

Determination of the Rate Constants k_1 and k_2 of the Linderstrøm-Lang Model for Protein Amide Hydrogen Exchange

A Study of the Individual Amides in Hen Egg-white Lysozyme

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The pH dependence of the amide/solvent hydrogen exchange of individual amide groups in hen egg-white lysozyme has been studied by nuclear magnetic resonance spectroscopy. Lysozyme has been used here as a model for a globular protein to re-examine the hypothesis for the amide/solvent hydrogen exchange reaction proposed by K. Linderstrøm-Lang and described in detail by Hvidt and Nielsen. The work has been focused on the most slowly exchanging amide at the temperature of 21°C and in the pH range between 4 and 8. Exchange rates have been measured for 64 of the 126 amide protons and the pH dependence has been determined for 52 of these.

The amides examined represent a sample that includes all the types of secondary structure and they are placed in the globular structure in a range of 3.2 Å to 8.5 Å from the closest water molecule on the surface. The measured exchange rates at pH 6 have been compared to these structural parameters and the results suggest that the rate constants are determined partly by the distance to the surface and partly by the type of secondary structure the amide is engaged in. Near the surface and in the very interior the distance to the surface seems to be rate-determining. Between the extremes the type of secondary structure is rate determining.

The pH dependent exchange of the examined amides was shown to be in agreement with the Linderstrøm-Lang model. For each of the amides examined the rate constants for the opening and the closing reaction in the first reaction step of the Linderstrøm-Lang model has been calculated and compared to structural parameters.

Keywords: pH dependence; hydrogen exchange models; electrostatics; protein; nuclear magnetic resonance

1. Introduction

The exchange of solvent and amide hydrogens in a protein occurs with a reaction rate that depends on the chemical environment and the structural and dynamic properties of the individual amide hydrogen. The kinetics of the individual exchange reactions can be examined directly by ^1H n.m.r.§ spectroscopy and this allows a mapping of the

hydrogen exchange rates at the individual sites in the protein. Amide hydrogens on the surface of protein molecules typically exchange fast with exchange times in the range of seconds, whereas amide hydrogens involved in the secondary structure of proteins exchange over periods of time ranging from fractions of a day to years. It is relevant to study the kinetics of these individual reactions because they may provide information about the dynamic processes in the protein responsible for the exchange reaction. The interpretation of the exchange rates for amides in the secondary structure, however, depends much on the mechanism of the molecular event preceding the exchange reaction. The question being, how does a water

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§ Abbreviations used: n.m.r. nuclear magnetic resonance; d_{hwm} distance to closest hypothetical water molecule.

Table 1
List of sampling dates for the hydrogen measurements for the individual pH series

pH	Series I			Series II			
	Days of exchange			Days of exchange			
4	0	2	4	10	32	62	92
5	—	2	4	10	22	43	62
6	1	1	2	4	15	29	43
7	—	1	2	4	15	29	43
8	—	1	2	4	15	29	43

by dissolving lysozyme at the concentration of 100 mg/ml in deionized water and adjusting pH to the wanted value. Subsequently the samples were lyophilized and redissolved in deuterium oxide where the pH was checked and if necessary readjusted to the wanted value.

Aliquots of 0.5 ml of the samples were placed in tubes and sealed to avoid contamination with atmospheric water. The exchange was performed at thermostated conditions at 21°C and measured over a period of 92 days for the pH 4.0 samples, 63 days for the pH 5.0 samples and for 43 days for the pH 6.0, 7.0 and 8.0 samples. Two series of experiments were performed at each pH at sampling days as given in Table 1. Each sampling was performed firstly by checking the pH and subsequently diluting the sample to a protein concentration of 50 mg/ml and adjusting pH to 3.8. The samples were kept at -20°C until recording of the n.m.r. spectrum. ¹H nuclear Overhauser enhancement spectra (Jeener *et al.*, 1979; Anil-Kumar *et al.*, 1980, 1981) were recorded with a mixing time of 150 ms at 308 K using gated decoupling to suppress the remaining water signal, applying the hyper-complex method (States *et al.*, 1982) recording 2048 complex t_2 and 512 t_1 values. Prior to Fourier transformation, the free induction decays were multiplied with a Gaussian window function in t_2 and a squared sine bell in t_1 followed by zero-filling both in the t_2 and the t_1 dimension to give spectra with 4096 and 2048 points, respectively, in the two dimensions.

The program MNMR (Pronto Software Development and Distribution, Copenhagen, Denmark) was used to process the n.m.r. data and the software package for analysis of n.m.r. data, PRONTO (Pronto Software Development and Distribution, Copenhagen, Denmark) was used for computerized cross-peak identification (Hoch *et al.*, 1987), integration, baseplane correction (Dietrich *et al.*, 1991), data management, and data registration in a database (Kjær *et al.*, 1991). Assignments of ¹H resonances were obtained from Redfield & Dobson (1988).

