Comparison of Protein Structure in the Crystal and in Solution. III. Tritium–Hydrogen Exchange of Lysozyme and a Lysozyme–Saccharide Complex*

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ABSTRACT: The tritium–hydrogen exchange of lysozyme was examined at long and short times of reaction, to determine the effects of substrate binding and crystallization. Solution and crystal showed identical exchange. Binding of tri(*N*-acetylglucosamine) decreased slightly the instantaneous exchange at pH 4.5, but had no effect at pH 9 or after 24 hr at pH 4.5.

The crystallographic structure that has been reported for lysozyme (Blake *et al.*, 1967b) lends interest to the chemistry of this protein. The β-1,4-linked trisaccharide of *N*-acetylglucosamine has been used to investigate the enzymic properties of lysozyme in the crystal (Blake *et al.*, 1967a) and in solution (Rupley and Gates, 1967). The following experiments concerning the rates of tritium–hydrogen exchange were designed to examine the effects of crystallization and of saccharide binding on the conformation of the molecule. Similar experiments with insulin were reported in the preceding paper (Praissman and Rupley, 1968), which also considered the background to exchange measurements and to the problem of crystal–solution comparison. Hvidt and Kanarek (1963) have studied in detail the rate of hydrogen exchange of lysozyme in solutions of pH between 3 and 8.7, at temperatures between 0 and 60°.

Materials and Methods

Hen egg-white lysozyme (mol wt 14,307; Canfield, 1963; Jolles *et al.*, 1963) was purchased from Worthington (lot LYSD-641) and stored at 5°. Crystals were grown by mixing equal volumes of solutions of 10% NaCl and 8% lysozyme (pH 4.7) and letting stand 24 hr at room temperature (Alderton and Fevold, 1946; D. C. Phillips, personal communication). After fragmentation by stirring, the crystals were approximately 10 μ in size. For exchange studies in solution, the protein was dissolved, centrifuged to remove debris, and exhaustively dialyzed against deionized water (four changes). All preparations were stored at 5°. Lysozyme concentration was determined from the optical density of an appropriate dilution, using $E_{280}^{1\%}$ 25.5 (Hartdegen, 1966). The β-1,4-linked trisaccharide derived from *N*-acetylglucosamine was prepared as previously described (Rupley, 1964). Other chemicals were of the highest purity commercially available.

The lyophilization procedure and apparatus described in the preceding paper (Praissman and Rupley, 1968) were used for measurement of the hydrogen exchange, with the following modifications. Dialyzed lysozyme solutions were adjusted to approximately 10 or 20 mg/ml of protein and the desired ionic strength, and then brought to either pH 4.7 or 9.5. Concentrated crystalline suspensions of lysozyme (>50 mg/ml at pH 4.7) were diluted to the appropriate concentration of salt, and were used at pH 4.7 or adjusted to pH 9.5 with 50% NaOH (this was done rapidly in order to avoid dissolution); the concentration of the suspension was then adjusted, usually to approximately 10 or 20 mg/ml. All samples were prepared using conductivity water and were stored at 3° for 12–18 hr before exchange was begun; 5 mg of dried protein was necessary for analysis of tritium incorporated through exchange; for the lysozyme concentrations studied, between 10 and 80 mg/ml, 0.5–0.05-ml aliquots were transferred into the donor bulbs of the lyophilization system. A bulb was removed from the vacuum train after 7-hr drying and the protein sample was dissolved immediately in 2 ml of water. Determination was made of the pH of the reaction mixture and of the concentrations and tritium contents of the reaction mixture and of each dried and redissolved aliquot. Three aliquots were analyzed and the results were averaged for each time of sampling of the reaction; the average deviation is reported with the data. A correction that averaged 3.6% was made for the greater absorbance of lyophilized over unlyophilized lysozyme; this was determined as for insulin (Praissman and Rupley, 1968). Precipitation of protein occurred upon mixing sample and scintillation solvent. In a control experiment the precipitate was thoroughly dispersed in the vial, and the sample was counted at 1-min intervals over the 5 min during which protein settled. There was no change in the counting rate over this time, and there was none after longer times of standing.

With the exception of one experiment, all exchange

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TABLE I: The Exchange of Lysozyme in Solution and in the Crystal, at pH 4.7–4.8 and 0°.

