above for hydrogen exchange from protein powders. The values of H_{obsd} (column 6) were corrected as described above to obtain H_{rem} (column 7). It is important

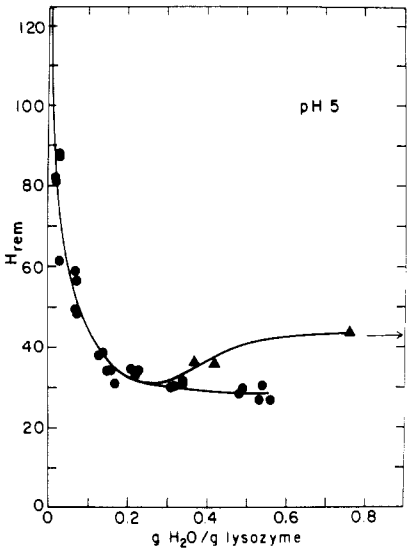


FIGURE 4: Hydration dependence of powder hydrogen exchange at pH 5. Individual samples of pH 5 fully labeled, lyophilized lysozyme were hydrated at 25 °C for 24 h to various water contents by isopiestic equilibration (●) or by addition and mixing of solvent (▲). The samples were then dissolved to a concentration of 20 mg/mL, and 100-μL aliquots were analyzed by gel filtration. The arrow (→) indicates the 24-h solution H_{rem} end point.

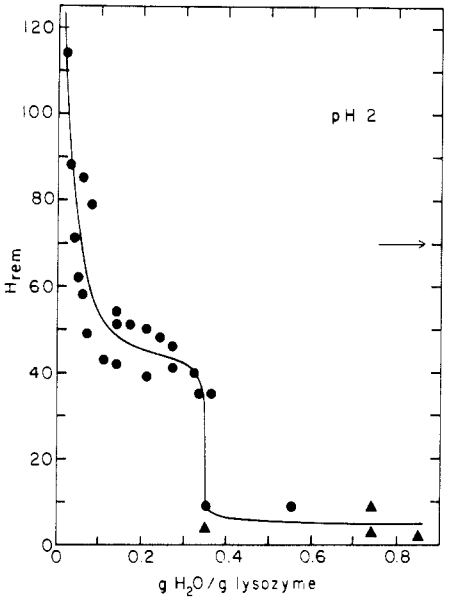


FIGURE 5: Hydration dependence of powder hydrogen exchange at pH 2. Conditions as described for Figure 4.

to note that even under the condition of slowest exchange between surface water and sink, the correction used to obtain H_{rem} is small, about five protons.

The hydration dependence of H_{rem} at pH 5 is illustrated in Figure 4. For isopiestically equilibrated samples (circles), the value of H_{rem} drops strongly with increase in hydration level in the first stage of hydration and reaches a constant value at about 0.15 *h*. This level of H_{rem} (28 H) is significantly below the solution value (43 H). As an alternative method for hydration levels above about 0.3, water could be added and mixed well with the powder at the start of the experiment to obtain the final hydration level quickly. When this was done, the 24-h value of H_{rem} was always higher than that for the isopiestically equilibrated samples and above 0.4 *h* was equal to the solution value.

Lysozyme Powder Exchange as a Function of pH. Figures 5, 6, 7, and 8 show the hydration dependence of the 24-h values

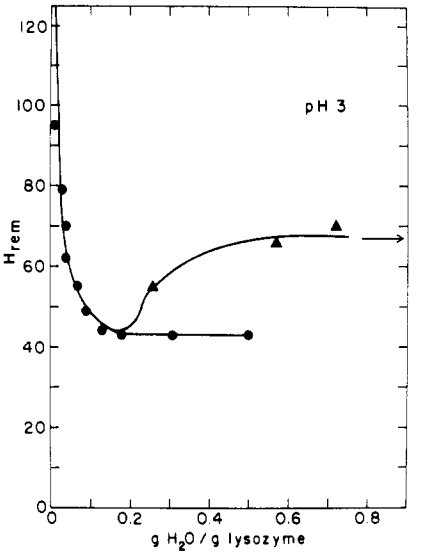


FIGURE 6: Hydration dependence of powder hydrogen exchange at pH 3. Conditions as described for Figure 4.

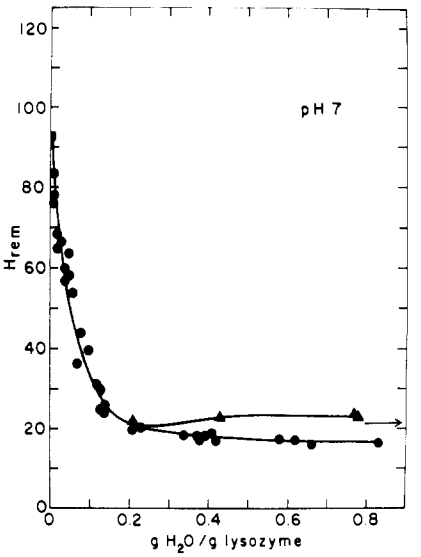


FIGURE 7: Hydration dependence of powder hydrogen exchange at pH 7. Conditions as described for Figure 4.

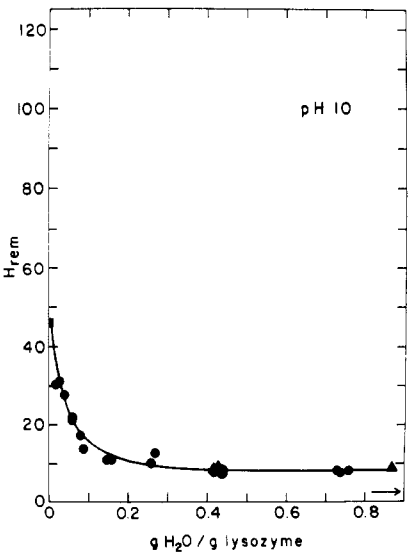


FIGURE 8: Hydration dependence of powder hydrogen exchange at pH 10. Conditions as described for Figure 4.

