

Studies of Triose Phosphate Isomerase by Hydrogen Exchange

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The ^3H -H exchange of chicken muscle and rabbit muscle triose phosphate isomerases was studied. Their behaviour was mostly very similar. 'Exchange-in' (acquisition of radioactivity when protein was incubated in $^3\text{H}_2\text{O}$) was measured at 37°C and at pH 7.5, and the rates of exchange of the native and liganded enzymes were compared. Inhibitors and substrates retarded exchange, substrates showing the most marked effect; structural rearrangements in the enzyme may thus play some part in catalysis. The inhibitor phosphoglycolate affected the rabbit enzyme, but had little or no effect on the chicken enzyme. 'Exchange-out' (loss of radioactivity from protein previously labelled by incubation in $^3\text{H}_2\text{O}$) was measured by hollow-fibre dialysis. When ligand was removed during the course of dialysis (by replacing buffer that contained ligand with buffer that lacked ligand) there was a prompt decrease in the number of labelled H atoms of the protein. Analysis of the curves provides some information about the number and half-lives of the responsive H atoms. Ligands decrease the motility of the protein and affect about one-fifth of the chain. Low concentrations of glycerol 3-phosphate have an effect that is greater than expected.

Information on protein structure in solution is valuable, but hard to obtain. Particularly valuable are signals of changes in structure when proteins combine with ligands and enzymes with substrates. This information goes some way towards bridging the gap between the detailed but non-dynamic results from crystallography and the dynamic but structurally uninformative results from kinetics. H exchange is a technique well-suited to provide such information, and the present paper illustrates its application to triose phosphate isomerase (EC 5.3.1.1). This is an enzyme now much studied; the work discussed here utilizes the enzyme from rabbit muscle or from chicken muscle. The rabbit enzyme is a dimer (Johnson & Waley, 1967) and the primary structure has been reported (Corran & Waley, 1973). The close homology of the chicken and rabbit enzymes follows from the comparison of their tryptic peptides (Furth *et al.*, 1974; Miller & Waley, 1971a; Corran & Waley, 1974), and the more recent work on the crystal structure (Banner *et al.*, 1971) and the mechanism (Knowles *et al.*, 1971; Plaut & Knowles, 1972) has used the chicken enzyme. One of the aims of the present work is to compare the rabbit and chicken enzymes, another is to see if the structure changes when the enzyme acts. Consideration of steric aspects of the reaction catalysed by triose phosphate isomerase led Miller & Waley (1971b) to suggest that changes in conformation of the enzyme might accompany catalytic action. Crystallography of the enzyme and the enzyme-substrate complex may eventually provide firm evidence about this; mean-

while, reactivity has to be used as a guide to structure, and this is the role of H exchange.

H exchange (Linderstrom-Lang, 1955; Hvidt & Nielsen, 1966; Englander *et al.*, 1972) of proteins in neutral solution mainly measures internally hydrogen-bonded peptide groups. Other chemical methods monitor side-chain reactivity, but here it is the backbone that is being scrutinized. In the method used in the present paper, the protein is labelled by incubation in $^3\text{H}_2\text{O}$ ('exchange-in') and then separated from bulk solvent by gel filtration (Englander, 1963). The measured extent of replacement of H by ^3H depends on the number of ^3H atoms gained in the first step and not lost in the second one. A perfectly rigid protein would presumably gain no ^3H atoms (except surface ones) in the first step; a perfectly flexible one would shed them all in the second step; H exchange shows proteins to be resilient.

In the work to be described, the course of 'exchange-in' and of 'exchange-out' have both been followed. Marked effects of ligands on the enzymes have been found, and, for one inhibitor, a different response from the rabbit and chicken enzymes. A preliminary account of some of this work has been given (Browne & Waley, 1973a).

Materials and Methods

Materials

Rabbit muscle triose phosphate isomerase was from Boehringer Corp. (London) Ltd., London

W5 2TZ, U.K.; chicken muscle triose phosphate isomerase was prepared by the methods of Putman *et al.* (1972) and McVittie *et al.* (1972) in the Oxford Enzyme Group Laboratory. Dihydroxyacetone phosphate and sodium DL-glycerol 3-phosphate were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; DL-glyceraldehyde 3-phosphate, the dehydrogenases and triethanolamine hydrochloride were from Boehringer Corp. (London) Ltd. 2-Phosphoglycollic acid was from General Biochemicals Ltd., Cleveland, Ohio, U.S.A. Sephadex G-25 was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K., and the $^3\text{H}_2\text{O}$ was from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Radioactivity (owing to ^3H) was determined with a Nuclear-Chicago Unilux IIA liquid-scintillation counter. The scintillation fluid for aqueous samples was toluene-dioxan-2-ethoxyethanol (1:3:1, by vol.) containing 8% (w/v) of naphthalene, 1% of 2,5-diphenyloxazole and 0.05% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene; the reagents, from BDH Chemicals Ltd., Poole, Dorset, U.K., were 'scintillation grade'. Aqueous samples (usually 0.1 ml) were counted for radioactivity (usually for 10 min) in 3 ml of scintillation fluid; the efficiency for ^3H was about 25%, and wherever possible constant-volume sets of constant chemical composition were counted to minimize differences in quenching.