	Hydrogens Exchanged/Molecule				
	Solution			Crystal	
Time	0% NaCl	1% NaCl	2% NaCl	3.5% NaCl	5% NaCl
Zero time	159 ± 2.0	144 ± 3.0	140 ± 4.0	127 ± 2.6	123 ± 2.6
24 hr	188 ± 2.5	200 ± 4.3	197 ± 3.3	192 ± 0.2	180 ± 1.6

TABLE II: Exchange of Lysozyme and a Lysozyme–Trisaccharide[a] Complex at pH 9 and 0° in the Crystal.

	Hydrogens Exchanged/Molecule		
	Free Enzyme		Complex[b]
Time	pH 9.2 2% NaCl	pH 9.0 3% NaCl	pH 9.1 3% NaCl
Zero time	237 ± 1.6	236 ± 1.3	234 ± 3.3
24 hr	243 ± 1.6	255 ± 3.3	256 ± 2.3

[a] The β-1,4-linked trisaccharide of *N*-acetylglucosamine. [b] Corrected for the contribution of saccharide.

studies were in solutions containing NaCl, and some water remained bound to the salt after drying. Correction was made as in the preceding paper (Praissman and Rupley, 1968) for this contribution to the total tritium content of the sample; the radioactivity retained was 345 ± 50 cpm/per % NaCl. The count rates of protein and salt together were 14,000–25,000 cpm, and the error introduced through uncertainty in the salt blank was no more than 1%, even at the highest salt concentration used.

Results

Exchange of Lysozyme as a Function of Salt Concentration. The hydrogen exchange of lysozyme was examined at pH 4.7 and 0° in solutions of 0–5% (0.85 M) NaCl. The protein was soluble at salt concentrations of 2% or less, and it was crystalline at higher concentrations. The solubilities of crystalline samples were about 10% of the total protein present, unless otherwise noted. The data given in Table I and Figure 1 show that exchange was dependent on salt concentration. The zero-time values decreased steadily with increasing ionic strength, but without discontinuity between solution and crystal. The 24-hr values behaved similarly, except for less exchange at 0% compared to 1% NaCl.

Exchange of lysozyme at high pH was examined in solutions of 2 and 3% NaCl (Table II), conditions under which the protein was crystalline and only slightly soluble. pH was determined shortly after the start of the reaction, and it was 9.0–9.2.

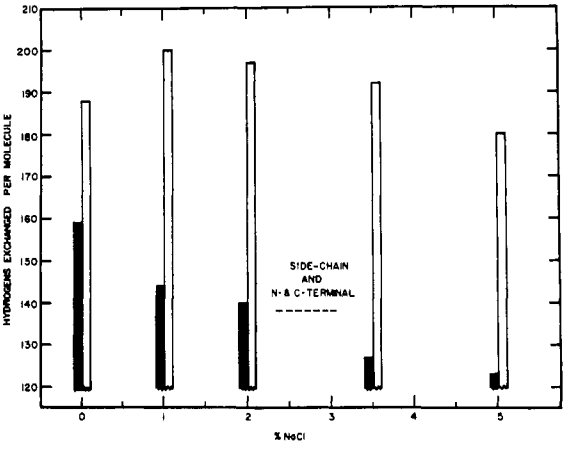

FIGURE 1: Hydrogen exchange of lysozyme as a function of salt concentration, at pH 4.7–4.8 and 0°. Solid bars: zero-time values; open bars: 24-hr values. The protein was crystalline at above 3% NaCl. The dashed line indicates the number of exchangeable hydrogens contributed by the side-chain terminal groups.

Effect of Temperature. Lysozyme in 2% NaCl solution exchanged 218 ± 2.5 hydrogens after 24 hr at 25° and pH 4.7, 21 more than the number at 0° (Table I) and 46 less than theoretical maximum exchange.[1] Crystalline lysozyme (81 mg/ml) suspended in 5% NaCl at 25°, pH 4.7), exchanged 219 ± 1.5 hydrogens after 24 hr, 39 more than at 0°. There was good agreement between the calculated (264) and measured (268 ± 2.3) values for maximum exchange (determined after 12-hr reaction in solution containing 2% NaCl, at 60° and pH 4.9; these conditions have been shown adequate to obtain full exchange (Hvidt and Kanarek, 1963)).