Table V: Hydrogen Exchange for Lysozyme Powders of pH 5[a]

h[b]	expt	vol % H_2SO_4[c]	vapor pressure[d]	rel sp act.[e]	H_{obsd}[f]	H_{rem}
0.02	19	50	2.30	0.079	84.8	80.8
0.02	19	50		0.069	85.7	82.2
0.03	27	50		0.111	93.1	88.3
0.03	27	50		0.083	91.3	87.6
(0.03)	20	40	6.05	0.023	63.3	61.7
0.03	20	40		0.025	63.3	61.6
0.07	19	35	8.60	0.008	58.8	58.2
0.07	19	35		0.011	57.4	56.5
0.07	20	30	11.33	0.006	49.9	49.3
0.07	20	30		0.007	48.9	48.3
0.13	19	20	17.18	0.001	38.2	38.1
0.14	19	20		0.002	38.8	38.6
0.15	29	15	19.57	0.001	34.2	34.1
0.16	29	15		0.000	34.0	34.0
0.17	37	15		0.005	31.5	30.9
0.17	37	15		0.002	30.8	30.7
0.21	36	10	21.35	0.000	34.4	34.4
0.22	19	10		0.002	33.4	33.2
0.23	19	10		0.001	33.8	33.7
0.31	20	5	22.62	0.002	29.9	29.7
0.32	20	5		0.002	30.6	30.4
0.34	19	5		0.001	31.4	31.3
0.34	19	5		0.002	31.0	30.8
0.37, m[g]	20	5		0.002	36.7	36.4
0.42, m	20	5		0.001	36.0	36.0
0.48	19	0	23.756	0.000	28.5	28.4
0.49	19	0		0.001	29.0	28.9
0.53	20	0		0.001	27.4	27.2
0.54	36	0		0.001	30.7	30.6
0.56	20	0		0.002	27.4	27.2
0.76, m	36	0		0.001	44.2	44.1
1.03, m	36	0		0.001	43.7	43.6
1.55, m	36	0		0.002	43.3	43.2
2.00, m	36	0		0.003	44.3	44.1
2.96, m	36	0		0.003	43.3	43.0
4.02, m	36	0		0.005	42.8	42.4
10.08, m	36	0		0.006	42.8	42.3
25.18, m	36	0		0.004	41.9	41.5

[a] All samples were isopiestically equilibrated at 25 °C for 24 h. Vapor pressure was controlled by H_2SO_4 solutions. [b] Hydration level expressed as grams of H_2O per gram of lysozyme. [c] H_2SO_4 solutions were used within 15 min of preparation. Samples at 0% H_2SO_4 were equilibrated with pH 5 titrated deionized water. [d] Vapor pressures were obtained, for each volume percent H_2SO_4, from data in the *International Critical Tables* (1928) and are expressed in millimeters of mercury. [e] Relative specific activity is the specific activity of tritium in the hydration water divided by the specific activity of tritium in the starting protein. [f] H_{obsd} values are the experimental data, which must be corrected to yield H_{rem}. See text for details. [g] Mixed hydration sample: pH 5 titrated deionized water, to give the final hydration value, was added directly to the protein powder and mixed. The bottle was closed, and the contents were equilibrated isopiestically like a regular sample for 24 h.

of H_{rem} for pH 2, 3, 7, and 10, respectively.

The data for pH 2 (Figure 5) apparently reflect processes in addition to hydrogen exchange of partially hydrated native protein. Agreement between experiments was not as good as for other pHs. The sharp drop in H_{rem} above 0.3 h suggests there is a hydration-dependent change in the protein. Partially hydrated samples of near 0.3 h did not dissolve as readily as less or more highly hydrated samples. Solutions of redissolved protein were found to have a pH below 2, probably because lysozyme lyophilized from solution with 0.010 M HCl contains residual HCl. Calculation based on the pH of 20 mg/mL solutions placed the HCl concentration of 0.3 h powders at 1.6 M. Denaturation is expected at this acid concentration. The low value of H_{rem} at high levels of hydration (above 0.4 h) is consistent with exchange from denatured protein. Because protein unfolding is inhibited at low levels of hydration, a transition between folded and unfolded states is expected

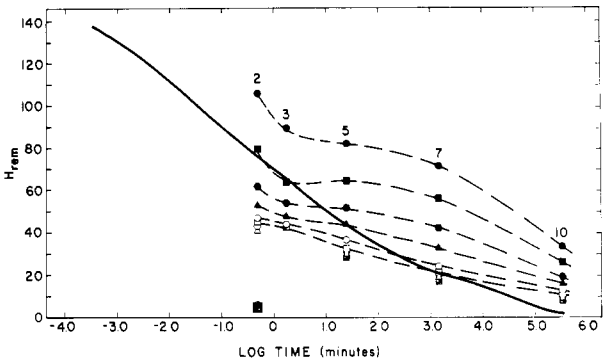


FIGURE 9: Comparison of solution and powder hydrogen exchange. The solid line is the composite solution profile for pH 7 constructed from data in Table I as described under Results. The points are powder 24-h H_{rem} values for pH 2–10, picked from the curves of Figures 4–8 for hydration levels of 0.04 (●), 0.06 (■), 0.08 (◉), 0.10 (▲), 0.15 (○), 0.20 (□), 0.25 (◎) 0.30 (△), 0.35 (◑), and 0.40 (box, left solid) g of H_2O/g of protein. The powder values for each pH were placed on the log time axis at the location of the 24-h solution value for the corresponding pH. The dashed lines are hydration contours.

to occur near full hydration (h = 0.38). The sharp drop in H_{rem} (Figure 5) can be explained as a shift from exchange of native to exchange of denatured lysozyme.

Data for pH 3 and 7 (Figures 6 and 7) were similar to the data for pH 5 (Figure 4). H_{rem} exhibited the same strong drop out to 0.15 h, after which the isopiestic and mixed sample curves divided. Again, the mixed hydration samples gave solution values of H_{rem} for h above 0.4. The differences between isopiestically equilibrated and mixed samples of the same hydration level decreased with increasing pH.

Exchange data for pH 10 (Figure 8) showed the same strong decrease in H_{rem} at low hydration, with H_{rem} leveling off at 0.15 h. However, isopiestically equilibrated and mixed samples gave the same H_{rem} values, and for both types of sample, the solution rate was never reached. Isopiestically equilibrated hydration samples between 0.2 and 0.8 h were more than 50% insoluble. This was not a problem with the mixed hydration samples in the same range. A possible explanation of this behavior is intermolecular disulfide exchange. In any case, the agreement among data for separate experiments suggests that hydrogen exchange of the soluble part of the protein powder was unaffected by whatever produced partial insolubility.

To show the pH dependence of exchange in partially hydrated powders and its relationship to solution hydrogen exchange, values of H_{rem} taken from curves for isopiestically equilibrated powders (Figures 4–8) are plotted in Figure 9 for selected hydration levels. The dashed lines connect H_{rem} values for powders of the same hydration level but of different pH. The difference between the high hydration limit for exchange of isopiestically equilibrated powders and the solution is clearly seen.

The principal conclusions to be drawn from comparison of the data of Figures 4–8 are the following:

(1) At all pHs, the hydrogen exchange rate as reflected by the 24-h H_{rem} value for isopiestically hydrated powder levels off at 0.15 h. We understand this behavior to mean that the effect of increasing hydration on hydrogen exchange is complete at 0.15 h.