Three types of H-exchange experiment were carried out: exchange-in, exchange-out by the two-column method, and exchange-out by hollow-fibre dialysis. When the course of exchange-in was followed, 10 μl of 100 mCi of $^3\text{H}_2\text{O}$ /ml was added to about 3 mg of rabbit or chicken enzyme in 1 ml of 20 mM-triethanolamine hydrochloride, pH 7.5, and the sample kept at 37°C for 0.25–2 h. After withdrawing 0.1 ml for counting for radioactivity, the sample was applied to a column (12 cm \times 2.5 cm diam.) of Sephadex G-25 (coarse grade) equilibrated with 0.1 M-sodium acetate buffer, pH 5.5, at 4°C. Fifty fractions of 1 ml were collected at flow rates of up to 900 ml/h; the protein emerged after 2–5 min. The E_{280} of the fractions containing protein were measured and 0.1 ml was taken for counting for radioactivity. Portions of the withdrawn sample were diluted and counted to obtain a value for the specific radioactivity of the medium. The enzymic activity was measured before the addition of $^3\text{H}_2\text{O}$ and at the end of the incubation.

When the course of exchange-out was followed by the two-column method, exchange-in was carried out in 20 mM-imidazole hydrochloride, pH 6.6, at 4°C or at 37°C, or in 0.2 M-sodium borate, pH 9.2, at

37°C. The separation of the protein from $^3\text{H}_2\text{O}$ was carried out on columns of Sephadex G-25, equilibrated with ligand if exchange-out in the presence of ligand was being followed. The pH 6.6 buffer, at 4°C, was used for the columns for the exchange-out; fractions containing protein were pooled after the first column, kept at 4°C for (usually) 24 h, and then put down the second column. Often a pair of 'second columns' was used, one with and one without ligand in the buffer. The difference between the two then reflected effects of the ligand.

Hollow-fibre dialysis in a Bio-Fibre 50 Beaker Dialyser unit (from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K.) was also used for following the course of exchange-out (Browne & Waley, 1973b). This gave results agreeing with those obtained by the use of two columns but was much more convenient. Exchange-in was for 1 h in 20 mM-triethanolamine hydrochloride, pH 7.5, at 37°C; the protein was separated from $^3\text{H}_2\text{O}$ on Sephadex G-25 in the pH 6.6 buffer at 4°C, and then introduced into the hollow-fibre dialyser. Ligands were present in the buffer when required, and were removed by replacing the outer fluid (volume about 100 ml) with buffer that lacked ligand. The concentration of the protein within the fibres remained constant throughout the experiment.

The values of H atoms/molecule were arrived at from:

$$\text{Apparent H atoms/molecule} = \frac{(\text{Radioactivity of enzyme})/(\text{concn. of enzyme})}{(\text{Radioactivity of } ^3\text{H}_2\text{O})/(\text{concn. of } ^3\text{H}_2\text{O})}$$

The concentration of enzyme was calculated with values of $E_{280}^{1\%} = 12$ and of mol.wt. 50000, and the concentration of $^3\text{H}_2\text{O}$ was taken as 110 M. The apparent values of H atoms/molecule were divided by 1.2 to allow for the equilibrium isotope effect for peptide H atoms (Englander & Poulsen, 1969). Although the isotope effect is rather different for side-chain amides, there were relatively few of these, and it is uncertain how many were detected in the present experiments. The rate of H exchange did not vary with the concentration of the protein.

Enzymic assays were carried out as described previously (Waley, 1973). The inhibition by sodium DL-glycerol 3-phosphate was carried out under the conditions used in exchange-out experiments, namely in 20 mM-imidazole hydrochloride, pH 6.6, at 4°C with concentrations of inhibitor of 0.2, 2 and 20 mM (as used in the H-exchange experiment of Fig. 2). The value of K_i was 0.65 ± 0.1 mM. This was determined from the Dixon (1953) plot of 1/rate against concentration of inhibitor, which gave a slope of 10.3×10^{-4} min/mM and vertical intercept of 12.7×10^{-4} min, and $1/k_{\text{cat}}$ of 6×10^{-4} min (for the conversion of dihydroxyacetone phosphate into glyceraldehyde phosphate).