Lysozyme–Saccharide Complex. The hydrogen exchange of soluble and crystalline lysozyme was determined in the presence of the trimer of *N*-acetylglucosamine at 0° and pH 4.7 or 9. The mole ratio of trimer to protein was between 1 and 1.5, sufficient under the conditions of these experiments to saturate the enzyme without significant hydrolysis of the substrate (Rupley

[1] The theroetical maximum exchange, calculated from amino acid composition (Jolles *et al.*, 1963; Canfield and Liu, 1965) and titration data (Tanford and Wagner, 1954), is 254 at the isoionic pH, 260 at pH 9, and 264 at pH 4.7. These numbers might be increased slightly if water molecules are part of the internal structure of lysozyme (there may be two; D. C. Phillips, personal communication).

and Gates, 1967). To ensure equilibration throughout the crystal, saccharide was added to suspensions 1 hr before exchange was started. The trisaccharide contains eleven exchangeable hydrogens, eight in hydroxyl groups and three in amides. In a control experiment the trimer in 3% NaCl exchanged 11.5 and 13.0 hydrogens, respectively, at zero time and 24 hr (single analyses). The contribution of these hydrogens was subtracted from the data for the complex. For both crystal and solution there was no significant effect of trisaccharide on the 24-hr exchange at pH 4.7 and 0° (Table III).

TABLE III: Exchange of Lysozyme and a Lysozyme–Trisaccharide[a] Complex at pH 4.5–4.7 and 0°.

	Hydrogens Exchanged/Molecule			
	Solution, 2% NaCl		Crystal, 4.2% NaCl	
Time	Complex[b] pH 4.5	Free Enzyme[c] pH 4.7	Complex[b] pH 4.5	Free Enzyme[c] pH 4.7
Zero time	132 ± 1.6	140	115 ± 4.3	125
24 hr	202 ± 0.3	197	188 ± 1.0	187

[a] The β-1,4-linked trisaccharide of *N*-acetylglucosamine. [b] Corrected for the contribution of saccharide. [c] From Table I; value at 4.2% estimated by interpolation between 3.5 and 5%.

However, zero-time exchange in the presence of substrate was lower by 8 in solution and by 10 in the crystal. Exchange of the crystalline protein at pH 9 and 0° was not affected by the binding of saccharide (Table II).

Discussion

Comparison with Previous Work. Hvidt and Kanarek (1963) have reported[2] that the maximum number of exchanging hydrogens was 269 for the isoionic protein (determined at pH 3–8, 60°), 15 above theory.[1] These authors also found that in 0.1 M KCl, at pH 4.7 and 0°, there were approximately 19 more hydrogens exchanged at zero time and 13 more at 24 hr than found in this work.[3] Comparison could not be made at pH 9, because the salt concentrations used in the two studies did not overlap. The discrepancy might originate in differences between lots of protein. In this connection, Hvidt and Kanarek (1963) have observed that exchange depended upon the age as well as the source of the sample. Alternatively, there may have been differences of technique. In the earlier work the protein was dried before exchange, with a possible effect on conformation. Also, drying may have been incomplete (Hvidt and Nielsen, 1966), a possibility favored by the substantial difference between theoretical maximum exchange and that measured by Hvidt and Kanarek (1963). It must be emphasized that the differences between the present data and those of Hvidt and Kanarek (1963) are essentially unimportant with regard to the interpretation of the exchange behavior of lysozyme. Indeed, the previous study is implicitly relied upon in the comments that follow.

Effect of Ionic Strength. In solution, increased salt concentration decreases zero-time exchange, and first increases (between 0 and 1% NaCl) and then slightly decreases the 24-hr value (Figure 1). Both zero-time and 24-hr exchange continue to decrease as the salt concentration is raised further, into the range in which crystals are stable. Benson *et al.* (1964) found that the 1.5-hr exchange of bovine serum albumin was reduced by about 5% in solutions of 0.11 compared to 0.01 ionic strength; there was little effect at zero time. Soluble insulin (Praissman and Rupley, 1968) was not affected significantly by the addition of salt, but exchange in the crystal was substantially increased. Factors that might contribute to an effect of ionic strength have been discussed in the preceding paper (Praissman and Rupley, 1968). An explanation that accounts for all data cannot be advanced at this time. It is of interest that the number of hydrogens exchanging at zero time in 3.5% NaCl in the crystalline protein is less than the number of side-chain and α-amino hydrogens; the decrease is even more pronounced in 5% NaCl, where at least 15 have not reacted (Figure 1). It is understood that side-chain hydrogens generally exchange rapidly, before zero time (Hvidt and Nielsen, 1966). The observed discrepancy between the side-chain and zero-time values is greater than the number (10) of labile hydrogens that participates in intermolecular contacts in the crystal (Blake *et al.*, 1967a). Moreover, there was no effect of crystallization on the zero-time exchange in 2% NaCl (see below), and the intermolecular contacts in crystalline insulin do not themselves contribute to differences in exchange between crystal and solution (Praissman and Rupley, 1968). It is therefore not likely that hydrogens involved in contacts are the only source of the low exchange. Increased ionic strength perhaps lowers the reaction rate of some side-chain hydrogens into the measurable range.