3. Results

The hydrogen exchange rates for each amide hydrogen measured in lysozyme are listed in Table 2. The accuracy of the rate constants obtained here was estimated using a Monte Carlo simulation and found to be less than 15% of the found values. The results represent a sample of exchange rates of three orders of magnitude ranging from 8.9×10^{-5} to 1.5×10^{-8} per second. The reaction rates have been measured at five pH values, however, due to the pH dependence, most of the amides were only measureable at three or four pH values. A unique pattern with respect to both the

value of the rate constant and the pH dependence exists for each of the individual amide hydrogens. Only a few amides show essentially no pH dependence for the exchange reaction and they are also characterized by very small rate constants. For the remaining majority of amides the pH dependence monitored as the increase in $\log_{10}k_0$ per pH unit varied from 0.1 to 1 (see Table 2), where those amides that have a pH dependence close to 1.0 were measured in the range of pH 4 to pH 6, and those with a pH dependence below 0.6 were measured in the range between pH 6 and pH 8 in agreement with the predicted pH dependence (see Fig. 1).

4. Discussion

(a) Correlation between exchange rate and structure

The amide exchange kinetics have been analysed for possible correlations to the structural properties of the individual sites of exchange. For example, the secondary structure and the distance from the amide to the closest water molecules on the surface of the protein molecule. In Figure 2, the exchange rate constant at pH 6.0 for each individual amide is shown in relation to the amino acid sequence of lysozyme. The Figure reveals that the amides can be roughly divided into three groups on the basis of their exchange rate constants, one with rate constants between 10^{-8} and 10^{-5} per second, which is associated with the three α -helices and the interior of the β -sheet region of the protein, one with rate constants between 10^{-5} and 0.001 per second mainly from amides hydrogen bonded in turns and to side-chain receptors, and a third group with rate constants between 0.001 and 100 per second exclusively from amides on the surface of the protein with no intramolecular hydrogen bonds. The correlation between exchange rates and distance to the surface for the individual amides is shown in Figure 3. The diagram shows that amides that are less than 4.3 Å (1 Å = 0.1 nm) from the nearest possible water molecule exchange with rates in the range between 10^{-6} and 10^{-4} per second and amides that are more than 7 Å away from the surface solvent are all in the range between 10^{-6} and 10^{-8} per second. In the distance range of 4.3 Å to 7 Å the diagram shows that distance to the surface is not the rate determining parameter. Here the type of secondary structure determines the exchange rate and divide the amides into the two ranges of rate constants as already shown in Figure 2.

The results discussed here are in very good agreement with a similar set of results for lysozyme obtained at 37°C at pH 7.5 reported by Radford *et al.* (1992a).

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The results for the individual amide exchange have shown that essentially all amides have pH dependent exchange reactions and the increase in $\log_{10}k_0$ per pH unit was seen to vary between 0 and

Table 2
Summary of results and analysis data for the hydrogen exchange study for the individual amides