Results

Course of exchange-in

Exchange-in is the first step in an H-exchange experiment, and so it is clearer if the experiments on the course of exchange-in are discussed first. The protein was incubated for various times in $^3\text{H}_2\text{O}$ at pH 7.5, 37°C, and the labelled protein and $^3\text{H}_2\text{O}$ were then separated on Sephadex G-25 at pH 5.5, 4°C; the conditions of separation were such that peptide groups that are exposed to the solvent exchange with a half-life of about 0.5 min (Englander & Staley, 1969) and the separation took about 2 min. Thus most of the peptide groups that are exposed to the solvent will lose ^3H during the separation, but most of the peptide groups that are appreciably stabilized should be detected. Exposed peptide groups exchange about 30-fold faster at 37°C than at 4°C, and 100-fold faster at pH 7.5 than at pH 5.5, and, to the extent that the EX₂ mechanism (Hvidt & Nielsen, 1966) holds, which is suspected of being quite generally for proteins (Englander *et al.*, 1972), these factors apply to all peptide H atoms. Hence the conditions of exchange-in were sufficiently vigorous to label, during an incubation of hours, H atoms with half-lives about 10^5 times those of exposed H atoms. These conditions then seemed to offer a chance of 'seeing' most of the structure, and the results are shown in Fig. 1. Each point in Fig. 1 comes from 10–25 estimations of specific radioactivity, derived from several columns and several fractions from each column, and is probably reliable to 5–10%. The results with the native (unliganded) enzymes will be discussed first, and then the effects of ligands.

Although the curves seem to plateau in Fig. 1, in fact, incubation of the chicken enzyme for 20h gave 370 ± 20 H atoms, and for 96h gave 430 ± 10 H atoms. There are approx. 550 amide H atoms/molecule of rabbit or chicken enzyme, derived from about 480 peptide bonds and about 35 side-chain amide groups; the values are approximate because the rabbit and chicken enzymes have slightly different compositions. Some of the side-chain amide H atoms will be lost during gel filtration, and so a reasonable upper limit is perhaps about 500 H atoms, which would leave 500–430 (i.e. 70) peptide groups exposed to the solvent and freely accessible. These exposed peptide groups may be in reverse turns (Crawford *et al.*, 1973) which are often at the surface (Kuntz, 1972).

There was no consistent difference between the H-exchange curves of unliganded rabbit and chicken enzymes (Fig. 1) and this may be taken as evidence that their structures in solution are closely similar. Small differences between homologous proteins (pig and bovine insulin) have been detected by H-exchange measurements (Capaldi & Garratt, 1971) of greater precision than those shown in Fig. 1.

The effects of ligands on the course of exchange-in

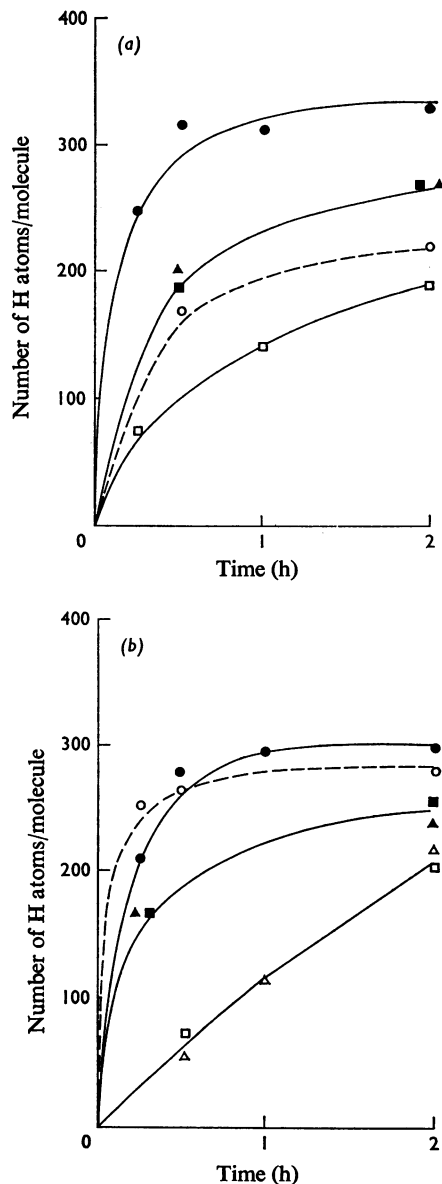


Fig. 1. Exchange-in: H^3H exchange of (a) rabbit and (b) chicken triose phosphate isomerase

Protein (3 mg) was incubated in 1 ml of 20 mM-triethanolamine hydrochloride, pH 7.5, containing 1 mCi of $^3\text{H}_2\text{O}$ (and the ligands specified below) at 37°C for the time given on the abscissa. The protein was then separated from the $^3\text{H}_2\text{O}$ on Sephadex G-25 at pH 5.5 and 4°C. Unliganded (●); 20 mM-sodium DL-glycerol 3-phosphate (■); 200 mM-sodium phosphate (▲); 0.5 mM-2-phosphoglycollate (○); 50 mM-DL-glyceraldehyde 3-phosphate (□); 50 mM-dihydroxyacetone phosphate (△).