(2) The H_{rem} value at high hydration for an isopiestically hydrated powder differs from the solution value. The difference is pH dependent, being largest at low pH and of the opposite sense at pH 10. Effectively instantaneous hydration to a high level, through the addition of water at the start of an experiment, results in an H_{rem} value at high hydration equal

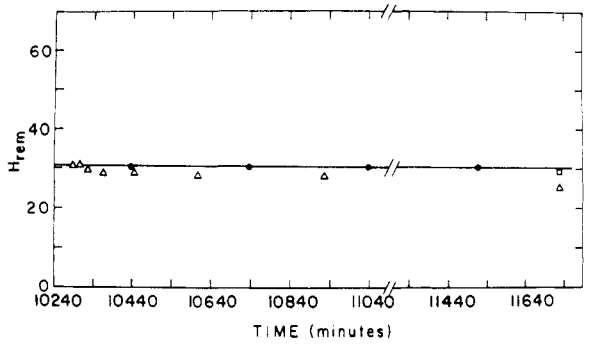


FIGURE 10: pH 5 powder to solution jump. Data for a powder sample jumped to pH 5.01 buffered solution (△) are compared with solution control data adjusted to pH 5.01 (●). pH 5 fully labeled, lyophilized lysozyme was isopiestically equilibrated at 25 °C for 24 h and then jumped to solution by dissolving to a concentration of 20 mg/mL in 0.100 M acetate buffer solution, pH 5.01. Equilibration continued at 25 °C for another 24 h, with 100-μL aliquots taken for analysis by gel filtration at times for 1.0 min to 24 h. For this comparison, the time-zero H_{rem} value of the jumped sample was located at the corresponding H_{rem} point of the control curve. The correction for the ionic strength contribution is indicated for the 24-h point (□).

to the solution value at pH 3, 5, or 7. Thus, the difference in exchange between isopiestically equilibrated powders of high hydration level and the solution reflects events occurring at low hydration levels during powder equilibration. This powder – solution difference appears to be an artifact of the hydration procedure.

Poole & Finney (1983) have reported exchange measurements for partially deuterated lysozyme powder samples as a function of hydration level, for unspecified conditions of pH and ionic strength. These results show a break near 0.1 *h* in the hydration dependence of H_{rem}, as observed in the measurements described above.

Powder to Solution Jump. A jump experiment was carried out to establish the relationship between the rank order of exchange in solution and in the partially hydrated powder. A buffered jump at pH 5 was done with a hydration sample of 0.17 *h* having a 24-h H_{rem} value less than the solution H_{rem} value. These conditions were chosen in order to obtain maximum divergence between the isopiestically equilibrated powder and solution. The results of this jump, given in Figure 10, show a small difference between H_{rem} values of control (solution) and experiment (powder to solution jump). However, when the contribution to exchange from the ionic strength of the solution after the jump is taken into account, the difference disappears, as shown by the open square value at long time in Figure 10. Thus, the rank order of exchange is closely similar in the powder and in solution, even under conditions where there is substantially greater 24-h exchange in the powder than in solution.

For a powder to solution jump made in the absence of strong buffer, the exchange differed from solution exchange. Apparently, in a weakly buffered solution, the dissolving powder can control the local pH and ionic strength for a period of time long enough to affect the exchange. These results bear upon the observation of Baker et al. (1983), who found small differences in exchange behavior between lysozyme in solution and freshly dissolved powder samples.

Comparison of Powder and Solution Rates. The unexchanged hydrogens can be expressed as

$$H_{rem} = \sum_i e^{-k_{i,s}t_s} \quad (4)$$

for a solution experiment and as

$$H_{rem} = \sum_i e^{-k_{i,h}t_h} \quad (5)$$

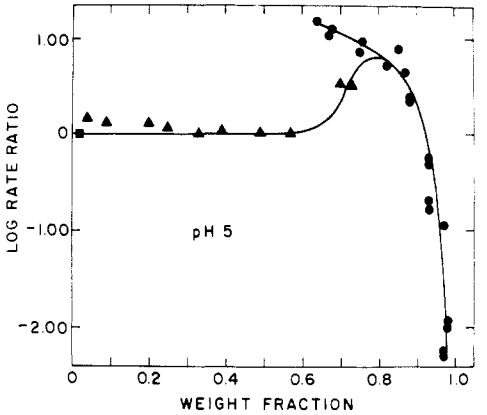


FIGURE 11: Dependence of log rate ratio of powder to solution exchange on weight fraction of protein at pH 5. The data show the log rate ratio dependence over the full range of system composition. Powder samples were hydrated by isopiestic equilibration (●) or by addition and mixing of solvent (▲). Protein solutions were of concentration 20 mg/mL, equivalent to 0.02 weight fraction (■).

for a partially hydrated powder experiment, where $k_{i,h}$ and $k_{i,s}$ are the rate constants for exchange of the *i*th labile proton in the powder and solution states, respectively, and t_h and t_s are the exchange times for the powder and solution experiments, respectively. At equal H_{rem}, the ratio of exchange times, t_s/t_h, is equal to the ratio of the average exchange rates, k_h/k_s, for the block of amide protons exchanging with characteristic times near t_s and t_h. The powder to solution jump experiment indicates that the rank order of exchange is the same for solution and powder states, and therefore at equal H_{rem}, the same protons are exchanging in solution and powder. Thus, the rate ratio k_h/k_s describes the properties of a particular small set of amide hydrogens, about 15, exchanging within a particular window of the 8 order of magnitude range of exchange rates. Rate ratios were calculated from plots of the data similar to Figure 9 as the ratio of the times required for solution and powder samples, respectively, to reach the same H_{rem} value at a given pH.

Log rate ratios as a function of hydration level for pH 5 are given in Figure 11. These results are typical of those for other pHs. The weight fraction display (Figure 11) shows that the hydrogen exchange rate can be measured continuously over the full range of system composition, from dry powder through solution. It also illustrates the close agreement between mixed powder and solution hydrogen exchange rates (closed triangles, 0–0.6 weight fraction of protein). The region of Figure 11 between 0.6 and 1.0 weight fraction of protein contains the data shown in Figure 4.

Dependence of Hydrogen Exchange Rate on Water Activity. The simplest dependence of the hydrogen exchange rate ratio (powder to solution) on water activity is

$$k_{r,obsd} = k_{r,0}(a_w)^n \quad (6)$$

where $k_{r,obsd}$ is the rate ratio, $k_{r,0}$ is the rate ratio for full hydration, a_w is the activity of water (equal to P/P_0), and n is the order of the exchange process in water. This expression implies that all exchanging protons are equivalent in their dependence on water activity.