a.a.r.	log (k_0)					$\Delta \log k_0 / \Delta \log \text{pH}$	log (k_1)	log (k_2)	log k_1/k_2	Molday factor
	pH 4	pH 5	pH 6	pH 7	pH 8					
F3	-6.87	-6.07	-5.24			0.82	4.81	-0.07	-4.7	1.3
G4	-5.76						>	>	-3.40	1.6
L8	-7.26	-6.49	-5.56			0.85	-4.86	0.30	-5.16	1.4
A9		-7.26	-6.37	-5.60	-4.90	0.78	-4.65	1.59	-6.23	1.0
A10			-6.87	-6.11	-5.29	0.80	-4.87	1.90	-6.77	1.0
A11	-7.54	-6.37	-5.62			0.96	-5.30	-0.27	-5.03	1.0
M12†			-7.36	-7.36	-7.13	0.12	-7.21	-0.59	-6.62	1.0
K13			-6.25	-5.29		0.97	-4.35	1.58	-5.93	2.0
R14	-6.74	-5.89	-4.90			0.86	>	>	-4.63	1.3
H15	-6.43	-5.61	-5.04			0.82	-4.88	-0.67	-4.21	17.8
L17	-5.86						>	>	-3.48	2.8
Y23	-6.07	-5.29	-4.51			0.78	-4.15	-0.22	-3.93	3.6
N27	-7.20	-6.30	-5.32			0.94	-4.03	0.94	-4.97	7.9
W28			-7.54	-7.17	-7.13	0.21	-7.11	-0.10	-7.01	2.5
V29†			-7.13	-7.41	-7.08	0.03	-7.08	-1.16	-5.92	1.0
C30			-7.07	-6.94	-6.86	0.10	-6.88	-0.55	-6.33	2.0
A31		-7.43	-7.24	-7.01	-6.63	0.27	-6.56	0.87	-7.43	3.2
A32			-6.91	-6.34	-5.90	0.51	-5.81	1.05	-6.86	1.0
K33			-6.88	-6.36	-5.93	0.47	-5.86	1.00	-6.86	2.0
F34			-6.82	-5.76		1.06	-4.40	2.03	-6.42	1.6
E35	-5.17						>	>	-2.85	1.1†
S36	-6.34	-5.51	-4.68			0.83	-4.25	-0.09	-4.16	2.8‡
N37	-5.71						>	>	-3.30	8.9
F38		-6.13	-5.34			0.79	-4.97	-0.18	-4.78	3.2
N39			-6.09	-5.10		0.99	-3.60	2.18	-5.78	2.2
T40	-6.18	-5.18	-4.18			1.00	-2.78	1.06	-3.84	5.0
A42	-7.03	-6.03	-5.17			0.93	-4.66	0.03	-4.69	1.4
N44	-7.46	-6.45	-5.29			1.24	>	>	-5.13	8.9
S50	-5.25						>	>	-2.88	5.6
D52			-7.23	-6.61	-6.08	0.57	-5.95	1.24	-7.19	3.6
Y53			-7.28	-6.96	-6.74	0.27	-6.73	0.37	-7.10	1.0
G54			-6.71	-6.03	-5.38	0.66	-5.18	1.46	-6.64	1.6
I55	-5.53						>	>	-3.09	2.8
L56	-5.64						>	>	-3.21	1.0
Q57			-6.99	-6.68	-6.50	0.25	-6.50	0.21	-6.71	1.4
I58				-6.37	-6.09	0.28	-6.06	0.69	-6.74	1.4
N59	-5.45						>	>	-3.04	2.8
S60			-7.49	-6.76	-6.17	0.60	-6.01	1.35	-7.36	5.0
R61	-6.58	-6.22	-5.65			0.47	-5.49	-0.70	-4.79	6.3
W63	-7.43	-6.20	-5.65			0.90	-5.49	-0.68	-4.80	1.0
C64			-7.02	-6.31	-5.68	0.67	-5.50	1.42	-6.92	2.0
N65	-7.31	-6.56	-5.60	-4.74		0.87	-4.22	1.05	-5.27	8.9
L75	-6.56	-5.88	-5.16			0.70	-4.91	-0.42	-4.49	2.5
C76	-7.24	-6.36	-5.56			0.84	-5.17	-0.15	-5.02	2.0
I78	-6.90	-6.04	-5.10			0.90	-4.29	0.41	-4.70	2.5
C80	-6.95	-6.05	-5.12			0.91	-4.36	0.37	-4.73	2.0
A82	-5.38						>	>	-2.96	3.2
L83			-6.64	-6.35		0.66	-6.30	-1.24	-5.06	1.0
L84	-5.90						>	>	-3.52	1.0
V92			-7.13	-6.65	-6.21	0.46	-6.12	1.02	-7.15	3.2
N93	-5.83						>	>	-3.46	2.8
C94	-5.99	-5.02	-4.05			0.97	>	>	-3.69	5.0
A95†			-7.42	-7.83	-7.48	-0.2	-7.53	0.03	-7.56	3.2
K96†			-7.23	-7.24	-7.12	0.03	-7.16	-0.24	-6.92	2.0
K97	-7.43	-6.46	-5.74	-5.00		0.80	-4.70	0.68	-5.38	2.5
I98			-7.05	-6.58	-6.13	0.46	-6.04	1.06	-7.10	1.3
V99		-7.40	-7.02	-6.28	-5.71	0.58	-5.56	1.31	-6.87	1.0
W108	-6.15	-5.42	-4.68			0.73	>	>	-4.06	1.0
W111	-7.01	-6.13	-5.49			0.76	-5.24	-0.46	-4.78	1.0
R112	-5.91	-5.15	-4.40			0.75	-4.09	-0.29	-3.79	2.0
C115	-6.68	-5.79	-5.00			0.85	-4.63	-0.17	-4.45	2.0
W123	-6.54	-5.51	-4.51			1.03	>	>	-4.20	1.0
I124	-6.95	-5.94	-5.07			0.94	-4.51	0.08	-4.59	1.0
R125	-5.86						>	>	-3.48	2.0

Column 1 lists the amino acid residue (a.a.r.) and number; columns 2 to 6 list the logarithm to the observed exchange rate constant k_0 (s^{-1}) at pH 4, 5, 6, 7 and 8, respectively; column 7 lists the increase in $\log_{10} k_0$ per pH unit; columns 8 and 9 list the logarithms to k_2 (s^{-1}) and $k_2(\text{app})$ (s^{-1}), k_3 used to calculate k_1 and $k_2(\text{app})$ from equation (2) is $2.0 \times 10^8 [\text{O}^{2-}\text{H}^-] \text{s}^{-1}$ (Gregory *et al.*, 1983); column 10 lists $\log k_1/k_2(\text{app})$; column 11 lists the Molday factors (Molday *et al.*, 1972). > Not determined.

† Residues with irregular pH dependence; their k_1 and $k_2(\text{app})$ were determined from k_0 at pH 7 and pH 8 only.