will now be described, in terms of a series of qualitative comparisons. Ligands retard exchange (Fig. 1). This applies, with one possible exception, to both chicken and rabbit enzymes, and both to the inhibitors (phosphate, glycerol 3-phosphate and phosphoglycollate) and to the substrates, as ligands. The rabbit and chicken enzymes behave similarly, with one exception. The exception is the inhibitor phosphoglycollate, which has a marked effect on the rabbit enzyme, but little (or no) effect on the chicken enzyme. This is a striking difference between these very similar enzymes, and has been confirmed in the exchange-out experiments described below. The inhibitors phosphate (Oesper & Meyerhof, 1950), glycerol 3-phosphate (Burton & Waley, 1968) and phosphoglycollate (Johnson & Wolfenden, 1970) are all regarded as competitive inhibitors and presumably interact with the substrate-binding site; where they have been compared, their K_i values for the rabbit and chicken enzymes do not differ appreciably. The inhibitors phosphate and glycerol 3-phosphate have similar effects on the course of exchange-in, although their sizes differ. Finally, when the substrates are compared with the inhibitors, the results in Fig. 1 show that, for both enzymes, substrates have the more marked effect. The substrates are interconverted rapidly and so both have the same effect; this also shows that the inert L isomer in the racemic glyceraldehyde 3-phosphate used was not contributing appreciably to the effects seen. Although the imprecision of the values leaves the shapes of the curves somewhat uncertain, many of the H atoms affected by the substrates appear to have relatively short half-lives. The extent of the difference amounted to about 100 H atoms after incubation for 2 h (Fig. 1), which is an appreciable fraction of the total.

Measuring the course of exchange-in has both advantages and disadvantages. One advantage was that the exchange could be done at pH 7.5, 37°C under more or less physiological conditions where the properties of the enzymes would be more likely to have some bearing on their functioning *in vivo*. The other, main, advantage was that the substrates could be used; the effects of the substrates on the course of exchange-out were not studied because of the relatively large volumes needed to equilibrate columns. The disadvantages of measuring the exchange-in were that the precision was low and the method laborious, since a separate incubation and gel filtration had to be done for each point in Fig. 1.

Course of exchange-out

Glycerol 3-phosphate as ligand. The experiments described now were carried out by incubation of the protein in $^3\text{H}_2\text{O}$ for 1 h at pH 7.5, 37°C and then separating the labelled protein by gel filtration at pH 6.6, 4°C and measuring the course of exchange-

out (under these conditions) by hollow-fibre dialysis (Browne & Waley, 1973b). The most informative experiments were those in which ligand was initially present during exchange-out, but was suddenly removed. Then structural changes (or, at least, changes in reactivity) are made manifest by abrupt changes in the H-exchange curves. The study of structure change by difference H-exchange methods has been developed by Englander & Rolfe (1973). The effects of three concentrations of glycerol 3-phosphate are shown in Fig. 2. The top curve shows the course of exchange-out in the presence of 2 mM- or 20 mM-glycerol 3-phosphate (the racemic mixture was used in all the experiments; it is the D isomer that is responsible for the inhibition). After 5 h the buffer surrounding the fibres was replaced by buffer lacking the ligand. There was an abrupt discontinuity (Fig. 2) in the H-exchange curve. Of the many different breathing motions that together give rise to H-exchange, some are responsive to the presence of ligand; removal of the ligand permits the execution of some hitherto hindered breathing process. We wish to focus attention on the responsive H atoms.

Since many of the responsive H atoms appear to have short half-lives in the 'fast' unliganded form, an experiment was carried out similar to that shown in Fig. 2 except that the ligand (2 mM-glycerol 3-phosphate) was removed after 1.25 h (Fig. 3) and, as

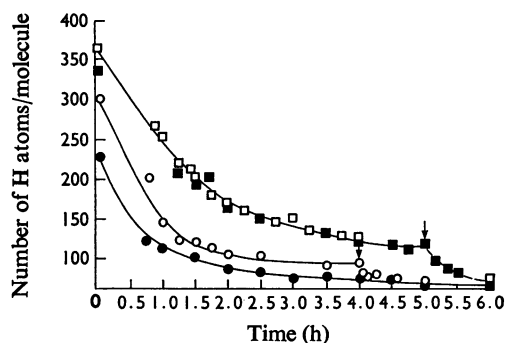


Fig. 2. Exchange-out of rabbit enzyme in the presence of glycerol 3-phosphate

Protein (5 mg) was labelled for 1 h as described in the legend to Fig. 1, and the protein was then separated from $^3\text{H}_2\text{O}$ on Sephadex G-25 at pH 6.6 in 20 mM-imidazole hydrochloride and introduced into the fibres of the dialyser unit, and the surrounding jacket was filled with the pH 6.6 buffer; samples of the solution containing protein were periodically removed for determination of radioactivity. The inhibitor, glycerol 3-phosphate, was present in the pH 6.6 buffer, both on the column of Sephadex G-25 and in the solution in the jacket, but at the times marked with an arrow the buffer in the jacket was replaced with buffer lacking inhibitor. The concentration of inhibitor was: zero (●); 0.2 mM (○); 2 mM (■); 20 mM (□).