Data for pH 2–10 were plotted (Figure 12) as log rate ratio vs. log (P/P_0). The lines of Figure 12 are for linear least-squares fits for each pH, with parameter values given in Table VI. Values of the least-squares slopes determined from the entire data set for each pH range from 2.5 to 3.1, with an average value of 2.9 ± 0.3 for the order of the water dependence. The best-fit values for $k_{r,0}$ are markedly pH dependent, reflecting the pH dependence of the difference between the

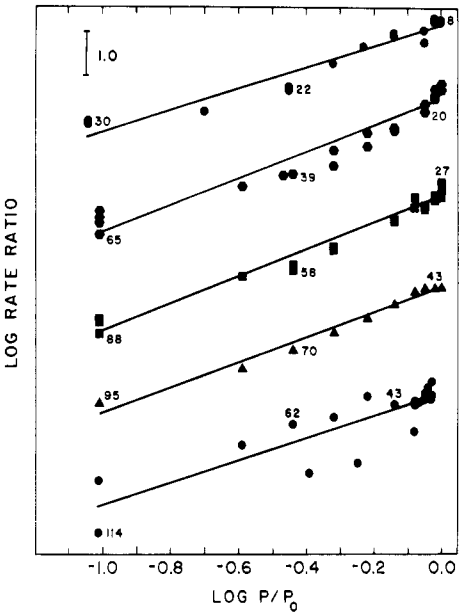


FIGURE 12: Dependence of log ratio of powder to solution exchange rate on log water activity. Log rate ratio data for pH 2 (bottom) to pH 10 (top) are given as a function of log (P/P_0). The slopes of the lines give the order of the protein exchange reaction with respect to water. The slopes from least-squares linear regression are the following: 2.57, pH 2; 2.90, pH 3; 3.14, pH 5; 3.14, pH 7; 2.53, pH 10. Displacement along the log rate ratio axis is arbitrary. Numbers indicate some of the H_{rem} values for which rate ratios were determined.

Table VI: Dependence on Water Activity of the Ratio of Hydrogen Exchange Rate in Powder to Solution at 25 °C[a]

pH	N	log $k_{r,0}$	n	rms error
2	20	1.47 ± 0.13	2.57 ± 0.35	0.46
	12	1.62 ± 0.16	4.6 ± 2.4	0.25
3	10	1.24 ± 0.06	2.90 ± 0.16	0.15
	5	1.31 ± 0.05	2.74 ± 0.69	0.08
5	28	0.95 ± 0.04	3.14 ± 0.09	0.17
	18	1.05 ± 0.04	4.53 ± 0.62	0.12
7	27	0.35 ± 0.07	3.14 ± 0.16	0.30
	15	0.59 ± 0.03	6.99 ± 0.54	0.09
10	18	−0.91 ± 0.08	2.53 ± 0.18	0.25
	10	−0.83 ± 0.06	2.53 ± 0.91	0.15
			2.9 ± 0.3[b]	
			4.3 ± 1.8[b]	

[a] For a single exponential fit of the rate ratios with the following equation: log $k_{r,obsd}$ = log $k_{r,0}$ + n log a_w. The first row of values for each pH is for the fit of all data given in Figure 12 for that pH. The second row is for the fit of data for log $a_w > -0.2$. N is the number of data points included in the fit. rms error is the root mean square error, calculated for the appropriate number of degrees of freedom. [b] Average values.

H_{rem} value at high powder hydration reached isopiestically and the H_{rem} value for solution measurements (Figure 9).

Also given in Table VI are parameter values for the fit of data with log (P/P_0) greater than −0.2, i.e., for the high hydration portion of the powder exchange data, with h greater than 0.1. The order in water averaged over all pHs for this subset of the data is 4.3 ± 1.8, with values for fixed pH ranging from 2.5 to 7. The considerable deviation among the individual values calculated from the high water activity data is not systematic and is in contrast to the consistency of the order (2.5–3.1) calculated from the full set of data for each pH. Scatter in the data for high hydration is expected, owing to the difficulty of establishing hydration equilibrium at P/P_0 greater than 0.9. Except for pH 5 and 7, the standard deviation of the estimate of the order from the high water activity data is large enough to include the more tightly defined estimate of the order obtained from the full set of data. Use of a two-exponential model substantiates the conclusion that the pH 5 and 7 data are the only sets for which the order is significantly greater than 3 at high hydration.

To summarize, the average order in water is 2.9 for the full range of water activity and 4.3 for high water activity. There is no pattern for deviations from the averages, comparing different pHs and thus different windows into the distribution of exchange rates. One can conclude that the order in water is less than or equal to 4.3 for all protons of lysozyme in the range of H_{rem} 114–8, the range covered by the data of Figure 12.

Discussion

Dependence of Exchange in the Powder on Level of Hydration. The principal observation made in this study is that at a hydration level of about 0.15 h the 24-h extent of amide hydrogen exchange of isopiestically equilibrated samples reaches a value that persists to the highest hydration that can be obtained isopiestically (0.6–0.8 h). This behavior holds for pH levels from 3 to 10. At pH 2, an inflection point in the exchange is reached near 0.15 h; at higher hydration, the 24-h value of H_{rem} falls sharply, reflecting denaturation or some other process not correlated with the dynamics of the native protein. Thus, the pH 2 data at low hydration also appear to be consistent with the data for other pHs.

Full hydration seen in thermodynamic and various other dynamic measurements is at 0.4 h. At 0.15 h, the surface of the protein is less than half covered with water.

The conclusion from the hydrogen exchange measurements is that the internal protein motions monitored by them are independent of hydration above the point where one-third to one-half of the surface is covered by water. This observation is remarkable, in light of the picture of the dynamics of the partially hydrated protein that has been obtained from other measurements.

Studies of several properties indicate that motion at the surface of the partially hydrated lysozyme molecule, both for solvent molecules and for protein groups, is restricted below 0.25–0.3 h. The dielectric constant of lysozyme powders increases with increased water content, rising gradually up to 0.25 h and steeply thereafter (Harvey & Hoekstra, 1972). Nuclear magnetic resonance cross-relaxation experiments with lysozyme powders indicate a rapid increase in relaxation rates above 0.25 h (Borah & Bryant, 1982). Electron spin resonance studies of lysozyme powders containing the nitroxide spin probe Tempone noncovalently bound at the protein surface show the rotational relaxation time remains unchanged through 0.2 h (Rupley et al., 1980). Enzymatic activity of lysozyme powders is undetectable below 0.2 h (Rupley et al., 1980) and above 0.25 h increases in parallel with the change in Tempone relaxation rate. Scanning calorimetry of partially hydrated lysozyme demonstrates that the unfolding process is inhibited at low hydration (Fujita & Noda, 1978).