‡ Molday factors for Glu35 and Ser36 when Glu35 is in the acid form, the Molday factors are 0.8 and 2.0, respectively.

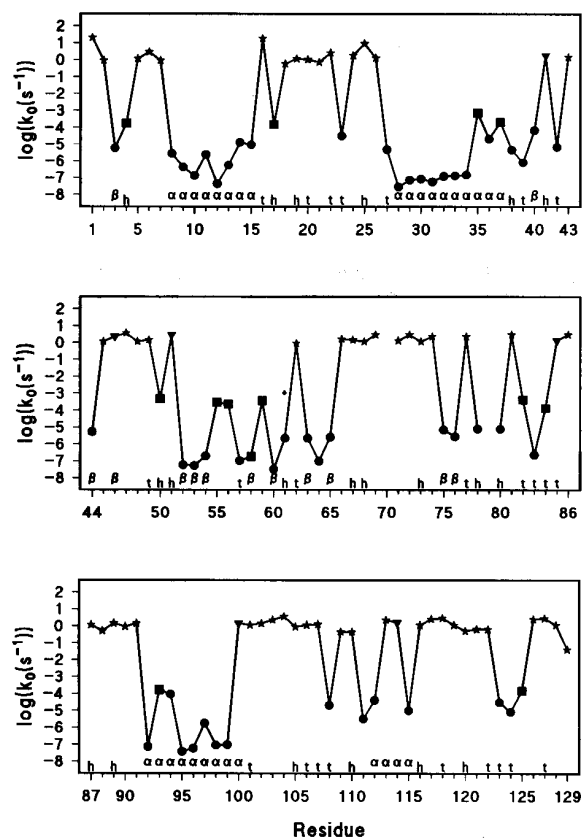


Figure 2. Amide exchange rates k_0 at pH 6 and 21°C for the amides in the lysozyme sequence. The secondary structure engagement of the amide hydrogen is annotated using α for α -helix, β for β -sheet, t for turn and h for amide hydrogens involved in other kind of hydrogen bonds. (●) k_0 measured at pH 6.0; (■) extrapolated from k_0 at pH 4.0 by assuming first order of catalysis by $[\text{OD}^-]$; (★) calculated from the primary structure effects on peptide group hydrogen exchange (Molday *et al.*, 1972) and $k_{\text{OD}} = 2.0 \times 10^8 \text{ s}^{-1} \times \text{mol}^{-1}$ (Gregory *et al.*, 1983); and (▼) reaction rate not measured but the α -NH cross-peaks were seen in NMR of freshly dissolved samples.

1. According to reaction scheme (1) amides that exchange within the EX1 limits should not have pH-dependent rate constants, because the pH-dependent k_3 is not involved in the expression, and k_1 is considered independent of pH. Amides that exchange according to the EX2 limits should have pH-dependent rate constants that have an increase in $\log_{10} k_0$ of 1.0 at pH values above the pH minimum of k_3 . A majority of the amides in lysozyme have pH-dependent rate constants between the two values, suggesting that they do not exchange according to either of the two mechanisms. In fact, Figure 1 reveals that a shift from EX2 to EX1 limits is expected between pH 4 and pH 8 in the range of k_1 and k_2 values examined there. This means that an analysis of the data will require the full expression for the rate constants (eqn (2)).

Given the reaction model (1) and the derived expression for the observable rate constant (2) each

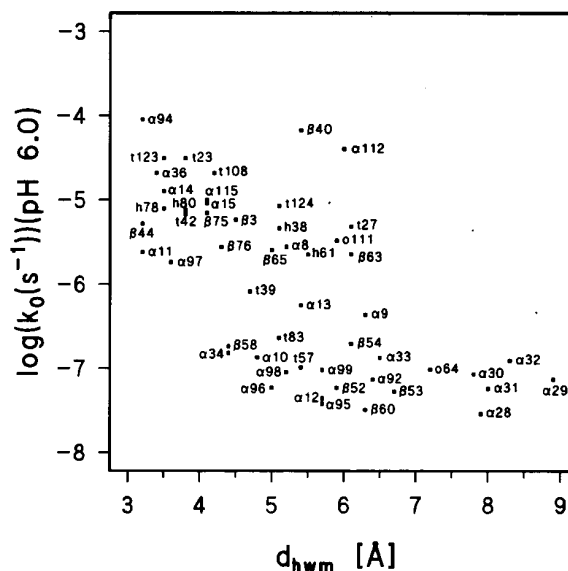


Figure 3. Comparison of the d_{hwm} in Å and observed amide exchange rate k_0 at pH 6. Each point is labelled with residue number and a signature for the secondary structure type is applied in Fig. 2; (o) amide hydrogens not involved in hydrogen bonding.

of the pH dependencies for the individual amide exchange reaction contains the information necessary to derive the two rate constants k_1 and k_2 . This can be done by fitting the measured results to equation (2) and making three assumptions: (1) that the two rate constants k_1 and k_2 are not pH-dependent; (2) that the exchange rate k_3 can be simulated using exchange rates calculated from exchange parameters obtained for model peptide amide exchange (Englander & Poulsen, 1969; Gregory *et al.*, 1983) in deuterium oxide (Covington *et al.*, 1966); and (3) that the hydroxide ion catalysed reaction is the predominant contributor to k_3 between pH 4 and pH 8 with no significant contribution to the exchange reaction from the other two processes (Gregory *et al.*, 1983).