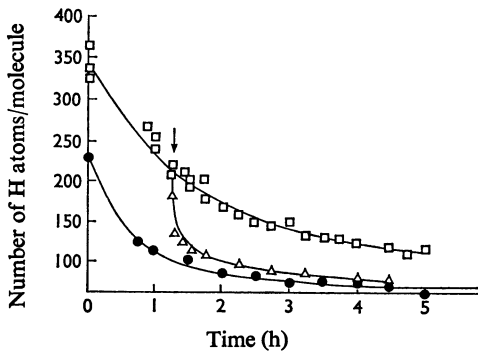


Fig. 3. Removal of glycerol 3-phosphate after exchange-out for 1.25 h

The exchange-out of rabbit enzyme was carried out in the presence of 2mM-glycerol 3-phosphate for 5h (□). In another experiment, the inhibitor was removed (see legend to Fig. 2) after 1.25h (△). The results are compared with those for unliganded protein (●). Other conditions are as in Fig. 2.

expected, a larger number of responsive H atoms was revealed. These results can be expressed quantitatively by analysis of the curves as discussed by Englander & Rolfe (1973).

We can focus attention on the responsive H atoms by subtraction of the background curve (Englander & Rolfe, 1973); a 'difference curve' is thus obtained. The background curve was obtained by keeping the protein in the fast (unliganded) form throughout the exchange-out. Although the responsive (as well as the indifferent) H atoms are labelled during exchange-in, they are largely lost during exchange-out by the time that the ligand is removed. Hence the background curve is largely composed of indifferent H atoms. The semi-logarithmic plot of the 'difference curve' gives the rate constant of the responsive H atoms in the fast (unliganded) form; kinetic classes can be roughly resolved by the sequential method, in which the slowest H atoms are treated first and then this set subtracted from the rest. This procedure gave the results in Table 1; the values for the 'slow' form were arrived at as follows.

Class analysis of the exchange-out curve in the 'slow' (liganded) form gave two classes, the half-lives being about 40min and about 20h (the earlier part of the exchange-out curves are too ill-defined to subdivide the faster H atoms). The H atoms of half-life about 40min would be nearly all lost by 5h, and so only the H atoms of half-life about 20h will be left. Now there were about 50 responsive H atoms when ligand was removed after 5h (Fig. 2), and the last two rows of Table 1 refer to these H atoms. Semi-logarithmic plots of the difference curves then

Table 1. Numbers and rates of H atoms responsive to glycerol 3-phosphate

The first column gives the approximate numbers of H atoms/molecule of rabbit enzyme that are affected by the presence of sodium DL-glycerol 3-phosphate. The second and third columns give the half-lives at pH 6.6, 4°C.

No. of H atoms/molecule	Half-life in:	
	Slow form (liganded)	Fast form (unliganded)
50	40min	4 min
30	20h	4 min
20	20h	40min

showed that, in the fast form, these 50 responsive H atoms fell into two classes, with half-lives of 4min and of 40min (second and third rows respectively of Table 1).

Now consider the experiment where the ligand was removed earlier, after 1.25h (Fig. 3). There were about 100 responsive H atoms, 50 more responsive H atoms than when the ligand was removed later (after 5h). Hence there were about 50 H atoms (here taken to be responsive H atoms) lost during the period between 1.25 and 5h of exchange-out; thus these H atoms will belong to the class with half-life of 40min in the 'slow' form (Table 1, first row). Semi-logarithmic plots of the difference curves in the experiment of Fig. 3 showed that about 80 of the responsive H atoms had a half-life of 4min (in the 'fast' form). This is also 50 more H atoms than were found when the ligand was removed later (Table 1, second row: 30 responsive H atoms with half-life of 4min in the fast form). We conclude that the kinetic class of 80 responsive H atoms characterized by a half-life of 4min in the fast form is derived partly from the 40min class and partly from the 20h class of the slow form. The values in Table 1 are quite tentative; the class sizes are uncorrected for responsive H atoms lost, and the shortest half-life is an upper limit as equilibration in the dialyser takes about 1min. The values are consistent with those from the exchange-in experiments (Fig. 1), and also with the results from 'two-column' experiments carried out above. For example, both rabbit and chicken enzymes after exchanging-in for 45h at pH 6.6, 37°C and then exchanging-out at 4°C for 24h in pH 6.6 buffer containing 20mM-glycerophosphate lost about 20 H atoms/molecule on gel filtration and left 15-20 H atoms still unaccounted for.

2-Phosphoglycollate as ligand. Three types of experiment with 2-phosphoglycollate as ligand were carried out: the first type, in which the course of exchange-in was followed, has already been described; the second type utilized two gel-filtration columns, and the third type utilized hollow-fibre dialysis. The

Table 2. *Effects of 2-phosphoglycollate on rabbit and chicken enzymes*

The values at the tails of the arrows are the H atoms/molecule after exchange-out for 24 h at pH 6.6, 4°C in the presence of 0.5 mM-2-phosphoglycollate; at the head of the arrows are the values obtained after gel filtration on columns lacking ligand. In the first series (Series I) exchange-in was for 95 h at pH 6.6, 4°C; in Series II exchange-in was for 45 h at pH 6.6, 37°C; in Series III for 20 h at pH 9.2, 37°C. The values of H atoms/molecule have coefficients of variation of about 5%.