Spectroscopic and thermodynamic measurements suggest an explanation for the increase in surface motion at high hydrations. Infrared spectroscopy of lysozyme powders and films shows that there is saturation with water of the carbonyl and carboxylate sites between 0.2 and 0.25 h (Careri et al., 1980). Completion of the hydration shell by covering the remaining regions of the surface that interact more weakly with water is a cooperative process, as seen in heat capacity measurements (Yang & Rupley, 1979). It appears that surface motion develops in conjunction with this cooperative

establishment of weak surface–water interactions.

We would expect that, to the extent there is linkage between the surface motions and the internal motions controlling access of water to the exchangeable hydrogens of lysozyme, the surface and internal events should be affected in parallel by hydration. Instead, the great difference between the hydration dependence of hydrogen exchange and of properties that monitor the surface indicates that the internal motions that determine hydrogen exchange rate are not strongly coupled to the surface. One can conclude that the interactions of protein with solvent affect the protein surface and the protein interior differently. These differences have significance for understanding the physical and functional properties of proteins in solution. In particular, one can conclude that the enzymatic activity of lysozyme depends more strongly on surface properties than on internal motions of the enzyme. The rate of ligand binding by myoglobin has been shown to depend on solution viscosity (Beece et al., 1980), and these results are interpreted in terms of a dynamic model in which ligand motion into and inside myoglobin is controlled by conformational fluctuations. By viewing these observations in light of the hydrogen exchange results on lysozyme, one can suggest that the preequilibrium steps of the myoglobin reactions involve portions of the protein near or at the protein surface.

Difference between Exchange of Lysozyme in Solution and High Hydration Powders. For measurements at pH 2–7, the 24-h H_{rem} values for powder samples of high hydration (0.6–0.8) obtained through isopiestic equilibration were lower than the values reached in solution: by 35 H at pH 2, 24 H at pH 3, 15 H at pH 5, and 5 H at pH 7. The difference decreases strongly with increased pH and is of the opposite sign, although small (6 H), at pH 10. In contrast, exchange of high hydration samples prepared by mixing at pH 3, 5, or 7 gave the solution value of H_{rem} if the hydration level was above 0.3–0.4 h.

One can conclude that the difference between isopiestically equilibrated samples of high hydration and the solution reflects (1) events that are limited to the low hydration region (below 0.15 h), because mixed samples of high hydration (above 0.3–0.4 h) behave like the solution and exchange is constant for isopiestically equilibrated powders of hydration above 0.15 h, and (2) events that are pH or charge dependent, in view of the strong decrease in the difference with increase in pH.

Three factors can contribute to a high rate of exchange at hydration below 0.15 h: (1) a loose or unfolded conformation at low hydration; (2) a high effective ionic strength; and (3) a high effective pH. These factors are considered separately below.

Various observations argue against significant conformational differences between powder samples and the solution state. Considerable evidence, summarized in Yang & Rupley (1979) and Careri et al. (1980), indicates that the time-average conformation is invariant to hydration above 0.07 h. Also, unfolding is inhibited at low hydration (Fujita & Noda, 1978), and some conformational fluctuations are also inhibited below 0.2 h (Bone & Pethig, 1982). With regard to hydrogen exchange measurements, a powder to solution jump experiment at pH 5 (Figure 10) showed that the rank order of exchange is conserved and thus the principal features of the protein conformation must be closely similar for solution and isopiestically equilibrated powder.

Because of the counterions and the salt from pH adjustments present in the protein solutions from which the powder samples were prepared, significant amounts of salt remain in the powder after lyophilization. For example, at pH 5, lysozyme has a net charge of +12, and at 0.2 h, it has 160 water molecules bound, giving an apparent ionic strength of 4.2 (assuming 1:1 electrolyte behavior for counterion and protein). A 20 mg/mL solution of this protein sample would have an apparent ionic strength of 0.016. Thus, ionic strength effects could be important for the exchange in the powder state. Solution measurements on lysozyme at pH 2 and 5 (see Results) show increased exchange at high ionic strength (above 0.5), consistent with what was found for powder samples relative to the solution.

At low hydration, the pH of a powder sample will depend strongly on hydration level and be higher than the pH of the solution from which the powder was obtained by lyophilization. Two factors contribute to this effect. First, Careri et al. (1980) have shown that about half of the carboxyl groups of nearly dry lysozyme powders and films have the pK shifted strongly upward, to above the pK of basic groups of the protein. In the hydration range 0.05–0.07 h, the pK order is normalized to the solution value. However, below hydration level 0.07 h, the effective pH of powder samples is determined by the amino ionizations, with a nominal pK of 9–10. Second, the high effective ionic strength at low hydration will produce, for a strongly positively charged protein like lysozyme, a pH higher than those found at higher hydration or in solution. Both the carboxylate pK anomaly and the ionic strength effect on pK should decrease as the pH is increased. Comparison of Figure 9 and the 24-h solution H_{rem} values of Table I shows that the transposition along the log time axis needed to bring the 0.2 h values of H_{rem} for the powders into agreement with the solution H_{rem} values is equivalent to a shift in pH to 5 for the pH 2 and 3 powders, to pH 6 for the pH 5 powder, to pH 9 for the pH 10 powder, and no shift for the pH 7 powder. Even the largest of these shifts is plausible for protein powders of low hydration.

The Appendix describes a model for the dependence of powder hydrogen exchange on water activity and hydration level. The basis of the model is that exchange occurring at low hydration can contribute to determination of H_{rem} for isopiestically equilibrated samples of high hydration because the rate constants for exchange are a function of hydration level and the final hydration level is reached relatively slowly. The principal conclusion from the treatment given in the Appendix is that the model can account for the following observations: (1) mixed samples, for which the hydration level is set instantaneously, having a hydrogen exchange rate equal to the solution rate above 0.2 h; (2) isopiestically equilibrated samples at acid pH having greater exchange than the solution at hydration levels of 0.1 and above; (3) isopiestically equilibrated samples having constant exchange above about 0.15 h.

The low hydration effects on exchange are probably complex, through reflecting more than one of the above factors. For example, the smaller extent of exchange for lysozyme powder compared with the solution at pH 10 can be explained by the weakening at high pH of the effects special to the protein powder, so that the effect of ionic strength found for the exchange of small amides and polypeptides can dominate.