The validity of these assumptions has been examined. First, with regard to the pH dependence of the rate constants k_1 and k_2 this can be examined by the pH dependence of k_0 . Inspection of Figure 1 reveals that changes by orders of magnitude in k_1 and k_2 as a function of pH will be reflected immediately on the pH dependence of k_0 . Minor pH dependencies of the two rate constants, however, would be difficult to identify. The origin of the pH dependence is the change in electrostatic potential at each site of an amide exchange reaction, and this depends on the pK of neighbouring ionizable groups. In general for lysozyme the electrostatic potential varies only little between 5 and 8. Only in the environment of the two residues His15 and Glu35 that have pK values of 5.4 and 6.2, respectively, the electrostatic potential may influence the exchange. No other groups in lysozyme have pK values in the range of pH 5 to pH 8. The assumption that k_1 and

k_2 do not vary with pH in this range is therefore likely to be valid for many of the exchange sites in the protein. For each of the amides that have been studied, the pH dependence of k_0 has been examined and those amides that reveal an unusual dependence has been marked in Table 2.

Secondly, by using the model peptide values for k_3 (Gregory *et al.*, 1983) in the fitting calculation the assumption is made that exchange occurs from a state where the amide is at a conformation where the physiochemical conditions are similar to those in solution. This rate is approximately the fastest the amide hydrogen exchange can occur by. However, as it is not known whether the opening reaction preceding the exchange step (1) brings the exchanging peptide segment in the protein into all the conformations that are allowed in the random coil model peptide, the application of the rate constants for the model peptide is an approximation. If the exchange occurs from a semi-exposed conformation, the rate constant k_3 is likely to be considerably smaller than in the fully exposed model peptide. Therefore, by using an approximated upper limit value for k_3 the values obtained for k_2 will also represent an upper limit rate constant for the closing process. Variation of k_3 up to four orders of magnitude was found not to influence the size of k_1 , whereas the values obtained for k_2 were found to be almost directly proportional to the variation of k_3 in the fitting calculations. This implies that the reported values for k_1 as obtained by the fitting procedure applied here can be taken as relatively precise measures of the individual rate constants for the opening reaction preceding the exchange process. For these reasons it was decided not to use the factors determined by Molday *et al.* (1972) for the effect of the neighbouring amino acid residue as these would hardly influence the calculation of k_1 given that the factors are rarely one order of magnitude different for poly-D,L-alanine rates. Instead we have listed the Molday factors in Table 2. Robertson & Baldwin (1991) found that the hydroxonium ion catalysed hydrogen exchange for valine was two to five times slower than predicted by Molday *et al.* (1972). The observation is not included in the listed Molday factors.

(c) *Structural interpretation of individual k_1 and k_2 results*

All the exchange data have been analysed to obtain k_1 and k_2 values by fitting the experimental results to expression (2). The results are listed in Table 2. These data are reported in consideration of the assumptions discussed above. The k_1 results are considered to be good approximations to the real values, whereas the k_2 values obtained are considered as apparent and upper limit values and hence referred to as $k_2(\text{app})$. The correlation between the results obtained and the structural properties have been examined and compared to the model-free analysis of the exchange reaction rate measured at pH 6 for the individual amides. The k_1

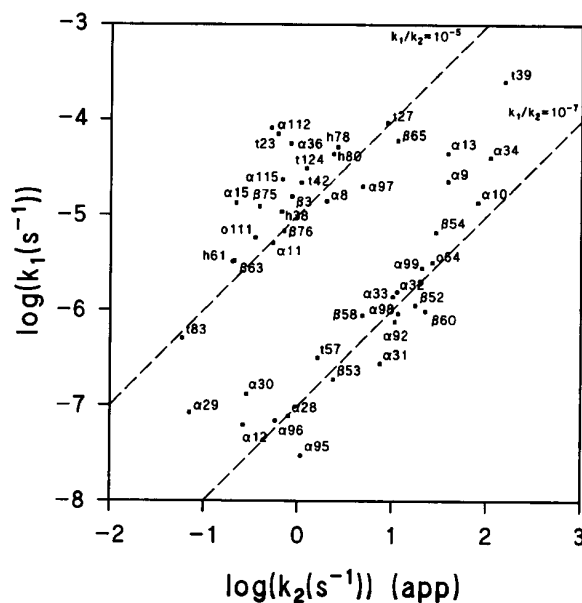


Figure 4. Comparison of the interdependent k_1 and $k_2(\text{app})$ for the individual amide groups in lysozyme. Each point is labelled with residue number and a signature for the secondary structure type as applied in Figs 2 and 3.