	Series I	Series II	Series III
Rabbit enzyme	22 → 14	83 → 60	
Chicken enzyme	41 → 41	72 → 77	105 → 104

two-column experiments (Table 2) involved exchange-out for 24 h in the presence of ligand and then separation from the ligand by gel filtration, a process that took several minutes. During this time the H atoms that now exchanged with half-lives of less than about 1 min were lost and the change, symbolized by the arrow in Table 2, is ascribed to the loss of these H atoms. These are H atoms that are relatively labile in the fast (unliganded) form. Similarly, those H atoms with half-lives of 6 h or less in the slow (unliganded) form would presumably have been lost during the 24 h exchange-out before the ligand was removed. The results in Table 2 show that the rabbit enzyme has at least 23 H atoms that have sufficiently short half-lives in the fast form and sufficiently long half-lives in the slow form to be revealed by this procedure. The lower values in Series I suggest that exchange-in at the lower temperature labelled fewer H atoms that have long enough half-lives to survive the exchange-out for 24 h.

The main feature of Table 2 is that the chicken enzyme seems to lack H atoms responsive to phosphoglycollate that are detected by the present procedure. The experiments in Series III were carried out in case exchange-in at the lower pH had failed to label the responsive H atoms, but still none were detected. This difference between the chicken and rabbit enzymes is presumably due to lack of responsive H atoms that are slow enough in the slow form, or fast enough in the fast form.

Hollow-fibre dialysis was used for further experiments with phosphoglycollate as ligand. The results (Fig. 4) now revealed some responsive H atoms in the chicken enzyme, but they confirmed the marked difference between the rabbit and chicken enzymes in their response to phosphoglycollate. There were about 80 responsive H atoms in the rabbit enzyme and about 20 in the chicken enzyme, values comparable with those obtained from measuring the course of exchange-in (Fig. 1). Moreover, inspection of Fig. 4(b) shows that there were particularly few

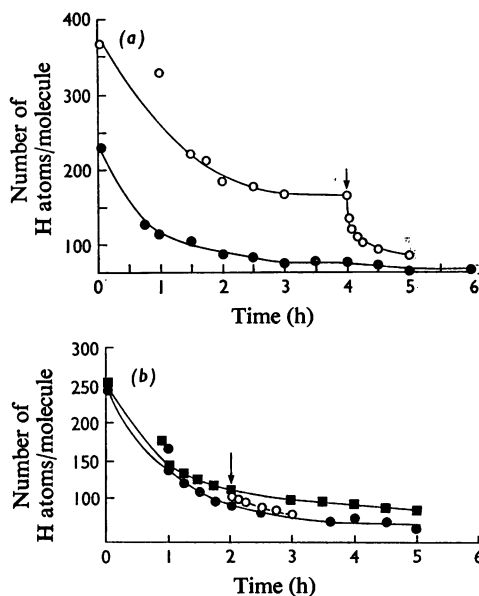


Fig. 4. *Effects of 2-phosphoglycollate on exchange-out of rabbit and chicken enzymes*

Exchange-out in the presence of 1 mM-2-phosphoglycollate is shown. In Fig. 4(a) rabbit enzyme was used and the inhibitor was removed after 4 h; in Fig. 4(b) chicken enzyme was used and the inhibitor was removed after 2 h. Other conditions are as in the legend to Fig. 2. In Fig. 4(a) the upper curve (○) refers to the experiment in which 2-phosphoglycollate was present; in Fig. 4(b) the upper curve (■) refers to an experiment in which 2-phosphoglycollate was present throughout, and the middle curve (○) to another experiment in which the inhibitor was removed after 2 h. In both Fig. 4(a) and Fig. 4(b) the lowest curve (●) refers to unliganded protein.

H atoms lost rapidly on removal of the ligand from the chicken enzyme, and so this partly explains why no effect was seen in the experiments reported in Table 2. Perhaps, as well, some of the responsive H atoms have relatively short half-lives in the liganded form and so are lost during exchange-out for 24 h.