Effect of Crystallization on Exchange Properties. The following data suggest that crystallization does not affect the hydrogen exchange of lysozyme. First, there is no discontinuity in exchange properties as the salt concentration is increased and the state of the protein changes from soluble to crystalline (Figure 1). Secondly, lysozyme was examined at high concentration (80 mg/ml) in 2% NaCl at pH 4.7 and 0°, conditions under which 48% of the protein was crystalline; 140 and 202 hydrogens had exchanged at zero time and 24 hr, respectively, in close agreement with the results (140 and

[2] The results of Hvidt and Kanarek (1963) were adjusted for comparison to the correct molecular weight (unknown in 1963).

[3] Comparison was for data obtained using protein from the same commercial source. Values for 0.1 M salt were determined from the data of Table I by linear interpolation between 0 and 1% (0.17 M) salt.

197, respectively; Table I) for fully soluble lysozyme under essentially identical conditions. Other experiments have shown that the average conformation of the protein is not affected by transfer into the crystal; specifically, several saccharides bind identically to crystalline and soluble lysozyme (Butler and Rupley, 1967). The exchange experiments therefore show that crystallization also does not affect motility, at least for those regions of the molecule that contain hydrogens with a measurable rate of exchange. Such regions embrace nearly half (57/128) of the peptide backbone at pH 4.7, and the exchange data therefore argue strongly for the identity of the crystal and solution structures. Furthermore, nearly full theoretical exchange was reached after 24 hr in 3% NaCl at pH 9 (theory value, 260 hydrogens; experimental value, 255), demonstrating that in the crystal all elements of the structure can be exposed to solvent, in accord with the insensitivity of exchange to crystallization. The lack of an effect of crystallization upon the exchange of lysozyme stands in contrast to results for insulin (Praissman and Rupley, 1968), which suggested that transfer of this protein into the crystal reduces motility without altering average conformation. Apparently, there is no uniform effect of crystallization on hydrogen exchange, a fact that was indicated some years ago by the data of Haggis (1957). Differences between proteins may reflect the nature and number of intermolecular contacts or the nature of the internal structure. In this connection, binding of substrate has little effect on the exchange of lysozyme (see below).

Effect of Binding of Trisaccharide. At pH 4.7 and 0°, lysozyme–saccharide complexes both in solution and in the crystal exchanged eight to ten fewer hydrogens at zero time than did the protein in the absence of substrate (Table III). However, there was no significant difference at pH 4.7 after 24-hr exchange, or at pH 9 at either zero time or 24 hr (Tables II and III). The effect of the trisaccharide is small and probably limited to hydrogens that are fully or nearly exposed. It may be that the decreased exchange at zero time principally reflects labile hydrogens that participate in bonds between enzyme and substrate (there are six of these; Blake *et al.*, 1967) or are covered and removed from contact with solvent when the complex forms (there are less than 4).[4]

The binding of trisaccharide is strong (ΔF° = 7.2 kcal), and it makes contact with a large portion of one face of the protein (approximately ten amino acid residues directly participate in the interaction).[4] Reversible unfolding, if it involves such large disorganization of the structure that the active site is disturbed, should be strongly affected by addition of trisaccharide (*i.e.*, the equilibrium constant for unfolding might be reduced by 10^5). Since this was not observed in exchange measurements, the kinetically important opening of the structure to permit access of a particular interior amide hydrogen to solvent (about 80% are buried)[4] must involve only a small part of the protein. Each region of the lysozyme structure apparently can unravel independently of others. This is plausible in that small conformational fluctuations should be much more probable than large ones (Linderstrom-Lang and Schellman, 1959). Similar exchange behavior appears in the polymerization of insulin (Praissman and Rupley, 1968) and the crystallization of lysozyme. Measurable but still only small effects of association have been reported for the binding of zinc by insulin (Praissman and Rupley, 1968) and the binding of pyridine nucleotides to yeast alcohol dehydrogenase (Hvidt and Kagi, 1963) and chicken heart lactic dehydrogenase (DiSabato and Ottesen, 1965). In contrast, a more substantial effect of association on exchange has been reported for the crystallization of insulin (Praissman and Rupley, 1968), the binding of substrates to luciferase (McElroy *et al.*, 1967), and the binding of cofactor to lactic dehyrogenase in the presence of detergent (DiSabato and Ottesen, 1965). It is noteworthy that substrate apparently affects the average conformation of luciferase (McElroy *et al.*, 1967). Perhaps association in solution is likely to alter exchange significantly only when it is correlated with rearrangement of the internal structure. If this is so, hydrogen exchange becomes a particularly interesting probe for the study of association reactions.