values found here for the amides of lysozyme were found to vary over a range spanning five orders of magnitude from 1.7×10^{-3} per second to 3.03×10^{-8} per second, and for $k_2(\text{app})$ values from 0.07 to 150 per second, giving a $k_1/k_2(\text{app})$ ratio variation varying between 1.4×10^{-3} and 2.8×10^{-8} . A comparison of the distribution of the corresponding k_1 and $k_2(\text{app})$ values for each of the individual amides is shown in Figure 4. The division into two groups of amides that could be made according to the exchange rate constants measured at pH 6 (Fig. 3), reappears in Figure 4, although here it is divided into groups according to their $k_1/k_2(\text{app})$ ratio. The group of amides with rate constants $k_0(\text{pH } 6)$ between 10^{-6} and 10^{-4} per second was seen to fall in the region of the diagram where the $k_1/k_2(\text{app})$ is around 10^{-5} with a relatively large concentration of amides with k_1 around 10^{-7} to 10^{-4} per second and $k_2(\text{app})$ around 1 per second. The other group of amides with very slow amide exchange rates k_0 between 10^{-6} and 10^{-8} per second is seen to have $k_1/k_2(\text{app})$ around 10^{-7} . For these the k_1 values are between 10^{-8} and 10^{-4} per second and span through a $k_2(\text{app})$ region of 0.1 to 100 per second. In this group most of the amides are from buried secondary structure in lysozyme. In the diagram one small subgroup can be identified that happens to consist of amides from the core of the three α -helices and the most buried amides in the structure. This group is further characterized by having the smallest k_1 values in the protein (less than 10^{-7} per second) and at the same time the smallest $k_2(\text{app})$ (between 0.07 and 1 per second). In other words, for these very slow amides exchange occurs

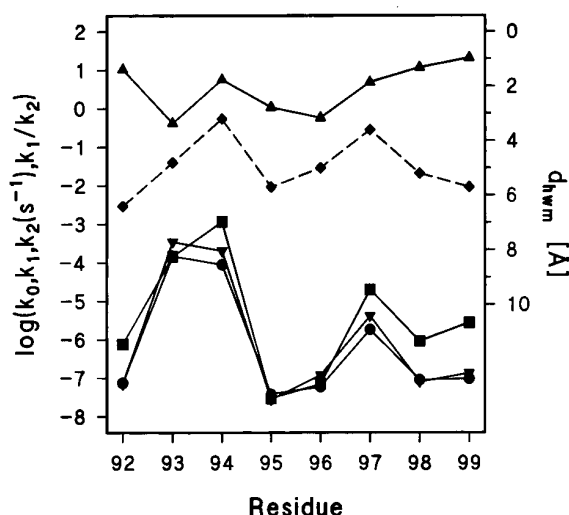


Figure 5. Summary for the exchange results in α -helix^(V92-S100) and the analysis results including \bullet — \bullet k_0 ; \blacksquare — \blacksquare k_1 ; \blacktriangle — \blacktriangle k_2 (app); \blacktriangledown — \blacktriangledown k_1/k_2 (app); \blacklozenge — \blacklozenge d_{hwm} .

only once or twice a year, corresponding to a similar rate for the opening reaction that precedes the exchange step, and a closing reaction that is complete within 1 to 10 seconds. Another subgroup is seen with k_1 around 10^{-6} per second and k_2 (app) between 10 and 100 per second, these are mostly from amides in buried secondary structure.

In other proteins similar values have been obtained for k_1/k_2 assuming EX2 limits for the exchange. Values around 10^{-6} were reported for residues in the centre of the β -sheets in tendamistat (Qiwen *et al.*, 1987) and desulphatothirudin (Haruyama *et al.*, 1989) at 20°C and 22°C respectively. The helical protons in cytochrome *c* have k_1/k_2 values in the range from 10^{-8} to 10^{-6} at 20°C, while the non-helical protons exhibit k_1/k_2 values from 10^{-6} to 10^{-4} (Jeng *et al.*, 1990).

(d) *Analysis of the rate constants*
 k_0 , k_1 , k_2 and k_1/k_2 in α -helix^(V92-S100)

The three major helices in lysozyme (Leu8 to His15), (Trp28 to Asn37) and (Val92 to Ser100) form a subdomain in lysozyme and include the so-called hydrophobic box. The first and the last helices are exterior amphipathic α -helices, whereas the second helix is essentially interior. Here we have chosen the third helix (Val92 to Ser100) to illustrate some of the hydrogen exchange properties and their correlation with the structural parameters. Figure 5 summarizes for each amide the structural parameters d_{hwm} (Pedersen *et al.*, 1991), the experimentally obtained exchange rates, k_0 , the calculated rate constants for the forward and reverse reactions of the reaction scheme (1), k_1 and k_2 (app), and the equilibrium constant k_1/k_2 (app). The helix contains two turns that have the amide nitrogens of residues