Discussion

Comparison of rabbit and chicken enzymes

The two enzymes studied here are closely homologous. It is the similarity in the sequences of the tryptic peptides of the rabbit and chicken enzymes that provides the evidence for the homology (Furth *et al.*, 1974; Corran & Waley, 1974) and permits the construction of a hypothetical sequence for the chicken enzyme by aligning the tryptic peptides in an order decided by reference to the complete

sequence (Corran & Waley, 1973) of the rabbit enzyme. The extent of homology is then about 86% between the two enzymes (Corran & Waley, 1974); there is much the same difference between the rabbit and coelacanth enzymes (Kolb *et al.*, 1974). The catalytic properties of rabbit and chicken enzymes are, as far as is known, identical, as are the hydrodynamic properties (McVittie *et al.*, 1972; Putman *et al.*, 1972); there are, however, some differences in the reactivity of the thiol groups. The H-exchange experiments reported in the present paper confirm both the general similarities in structure and the particular differences in reactivity. The one clear-cut difference between the rabbit and chicken enzymes lies in their response to phosphoglycollate, which has a much more pronounced effect on the rabbit enzyme than on the chicken enzyme. Previous work had failed to detect differences between the two enzymes in their response to this (or other) inhibitor; thus the dissociation constants for phosphoglycollate, as estimated from kinetics or from difference spectra, are the same for rabbit and chicken enzyme (Johnson & Wolfenden, 1970). The difference now detected is therefore a tribute to the sensitivity of H exchange. The detailed interpretation of the differences will have to await the results of crystallography and model-building.

Comparison of ligands

P_i (Oesper & Meyerhof, 1950) and glycerol 3-phosphate (Burton & Waley, 1968) are inhibitors of the enzymic reaction, but only the latter affects the u.v. absorbance of the rabbit and chicken enzymes (Johnson & Wolfenden, 1970). The difference between the free-energy changes on binding the two inhibitors is only about 4.2 kJ/mol (1 kcal/mol), out of a free total energy change of about 19 kJ/mol (4.5 kcal/mol), which suggests that much of the energy of binding of glycerol 3-phosphate can be ascribed to polar interactions with the phosphate group. These two inhibitors also have much the same effect on the H exchange. They are not distinguished by the exchange-in experiments (Fig. 1), but some experiments of the type of Series III in Table 2 indicated that all of the H atoms responsive to P_i , but only some of those responsive to glycerol 3-phosphate, were lost on gel filtration. Hence glycerol 3-phosphate has a slightly more pronounced effect than P_i , and there is thus a parallel with their binding energies.

Phosphoglycollate differs from the other two inhibitors in discriminating between the two enzymes, as discussed above. The effect of phosphoglycollate on the rabbit enzyme is somewhat more marked than that of the other two inhibitors (Fig. 1). Presumably the main qualitative effects of all three inhibitors are similar. That is, there are sets of segmental movements

of the chain (breathing motions) that expose previously internally hydrogen-bonded peptide groups to the solvent. The rate of H exchange is controlled by the population density of such partially everted chains, and this is diminished in the liganded enzyme. The magnitude of the effect suggests that perhaps the active site may be formed from two 'wings' brought together by the folding of the chain. The ligand embedded between the wings could then restrain their outwards motion and so affect a relatively large portion of the chain.

Effect of substrates

Of all the ligands, the substrates show the most marked effects (Fig. 1). There were about 100 H atoms/molecule affected, which is about one-fifth of the chain. This is a large enough change to suggest that there are appreciable structural differences when substrates are bound. It might perhaps have been expected that the stress of action would lead to lability, especially as there will be some movement to compensate for the differing geometry of the two substrates (Miller & Waley, 1971b). Instead, the substrates diminish breathing motions. The energetics of protein breathing may interact with the energetics of enzyme action. The rate-limiting step in the conversion of dihydroxyacetone phosphate into D-glyceraldehyde 3-phosphate is the dissociation of free aldehyde from the enzyme (Knowles *et al.*, 1971; Trentham *et al.*, 1969). It now seems likely that the expulsion of the aldehyde is accompanied by an appreciable structural rearrangement. Indeed, this rearrangement may take a time of the order of 10 ms and account for the relative slowness of the dissociation.

Unexpected effects of low concentrations of ligand

Two different concentrations of glycerol 3-phosphate gave the same result (Fig. 2): they were 2 mM and 20 mM. As the K_i is 0.65 mM under these conditions, the concentrations are about $3K_i$ and $30K_i$, and the unliganded protein is about 1/4 and 1/31 of the total protein. Clearly, H exchange from both liganded and unliganded protein can occur.

The lower curve in Fig. 2 shows the effect of 0.2 mM-glycerol 3-phosphate. Even at this concentration the ligand seems to have an effect.

As the ligand was removed after 4 h in this experiment, the protein was unliganded for 3 out of the 4 h, and so the H atoms present should have half-lives of hours, rather than minutes. The same unexpected feature is apparent in the experiments when the concentration of ligand was 2 mM; here the protein was presumably unliganded for about 1 h, and yet many of the responsive H atoms have half-lives of a few minutes. Again, other experiments, with both the