[4] Determined by examination of a model built through the kind cooperation of Drs. D. C. Phillips, A. C. T. North, C. C. F. Blake, and their colleagues.

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Study of a Sulfonyl Derivative of α-Chymotrypsin by Chlorine Nuclear Magnetic Resonance*

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ABSTRACT: The width of the ^{35}Cl nuclear magnetic resonance line provides a measure of the rotational motion of the inhibitor, *p*-mercuribenzenesulfonyl fluoride (RHgCl), attached to the active site of α-chymotrypsin in aqueous NaCl solution. The inhibitor appears to bind to the active serine at the catalytic site. If a single methionine residue on the enzyme is first alkylated with benzyl bromide, subsequent binding of RHgCl is still specific for the active site, but the observed ^{35}Cl resonance line is broadened relative to the inhibited native enzyme.

This indicates a more restricted rotational motion of the inhibitor attached to the *S*-benzylmethionine-chymotrypsin. In 8 M urea, however, the line width for RHgCl attached to *S*-benzylmethionine-chymotrypsin is the same as for RHgCl attached to native chymotrypsin. Apparently a well-defined secondary and tertiary structure at the active region is necessary for the alkyl group to affect motion of the inhibitor at the active site.

Attempts to correlate structure and function of enzymes in solution have drawn heavily from studies of chemical binding specificity and catalytic activity toward substrates. An enzyme commonly chosen for study is α-chymotrypsin since the binding and catalytic constants for a wide spectrum of substrates are known (Bender *et al.*, 1964). A common technique has been to examine binding or catalytic constants before and after some chemical modification of part of the enzyme. However any conclusions about steric relations between various residues at the active site and the various parts of a substrate require the testing of a substantial number of substrates, and the result is still often ambiguous.

It has recently been shown that relaxation and exchange of quadrupolar nuclei at suitable sites can provide a general method for nuclear magnetic resonance study of proteins in solution (Stengle and Baldeschwieler, 1966, 1967). In particular it is possible to obtain direct information about the accessibility of a particular region of a macromolecule to a solvent and also the degree of motional freedom of a label attached to a site on the macromolecule. In the present work, this technique is used to probe the active site of α-chymotrypsin by observing the effect of alkylating a particular methionine residue.

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Theory

For a nucleus of spin greater than one-half (*e.g.*, ^{35}Cl, ^{79}Br, ^{81}Br, ^{23}Na, or ^{127}I) the interaction of the nuclear electric quadrupole moment Q with the fluctuating electric field gradient q at the nucleus can provide a simple and dominant relaxation mechanism. In the extreme narrowing approximation ($\omega\tau_0 \ll 1$), the contribution to the nuclear resonance line width from quadrupole relaxation is

$$\Delta\nu = K(e^2qQ)^2\tau_c \qquad (1)$$

where $\Delta\nu$ is the full line width at half-height in cycles per second, (e^2qQ) is the quadrupole coupling constant in cycles per second, τ_c is the correlation time for molecular rotation in seconds, and K equals $2\pi/5$ for a nucleus of spin three-halves if the asymmetry parameter is neglected (Abragam, 1961). A large range of line widths is possible depending on the values of these quantities. For example, for a chloride ion in dilute aqueous solution, the solvation of the ion is essentially symmetric, and the electric field gradient at the nucleus is nearly zero. This results in a line width of 15–20 cps for the ^{35}Cl signal in aqueous solutions of NaCl. However when the chlorine atom is involved in covalent binding, the value of (e^2qQ) is quite large; the line width for CCl_4 is 14.5 kcps. Even greater line widths are expected for molecules larger than CCl_4 with longer τ_c.

If a quadrupolar nucleus can be located at different kinds of sites in solution, the resulting line shape depends on the relative concentrations of the various sites, the values of (e^2qQ) and τ_c at each site, and the rate of