Asn93 and Cys94 near the surface of the protein in the first turn and Lys97 in the second turn. Interior in the helix are the amide nitrogen of residues Val92, Ala95, Lys96, Ile98 and Val99. The amide hydrogen exchange rates do indeed reflect this. All the interior amides are slowly exchanging with exchange rates at 10^{-8} to 10^{-7} per second, whereas the exterior have rates two to three orders of magnitude faster. This result shows that within the α -helix the individual exchange reaction is not a result of a co-operative event involving the entire element of secondary structure. Furthermore, the result shows that neighbouring amides in an α -helix where the amide nitrogen atoms are only 2.7 Å to 2.9 Å apart can have exchange rates that are different by a factor of more than 1000, as seen for Val92 and Asn93, and Cys94 and Ala95 (see Table 2). A similar observation is made for a pair of amides in the β -sheet Asn44 and Asp52. Asn44 is right on the surface of the protein and at the same time the amide hydrogen and the carbonyl is involved in hydrogen bonding with the complementary groups of Asp52 in the β -sheet. The k_1 of Asp52 is 100 times smaller than that for Asn44 and the two k_2 (app) are approximately the same (see Table 2). This result also suggests that the exchange of the two amides is not co-operative and resembles the situation found in the α -helices.

The correlation of exchange with the distance to the closest water molecule seen here is very similar to that observed in the other two major α -helices in lysozyme. Furthermore, a comparison of the amide exchange observed here to the three segments of an amphipathic helix in a coiled-coil leucine zipper peptide (Goodman & Kim, 1991) shows a very similar behaviour with respect to the periodicity in the exchange and the sequence positions in the helix. These results imply that exchange from individual residues in α -helix and β -sheet can take place without destabilization of the hydrogen bonds around it, as suggested by Dempsey (1992) for Gly12 in [Ala14]Melittin.

(e) *Amides with a small and/or uncommon pH-dependence and electrostatic potentials*

Unusual pH dependence was observed for the amides of Met12, Trp28, Val29, Cys30, Ala95 and Lys96. These are all from the centre of one of the three α -helices and all belong to the group of the most slowly and most buried amides in lysozyme. The pH dependence is unusual in these cases because it is essentially absent. In terms of the model (1) this suggests that the exchange for these amides is close to the EX1 limit where $k_0 = k_1$ and little pH dependence is expected (see Fig. 1). From a structural consideration this is feasible as the most interior amides in the globular structure are expected to be exposed to solvent most rarely.

Electrostatic effects are expected to cause pH dependencies that reflect local pK values. These effects are primarily expected either if k_1 and k_2 are pH dependent and/or the exchange occurs from a

conformation similar to the native structure. The electrostatic potential for several amides in lysozyme, including Asp52, Tyr53, Val92 and Ala95, were seen to be influenced by the ionization of Glu35 (Delepierre *et al.*, 1987). However, both in the present work, which is performed at 21°C, and in the work of Delepierre *et al.* (1987) performed at 37°C, dependence reflecting the pK of Glu35 is not seen in the pH range between 5 and 8 for any amide.

(f) Energy of activation

Hydrogen exchange in lysozyme is known to follow two mechanisms (Wedin *et al.*, 1982). One, which dominates at higher temperatures, is characterized by a high energy of activation and is assigned to occur by co-operative global unfolding. The other occurs preferentially at lower temperatures and has a lower energy of activation. The mechanism of the latter is not known; however, several models for the mechanism of this reaction have been proposed (see Introduction). An estimate of the energy of activation using the present dataset and the results of Delepierre *et al.* (1987) shows that the energy of activation for most amides is relatively small, 5 kcal to 40 kcal mol⁻¹. This excludes that the exchange of the amides is taking place by an unfolding mechanism that is expected to have an energy of activation of 120 kcal mol⁻¹ (Wedin *et al.*, 1982). Furthermore it suggests that the individual exchange reactions occur by a local rearrangement with a relatively small energy requirement. This is in agreement with results obtained in our laboratory for a wider range of temperatures below 21°C (unpublished results).

(g) Comparison of $k_2(\text{app})$ to the folding kinetics

It has been of interest to compare the rate constants for the individual folding processes recently described by Radford *et al.* (1992b) with the rate constants $k_2(\text{app})$ for local closing obtained here. The comparison is made keeping in mind that the two types of measurements determine the rate constants of two different types of reactions. One method measures the rate of the folding of a segment in a denatured protein, the other measures the rate of closing a segment that is part of an otherwise folded protein. The folding processes are reported to occur in biphasic reactions where the fast reactions occur with rate constants of 500 to 40 per second and the slower reactions with rate constants in the range of 25 to 3 per second. An independent study (Chafotte *et al.*, 1992) has measured rate constants using stopped-flow far-u.v. circular dichroism and observed biphasic folding kinetics with rate constants of 50 to 2.5 per second. The individual $k_2(\text{app})$ found in this work range between 0.07 and 150 per second (see Fig. 4). These values are upper limits. It is seen that the ranges overlap; however, a comparison of the rates of the individual residues reveal significant differences as expected.