To summarize, a combination of pH and ionic strength effects can account for anomalously high exchange at low hydration, as well as the pH dependence of this anomaly. A contribution from conformation differences to exchange differences between powder and solution is possible but is viewed as unlikely. The exchange of isopiestically equilibrated powders, although complicated by the low hydration effects, can be interpreted in a straightforward manner for the hydration

Table VII: Contribution from the Time Dependence of the Hydrogen Exchange Rate Constants to the Integrated Extent of Exchange: Evaluation of F^*, $F^*(\tau = 0)$, and n^*[a]

$n(f)$	a_w	app ionic strength	τ/t	$[a/h(f)]^3$	$\{h(f)/[h(f) - \Delta h]\}^2$	F^*	F^*_0	n^*
0.04	0.096	126	0.025	166	1	175	167	−1.5
0.07	0.27	72	0.025	31.0	6.25	37	32	−1.5
0.12	0.56	42	0.05	6.2	25	13	7.2	−1.5
0.22	0.87	23	0.1	1.0	100	8	2.0	~0
0.42	>0.95	12	0.2	0.14	400	7.3	1.14	~0
0.82	>0.95	6	0.4	0.019	1600	7.5	1.02	~0

[a] The factor F^* is the time-dependent contribution to exchange in isopiestically equilibrated powders, and n^* is the corresponding contribution to the experimentally determined order of the exchange in water. Parameter values are set as follows for the model of eq A5 and A6a,b: $a = 0.22$; $h(f) - \Delta h = 0.02$; τ/t set according to experiment for $t = 24$ h. The dependence of $h(f)$, the final hydration level, on a_w, the starting hydration level, is from the sorption isotherm data of Hnojewyj & Reyerson (1961). Values of F^* and F^*_0 were calculated according to eq A6a,b. Values of n^* were determined graphically. The apparent ionic strength is calculated for protein powders of pH 5.

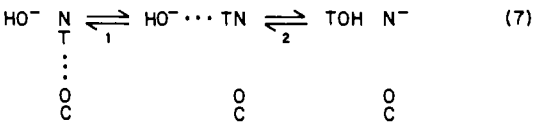
region above 0.15 h. This last point is important. It allows the conclusion that the leveling off of exchange in the powder defines a hydration level, 0.15 h, beyond which hydration has no substantial further effect on lysozyme internal motions as measured by hydrogen exchange.

Order of the Exchange Rate in Water. The dependence on water activity of exchange of isopiestically equilibrated powders over the pH range 2–10 is described in Figure 12 and Table VI. The order in water for all data of P/P_0 greater than 0.1 is 2.9 ± 0.3, averaged over all pHs. The average order in water for data of P/P_0 greater than 0.6, i.e., with a hydration level greater than 0.1, is 4.3 ± 1.8.

The model described in the Appendix for hydrogen exchange in isopiestically equilibrated powders predicts that at hydration levels below about 0.1 h there will be a contribution from environmental factors, such as ionic strength, pH, and conformation effects, to the order in water. The observed order in water is the sum of the true order in water and the order in water for the environmental factors. For hydration levels below about 0.1 h, the latter term will be negative, and the true order in water for the exchange process will be greater than the measured order. The size of the contribution from environmental factors depends on the shape of the sorption isotherm and on the dependence of the environmental factors on hydration level. For the model system described in the Appendix (Table VII), the environmental factor contribution to the order is 1.5 at hydration levels below 0.1 h and is zero at higher hydration levels. This is consistent with the results presented in Figure 12 and Table VI, for which there is a difference of 1.4 between the average orders calculated for data of hydration level above 0.1 and for the full set of data.

The best estimate of the order in water for hydrogen exchange in protein powders is the average value for the hydration range above 0.1 h, 4.3 ± 1.8. The foregoing discussion justifies this use of the high water activity data in determining the true order of the exchange with respect to water. The data given in Figure 12 and Table VI cover the range of pH 2–10. There is no systematic pH dependence of the order, regardless of whether it was calculated from the high water activity subset of the data or from the full set of data for the full range of water activity. This observation is significant, because it shows that the average order of 4.3 applies to all amide hydrogens exchanging in the H_{rem} range 114–8. Furthermore, the low hydration events that contribute to the discrepancy in H_{rem}, comparing 24-h values for exchange in isopiestically equilibrated powders of high hydration and in the solution, have the same order in water for all pHs, even though the magnitude of the effect varies (large at low pH, small at pH 7 and 10).

Mechanism of Amide Hydrogen Exchange in Lysozyme. Specific base-catalyzed hydrogen exchange of small amide compounds proceeds by a pathway (Perrin et al., 1981) that includes the following steps:



(7)

Step 1 is the formation of a hydrogen-bonded complex between catalyst and amide, and step 2 is an unfavorable proton transfer with rotation within the hydrogen-bonded complex and transient severing of the hydrogen bond. This is the rate-determining step. The fraction of successful transfers is determined by the preequilibrium proton transfer within the hydrogen-bonded complex. The mechanism for specific acid-catalyzed exchange is similar (Perrin & Johnston, 1981) in that it involves the same rate-limiting step.

The mechanism of hydrogen exchange for proteins [for review discussions, see Englander et al. (1972), Wuthrich & Wagner (1978), Woodward & Hilton (1979), and Richards (1979)] probably exhibits the same rate-determining step as for small amides. The difference between proteins and small amides is expected to be principally in the preequilibrium step 1, the formation of the hydrogen-bonded complex with a specific acid or base catalyst, although there might also be an effect of the protein on step 2. With regard to step 1, there must be approximation of the catalyst species to the amide group, followed or preceded by a rearrangement of the conformation that frees the amide for hydrogen bond formation with the catalyst. The distinction between the several views of hydrogen exchange (local unfolding, domain rocking, and penetration) is in the sequence of and structural models for the approximation and rearrangement events.

The rate of hydrogen exchange of an amide group in a protein, k_{obsd}, can be expressed as a function of the rate, k_{pep}, for a chemically similar group in a small amide compound or an unfolded polypeptide chain:

$$k_{obsd} = k_{pep}[HO^-]f_1f_2 \quad (8)$$

where f_1 is the effect of protein dynamic structure on step 1, the formation of the hydrogen-bonded complex, and f_2 is the effect of protein folding on the chemical steps of the exchange process of eq 7. The order of the protein exchange process in water is obtained by differentiating log k_{obsd} for the protein amide with respect to log water activity:

$$\frac{d \log k_{obsd}}{d \log a_w} = \frac{d \log k_{pep}}{d \log a_w} + \frac{d \log [HO^-]}{d \log a_w} + \sum \frac{d \log f_i}{d \log a_w} \quad (9)$$

The first term on the right of eq 9 is the order in water of the small amide exchange rate, likely to be small and certainly

not negative. The second term reflects the dependence of solvent species concentration on water activity and is certain to be near unity. The third term, the one of interest for protein hydrogen exchange, gives the dependence on water of all the protein effects on the steps of the exchange process, with the principal contribution presumably reflecting step 1.