rabbit and chicken enzymes, showed that there were responsive H atoms with half-lives of a few minutes after removal of 20mM-glycerol 3-phosphate after 24h exchange-out; here also, the protein should have been unliganded for about 1h. These effects of low concentrations of glycerol 3-phosphate are surprising. We now consider (and reject) various explanations. The assumption is that when 75% of the protein is unliganded then the protein may be regarded as unliganded for 75% of the time. This assumption will be true unless there is some slow change in conformation that occurs after the ligand has dissociated from the enzyme-ligand complex. What counts as slow in this context may depend on the mechanism of H exchange. In the EX₂ mechanism (Hvidt & Nielsen, 1966; Englander *et al.*, 1972) the equilibria governing 'breathing motions' are maintained and the slow process is that of exchange itself; under the present conditions this time-scale amounts to seconds. Thus a change in conformation taking seconds could invalidate the assumption given above. On the other hand, there is no other evidence for such a slow change in conformation and some evidence against it. For example, glycerol 3-phosphate behaves as a competitive inhibitor and this effectively entails rapid equilibration with the enzyme (Webb, 1963). Another possibility might be that H exchange detected a separate binding site with much higher affinity for the ligand; this possibility, however, has been ruled out by estimations of the dissociation constant by equilibrium dialysis which gave values not significantly different from the value obtained for K_t which is given under 'Methods' (P. H. Corran & S. G. Waley, unpublished work). Thus there does not seem to be any obvious explanation for the effects of low concentrations of ligand.

No comparable effects with other enzymes have come to our notice. With systems interacting with tightly bound ligands, such as haemoglobin (Englander & Rolfe, 1973) or antibodies (Liberti *et al.*, 1972), low concentrations naturally have a proportionate effect.

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References

Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C. & Pogson, C. I. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 151-155

- Browne, C. A. & Waley, S. G. (1973a) *Biochem. Soc. Trans.* **1**, 1155-1158
- Browne, C. A. & Waley, S. G. (1973b) *Anal. Biochem.* **56**, 289-291
- Burton, P. M. & Waley, S. G. (1968) *Biochim. Biophys. Acta* **151**, 714-715
- Capaldi, R. A. & Garratt, C. J. (1971) *Eur. J. Biochem.* **23**, 551-556
- Corran, P. H. & Waley, S. G. (1973) *FEBS Lett.* **30**, 97-99
- Corran, P. H. & Waley, S. G. (1974) *Biochem. J.* **139**, 1-10
- Crawford, J. L., Lipscomb, W. N. & Schellman, C. G. (1973) *Proc. Nat. Acad. Sci. U.S.A.* **70**, 538-542
- Dixon, M. (1953) *Biochem. J.* **55**, 170-171
- Englander, S. W. (1963) *Biochemistry* **2**, 798-807
- Englander, S. W. & Poulsen, A. (1969) *Biopolymers* **7**, 379-393
- Englander, S. W. & Rolfe, A. (1973) *J. Biol. Chem.* **248**, 4852-4861
- Englander, S. W. & Staley, R. (1969) *J. Mol. Biol.* **45**, 277-295
- Englander, S. W., Downer, N. W. & Teitelbaum, H. (1972) *Annu. Rev. Biochem.* **41**, 903-924
- Furth, A. J., Millman, J. D., Priddle, J. D. & Offord, R. E. (1974) *Biochem. J.* **139**, 11-25
- Hvidt, A. & Nielsen, S. O. (1966) *Advan. Protein Chem.* **21**, 287-386
- Johnson, L. N. & Waley, S. G. (1967) *J. Mol. Biol.* **29**, 321-322
- Johnson, L. N. & Wolfenden, R. (1970) *J. Mol. Biol.* **47**, 93-100
- Knowles, J. R., Leadlay, P. F. & Maister, S. G. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 157-164
- Kolb, E., Harris, J. I. & Bridgen, J. (1974) *Biochem. J.* **137**, 185-197
- Kuntz, I. D. (1972) *J. Amer. Chem. Soc.* **94**, 4009-4012
- Liberti, P. A., Stylos, W. A. & Maurer, P. H. (1972) *Biochemistry* **11**, 3312-3320
- Linderstrom-Lang, K. (1955) *Symp. Peptide Chemistry, Chem. Soc. Spec. Publ.* **2**, 1-24
- McVittie, J. D., Esnouf, M. P. & Peacocke, A. R. (1972) *Eur. J. Biochem.* **29**, 67-73
- Miller, J. C. & Waley, S. G. (1971a) *Biochem. J.* **122**, 209-218
- Miller, J. C. & Waley, S. G. (1971b) *Biochem. J.* **123**, 163-170
- Oesper, P. & Meyerhof, O. (1950) *Arch. Biochem. Biophys.* **27**, 223-233
- Plaut, B. & Knowles, J. R. (1972) *Biochem. J.* **129**, 311-320
- Putman, S. J., Coulson, A. F. W., Farley, I. R. T., Riddleston, B. & Knowles, J. R. (1972) *Biochem. J.* **129**, 301-310
- Trentham, D. R., McMurray, C. H. & Pogson, C. I. (1969) *Biochem. J.* **114**, 19-24
- Waley, S. G. (1973) *Biochem. J.* **135**, 165-172
- Webb, J. L. (1963) *Enzyme and Metabolic Inhibitors*, vol. 1, p. 189, Academic Press, New York