It was noted that the fastest peptide segments to be protected in the folding experiments at the same time are very slow closing after opening for the hydrogen exchange. These residues are in the centre of the three major helices, and in the globular structure are hidden inside the hydrophobic box. The results suggest that when these amides become accessible to solvent it is relatively difficult to bring the segment back into the regular structure. Comparison of the rate constants also reveals that the opening and folding rate constants at the N and C-terminal parts of the three major α -helices are very similar. This would suggest that the closing reaction and the folding reaction are determined by similar structural parameters, and implies that the open form of the segments in the otherwise folded protein is similar to the conformation of the segments in the protein during the folding process.

(h) The amide exchange models and the observed pH-dependencies

When the Linderstrøm-Lang model was first proposed, no three-dimensional structure of a protein had yet been determined, and presumably, therefore, the nature of the opening and closing mechanisms was not part of the proposed mechanism. It was known that the amide exchange was pH dependent and it was proposed therefore, that this reaction was similar to the exchange reaction of a peptide free in solution. When protein structures later became known, it was not obvious how water would get into the protein structure given the proposed mechanism of Linderstrøm-Lang, and given the compact nature of the protein structures. The reaction scheme (1) becomes universal for a two-step reaction when the exchange step of the scheme is not considered to be following the kinetics of the peptide in solution. With this presumption a penetration model can be proposed to account for the observation that interior amides could exchange their hydrogen with solvent without complete unfolding. Furthermore, when studies of individual amide exchange became possible by n.m.r. spectroscopy it was observed that the pH dependence for certain amides did not follow a regular path, and this again was interpreted as support for a mechanism of exchange where the first step was a penetration mechanism.

It is of interest to discuss the penetration model and the breathing model in relation to the results obtained in this work. Both models can be explained by a two-step reaction scheme, where the first equilibrium for the penetration model is the flux of water into and out of the reaction site, and the second step is the exchange step. For the breathing model, the first step in the reaction scheme is the reversible opening and closing of the exchange site, and the second step is the exchange step according to the exchange of model peptides in solution. The penetration model can only explain the results obtained for the four most slowly exchanging amides in the study. These were seen also to have an

unusual pH dependence. The results obtained for these amides could be explained by penetration of water into the four reaction sites where the direct and pH-independent exchange process with the water molecule could occur. The weak pH dependence could then be a consequence of a conformational change that reduced the flux of water into and out of the reaction sites. The majority of exchange sites have a pH dependence that is incompatible with this type of mechanism, as the results suggest that one or several of the reaction steps are pH-dependent. The observed pH dependence for this very large group of slowly exchanging amides in lysozyme clearly implies this.

5. Conclusion

The pH dependence of all the amides in this work can, in all cases, be explained by the reaction scheme proposed by Linderström-Lang. This is shown by the observation that, essentially, all the pH dependence data could be fitted to the predicted functions, with the exception of the data obtained for the group of amides from the very interior of the protein. In this work one further step has been taken to calculate the rate constants for the opening and closing reactions in the first of the reaction steps. Given the model, the physical meaning of the results obtained is a set of rate constants, k_1 and k_2 , where k_1 is the reaction that led to the complete opening of the local peptide segment to expose the exchanging amide to exchange as it would do had it been free in solution, and k_2 for the reaction that put it back into order. It can be claimed that certain peptide segments in the protein never get into this state, but into a semi-exposed state where the exchange kinetics follow different parameters from those found in the free state. There is no experimental evidence to eliminate this possibility entirely and this is the reason the obtained k_2 values are referred to in this work as apparent values, to indicate that they have been calculated under the assumption that exchange takes place from the fully open state.

We have shown in this work that the reaction scheme originally proposed by Linderström-Lang is applicable to the analysis of individual amides in hen egg-white lysozyme, and that the opening and closing reaction rates could be determined for each of the slowly exchanging amide segments. By comparing the amide exchange throughout the structure it has been possible to establish that the open/close reactions in most cases are non-co-operative with the reactions of other amides. The rate constants for the reactions seem to depend essentially on two factors: (1) the physical distance from the reaction site to the solvent; (2) the position of the amide in the secondary structure. In several cases the distance to the solvent seems to overrule the engagement in the secondary structure. Other factors, such as local electrostatic effects, seem to have very little effect on the pH dependence of the exchange rate in most cases. A preliminary analysis

suggests that the exchange reaction has a small energy of activation, and this has stimulated a series of experiments that will be reported separately. At present, the evidence for the mechanism of the hydrogen exchange could therefore be that a partial or full opening of the structure is responsible for the exchange. The exchange takes place in an environment that is very similar to the bulk solvent. The opening rate k_1 of these processes can be determined relatively precisely for these reactions and the upper limit for the closing rate k_2 can be only estimated because the amide exchange rate k_3 depends on the conformational state of the amide during the exchange and this is not known.

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