When the unit dependence of catalyst concentration on water activity is taken into account, the above analysis indicates that the effect of water activity on step 1 is of order one less than the order 4.3 obtained from the data of Figure 12. Thus, about three water molecules enter the transition state owing to the rearrangement and diffusion processes of step 1. This is a remarkably low number. It corresponds to 60 Å^2 of surface. It is 1% of the water of hydration of lysozyme. In comparison, thermal unfolding of lysozyme in aqueous solution increases by 100% the water of hydration, and unfolding to the random coil in a good solvent would require a 300% increase in water of hydration. Thus, there cannot be any substantial change in protein surface associated with exchange of the major portion of the amide hydrogens of lysozyme (the data of Figure 12 cover H_{rem} values from 114 to 8). It is also remarkable that the order in water is constant for pH 2–10. Apparently the effect of hydration on the approximation and rearrangement events for protein amide hydrogen exchange is substantially the same for both acid- and base-catalyzed exchange. More surprisingly, the effect of hydration is the same for rapidly and slowly exchanging hydrogens that may differ in distance from the protein surface and that exist presumably in different local conformational environments and require different local conformational rearrangements for exchange.

Among the several pictures of the mechanism of hydrogen exchange that are considered of possible importance for proteins, the results of this study support a penetration model, in that other pictures imply larger changes in protein surface exposed to solvent. The invariance of order in water to rank order of exchange is most simply explained by a penetration model, in which the environment for step 2 of eq 7 would be similar for all amide protons, regardless of how far from the surface they might be located or what the structural details of their environment might be. For proteins with a markedly different pattern of folding from lysozyme, the internal dynamics, and thus the hydrogen exchange behavior, may differ from those of lysozyme.

The low order in water of hydrogen exchange for partially hydrated protein powders is in contrast to the high order in water found for other protein dynamic properties. The correlation time for an electron spin resonance (ESR) probe at the protein surface and the rate constant for enzymatic activity of lysozyme change with hydration closely in parallel and show 15th order dependence on water activity (Rupley et al., 1983). This high order in water for the surface motions is expected, because the motions develop in parallel with a cooperative process, the completion of the hydration shell through condensation of water on the weakest surface sites. Apparently, the important events for hydrogen exchange of proteins do not involve the surface and are not highly cooperative in water.

Relation of Hydrogen Exchange in Powders to Exchange in Solution. The conclusions reached above, concerning the lack of coupling between internal and surface motions and the order in water for exchange in isopiestically equilibrated lysozyme powders, can be expected to hold also for lysozyme in solution, for the following reasons:

(1) The time-average conformation of lysozyme in the partially hydrated powder is closely similar to and possibly identical with the conformation in solution, as shown by measurements sensitive to conformation, for example, the following: the CD spectrum of the dry film is similar to the CD spectrum in solution (Chirgadze & Ovsepyan, 1972); Tempone spin–spin interaction is invariant to hydration (Rupley et al., 1980); enzymatic activity is observed at 0.2 *h* (Rupley et al., 1980); the thermal stability of lysozyme increases sharply with decrease in hydration below 0.3 *h* (Fujita & Noda, 1978). Thus, the high hydration conformation of lysozyme is frozen in as the hydration level is reduced. Although the dynamic and thermodynamic properties of the lysozyme–water system change with hydration, these changes appear to reflect interaction of the surface with solvent and not to involve measurable rearrangement of backbone or internal residues (Careri et al., 1980; Rupley et al., 1983).

(2) Isopiestically equilibrated powders show no change in exchange properties over the region 0.2–0.6 *h*, within which full hydration is obtained at 0.4 *h*, as measured by other properties (heat capacity, Tempone motion, sorption isotherm, etc.). The discrepancy between 24-h exchange values for isopiestically equilibrated powders of hydration above 0.15 *h* and the solution is of interest in itself, but it is not important for the conclusions reached here regarding the separation of internal and surface motions and the order of the exchange with respect to water. As discussed above, the discrepancy reflects low hydration events.

To summarize, the low order in water measured for the powder is expected to hold for the solution, and the break in the exchange properties at 0.15 *h* clearly separates the internal motions important for hydrogen exchange from the surface motions that come into play above 0.25 *h*.

Conclusions

Hydrogen exchange measurements with lysozyme powders have proven a sensitive technique for examining the effect of hydration on protein internal motion. The principal conclusion from these studies is that the dynamics of the protein interior, as measured by hydrogen exchange, are the same in both partially hydrated powder (above 0.15 *h*) and fully hydrated lysozyme. It is important that surface motions, as measured by ESR probe motion and enzymatic activity, are relatively frozen at 0.15 *h* and become free only upon the condensation event that leads to full hydration. Apparently the surface and internal motions of the protein are uncoupled. On this basis, one expects that simulations of protein internal dynamics carried out for the vacuum should not give results greatly different from those carried out with solvent present, and this seems to be true (van Gunsteren & Karplus, 1982).

The dependence of exchange rate on water activity is of low order, about 4, and independent of pH. Both slow- and fast-exchanging amide hydrogens show the same dependence of exchange rate on hydration; thus, the transition states for the relatively shielded and for the relatively accessible amide hydrogens are similar in involving few water molecules. These observations support, but of course do not prove, a solvent penetration model of protein hydrogen exchange.

It is not possible to present a detailed molecular model that explains hydrogen exchange being about fourth order in water. The determination of reaction order is a thermodynamic treatment of the transition state. For a system as large and complex as a protein, linked effects may be important, and it may not be that three molecules of water in addition to one molecule of hydroxide ion are near neighbors in the transition-state structure. The important point that bears on the mechanism of hydrogen exchange in solution is that so few waters are formally involved and that the number of waters

is independent of the extent to which an amide is hindered from exchange by the protein. These observations indicate that exchange requires no large conformational rearrangement.

APPENDIX

Model for the Dependence of Hydrogen Exchange in the Powder on Water Activity and Hydration Level: Analysis of Hydrogen Exchange with Time-Dependent Rate Constants. Because of the significant time required to establish hydration equilibrium and hydration dependence of the exchange rate, the rate constant for exchange in isopiestically equilibrated powders is time dependent. For a single amide hydrogen exchanging with the time-dependent rate constant $k_{i,t}$

$$dH^i_{rem}/dt = k_{i,t}H^i_{rem} \quad (A1)$$

Integrating and summing over all exchanged hydrogens yields

$$H_{rem} = \sum_i \exp\left(-\int_0^t k_{i,t}\, dt\right) \quad (A2)$$

Assuming that the effect of hydration is the same for all exchanging hydrogens, the ith rate constant can be expressed as

$$k_{i,t} = k_{i,0}(a_w)^n g[t, h(f), \tau] \quad (A3)$$

and the integral of the exponential of eq A2 as

$$I = \int_0^t k_{i,t}\, dt \quad (A4a)$$

$$I = k_{i,0}(a_w)^n \int_0^t g[t, h(f), \tau]\, dt \quad (A4b)$$

The constant $k_{i,0}$ is the rate measured for unit water activity and for conditions where ionic strength and similar effects are absent (infinite hydration with instantaneous mixing); thus, $k_{i,0}$ is the intrinsic solution rate constant. The factor $(a_w)^n$ expresses the dependence of the rate on water activity in the absence of time dependence and in the absence of special environmental effects (pK, ionic strength, and conformation). The factor $g[t, h(f), \tau]$ expresses the time dependence of the exchange rate. The final hydration level, $h(f)$, is measurable and is determined by the water activity, which for the isopiestically equilibrated powder is equal to the partial pressure of water in the vapor phase. The characteristic time, τ, for approach to $h(f)$ depends on the rate of water transport and the on and off rates for water at the protein surface and is measurable. For protein powders of low final hydration, τ is about 0.5 h, and for powders of high hydration, τ is 2 h or more.

The function $g[t, h(f), \tau]$ must have the following properties: (1) approach unity for a high level of hydration and a long time of hydration; (2) contain a term that can be large for a low level of hydration, in order to include the combination of pK, ionic strength, and conformation effects important at low hydration and low pH; (3) include time dependence through a relaxation term, $\Delta h \exp(-t/\tau)$, where Δh is the change in hydration between the start and end of equilibration.

To examine the consequences of a time-dependent rate constant that is large at low hydration, one can for convenience assume a third-order dependence of a correction term on hydration level, obtaining

$$g[t, h(f), \tau] = 1 + a^3/[h(f) - \Delta h \exp(-t/\tau)]^3 \quad (A5)$$

where a is an adjustable parameter. Integration of eq A5, neglecting small terms, gives

$$F = \int_0^t g[t, h(f), \tau]\, dt = tF^* \quad (A6a)$$

$$F^* = 1 + [a/h(f)]^3 + [\tau/(2t)][a/h(f)]^3\{h(f)/[h(f) - \Delta h]\}^2 \quad (A6b)$$

Table VII gives values of F^* calculated with the following assumptions: values of τ/t, for t = 24 h exchange time, increase with increase in the final hydration level, $h(f)$, and are set consistent with experiment. The starting hydration level, $[h(f) - \Delta h]$, is set at 0.02, the value measured for protein powders maintained over phosphorus pentoxide. The parameter a is set arbitrarily at 0.22, giving unit ratio $a/h(f)$ at $h(f)$ = 0.22.

The principal conclusions to be drawn from Table VII are the following: (1) the value of F^* falls strongly at low hydration to a limit of about 10 for $h(f)$ above 0.1; (2) the value of F^*_0, for instantaneous mixing (τ = 0) to give the final value of $h(f)$, also falls strongly at low hydration but approaches unity at high hydration. A nonunit ratio, F^*/F^*_0, accounts for the difference in H_{rem}, comparing isopiestically hydrated powder samples to solution or powder samples of the same high hydration obtained by mixing. A value of the ratio near 10 corresponds to the transposition of about 1 unit in log time required to bring the pH 5 high hydration values of Figure 9 onto the solution curve.

Because the factor F^* depends on $h(f)$, which in turn depends on the water activity, the experimentally determined dependence on water activity of the exchange rate for isopiestically equilibrated powders will contain a contribution from the factor F^*. From eq A4a,b and A6a,b, one can write the observed rate constant, i.e., the time-integrated rate constant, for exchange of the ith hydrogen of the isopiestically equilibrated powder as

$$k_{i,obsd} = k_{i,0}(a_w)^n F^* \quad (A7)$$

The observed order in water is then

$$n_{obsd} = d \log k_{i,obsd}/d \log a_w \quad (A8)$$

$$n_{obsd} = n + n^*$$

$$n^* = d \log F^*/d \log a_w$$

The term n^* reflects contributions to the rate from hydration-dependent environmental factors (pK, ionic strength, and conformation-related factors). It is of opposite sign to n, the true order in water, i.e., the water dependence of the exchange rate for a fixed environment.

From eq A8, one can evaluate n^* as

$$n^* = (a_w/F^*)[dF^*/dh(f)][dh(f)/da_w] \quad (A9)$$

$$dF^*/dh(f) = \partial F^*/\partial h(f) + (\partial F^*/\partial \tau)[d\tau/dh(f)]$$

It is simpler to evaluate n^* graphically, as the slope of a plot of log F^* vs. log a_w, and such values are given in Table VII.

The following conclusions can be drawn from the values of n^* given in Table VII. The observed order in water for the rate of amide hydrogen exchange in isopiestically equilibrated powders has a small but significant contribution from the hydration dependence of the environment. The order for constant environment, n, is greater than the observed order, n_{obsd}, for hydration levels below about 0.1. The magnitude of n^* at low hydration depends on the shape of the sorption isotherm [d log $h(f)$/d log a_w] and the order of F^*_0 in $h(f)$ (determined by the form of $g[t, h(f), \tau]$; order −3 for the model described in Table VII). The change to a near-zero value of n^* for hydration levels above 0.1 accounts for the upward curvature of the data of Figure 12 at high hydration (increase in n_{obsd} at high hydration).

Registry No. H_2, 1333-74-0; lysozyme, 9001-63-2.

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Orientation of Ferrochelatase in Bovine Liver Mitochondria†

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ABSTRACT: The orientation of ferrochelatase (protoheme ferro-lyase, EC 4.99.1.1), the terminal enzyme of the heme biosynthetic pathway, was examined in bovine liver mitochondria. The ability of a membrane-impermeable sulfhydryl reagent, 4,4′-dimaleimidylstilbene-2,2′-disulfonic acid, to inactivate ferrochelatase in intact or disrupted mitochondria and mitoplasts was examined. Using succinate dehydrogenase as an internal marker, it was found that ferrochelatase was inactivated only in disrupted mitochondria and mitoplasts, suggesting an internal location for the active site of the enzyme. In addition, antibodies raised against purified ferrochelatase were found to inhibit activity only in disrupted but not in intact mitoplasts. These data demonstrate that in bovine liver mitochondria ferrochelatase is located on the matrix side of the inner mitochondrial membrane. Data obtained with the membrane-impermeable amino reagent isethionyl acetimidate indicate that ferrochelatase physically spans the inner mitochondrial membrane with portions of the protein exposed on both sides of the membrane.

In eukaryotic cells, the enzymes of the heme biosynthetic pathway are distributed between both mitochondrial and cytosolic compartments [see Granick Beale (1978)]. The initial and rate-limiting enzyme in the pathway, δ-aminolevulinate synthase, is located in the mitochondrial matrix. Aminolevulinate is then transported into the cytosol where it is converted first into porphobilinogen, then uroporphyrinogen III, and finally coproporphyrinogen III. The terminal three enzymes responsible for formation of protoheme IX are located in the mitochondria, either associated with or bound to the inner mitochondrial membrane. While the position of coproporphyrinogen oxidase is known to be the cytosolic side of the inner mitochondrial membrane (Elder & Evans, 1978),

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