The Proton Exchange of the *pro-S* Hydrogen Atom at C-1 in Dihydroxyacetone Phosphate and D-Fructose 1,6-Bisphosphate Catalysed by Class-I and Class-II Aldolases

By ALPHONSE GALDES and H. ALLEN O. HILL*
*Inorganic Chemistry Laboratory, University of Oxford,
South Parks Road, Oxford OX1 3QR, U.K.*

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The efficacy of class-I and class-II aldolases in catalysing the C-1 proton exchange in fructose 1,6-bisphosphate and dihydroxyacetone phosphate was investigated. The rate of this reaction was at least two orders of magnitude slower in class-II than in the class-I aldolases. It is suggested that this difference reflects the formation of different intermediates in the reactions catalysed by the two classes of aldolase.

D-Fructose 1,6-bisphosphate aldolases (EC 4.1.2.13) have been subdivided into two main classes (Rutter, 1964). The class-I aldolases, typified by rabbit muscle aldolase, do not require a metal-ion cofactor and form a Schiff-base intermediate with their substrate (Horecker *et al.*, 1972). The class-II aldolases, typified by yeast aldolase, are metallo-enzymes inhibited by chelating agents and stimulated by K^+ (Rutter, 1964). Variants of each aldolase type have been proposed (Rutter *et al.*, 1963; Willard & Gibbs, 1968; Hill *et al.*, 1976). In particular the enzyme from *Bacillus stearothermophilus* is considered an atypical class-II aldolase in that its activity is not enhanced by K^+ ions (Hill *et al.*, 1976) and shows little sequence homology with yeast aldolase (Jack, 1973).

It has been well established (Rose & Rieder, 1958; Richards & Rutter, 1961*b*) that both classes of aldolase are capable of catalysing a rapid, stereospecific, proton exchange of the *pro-S* hydrogen atom at C-3 in dihydroxyacetone phosphate. In addition it has been reported (Lowe & Pratt, 1976) that rabbit muscle aldolase can catalyse the stereospecific exchange of the *pro-S* C-1 hydrogen atom in dihydroxyacetone phosphate and D-fructose 1,6-bisphosphate. The rate of this C-1 hydrogen exchange was at least 400 times slower than the C-3 hydrogen exchange in dihydroxyacetone phosphate (Lowe & Pratt, 1976).

In the present study the ability of class-I and class-II aldolases to catalyse the C-1 hydrogen exchange in dihydroxyacetone phosphate and D-fructose 1,6-bisphosphate was investigated by using rabbit muscle aldolase, yeast aldolase and *B. stearothermophilus* aldolase.

The exchange was investigated by following the 2H labelling of these substrates in 2H_2O by using 1H n.m.r. spectroscopy.

* To whom requests for reprints should be addressed.

Experimental

Rabbit muscle aldolase and yeast aldolase were prepared by established procedures (Taylor *et al.*, 1948; Hill *et al.*, 1976) in the Enzyme Preparation Laboratory of the Oxford Enzyme Group. *B. stearothermophilus* aldolase was prepared as previously reported (Jack, 1973; Hill *et al.*, 1976). All three enzymes were homogeneous by disc electrophoresis on a polyacrylamide gel containing 0.1% sodium dodecyl sulphate and were stored as the $(NH_4)_2SO_4$ precipitate at 4°C.

Glyceraldehyde 3-phosphate dehydrogenase and NAD^+ were from Boehringer (Mannheim), London W5 2TZ, U.K. D-Fructose 1,6-bisphosphate was purchased from Wessex Biochemicals, Bournemouth, Hants., U.K. Chlorohydroxyacetone phosphate was a gift from Dr. S. Waley, University of Oxford. 2H_2O (minimum isotopic purity 99.7 atom % 2H) was purchased from Merck and Co., Montreal, Canada.

Dihydroxyacetone phosphate was prepared (S. Waley, unpublished work) enzymically from D-fructose 1,6-bisphosphate by using rabbit muscle aldolase and chicken muscle triose phosphate isomerase (supplied by the Enzyme Preparation Laboratory of the Oxford Enzyme Group). The dihydroxyacetone phosphate was separated from the residual fructose 1,6-bisphosphate (after precipitation of the enzymes) on a column (20cm $\times$ 2.0cm) of Dowex-1 in the formate form. The column was eluted with 2 litres of 0.35M-ammonium formate, pH 3.0, 130 15-ml fractions being collected. The resulting material was judged to be pure by high-voltage paper electrophoresis [in 1% $(NH_4)_2CO_3$; developed for phosphate with ammonium molybdate] and 1H n.m.r. spectroscopy (Gray & Barker, 1970).

Aldolase activity was assayed by the coupled glyceraldehyde 3-phosphate dehydrogenase method

in 50mM-triethanolamine hydrochloride buffer, pH 7.3 (Hill *et al.*, 1976), and aldolase activity expressed as μmol of fructose 1,6-bisphosphate cleaved/min at 25°C.

B. stearothermophilus and rabbit muscle aldolase were prepared for n.m.r. work by extended dialysis against 20mM-sodium cacodylate buffer, containing 100mM-NaCl, pH 7.0, followed by repeated freeze-drying, the protein being taken up in $^2\text{H}_2\text{O}$. Yeast aldolase was prepared by dissolving the $(\text{NH}_4)_2\text{SO}_4$ precipitate in 5ml of 20mM-cacodylate buffer, containing 100mM-NaCl, made up in $^2\text{H}_2\text{O}$. This solution was then concentrated in a 10ml Amicon Ultrafiltration cell, by using a PM10 membrane, at 4°C to 1ml, and then remade up to 5ml with the cacodylate buffer and the process repeated twice. This procedure was necessary to minimize enzyme inactivation (Richards & Rutter, 1961a).

Pulsed Fourier-transform ^1H n.m.r. spectra were obtained at 270MHz by using a modified Bruker HFX-90 console, an Oxford Instruments superconducting magnet and a Nicolet 1085 computer of the Oxford Enzyme Group. Routinely 400 free-induction decays were collected, with a pulse-to-pulse time of 0.6s. Chemical-shift values are quoted in p.p.m. downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate as internal standard. $p^2\text{H}$ measurements were made with a Pye Ingold micro-

electrode ($p^2\text{H} = \text{pH meter reading} + 0.4$; Fife & Bruice, 1961).

In a typical experiment the reaction would be initiated by the addition of a small volume (10–50 μl) of the appropriate enzyme solution to a solution of the substrate in 20mM-cacodylate buffer, made up with $^2\text{H}_2\text{O}$, and containing 100mM-NaCl or -KCl as indicated, and 1mg of bovine serum albumin/ml to stabilize the aldolase at high dilution (Richards & Rutter, 1961b). This solution was adjusted to the required $p^2\text{H}$ in the n.m.r. tube by the addition of small quantities of 0.1M- NaO^2H or 0.1M- ^2HCl solutions before addition of the aldolase. The $p^2\text{H}$ was remeasured at the end of the experiment and was found not to differ from the initial reading by more than 0.05 $p^2\text{H}$ unit.

The aldolase activity in the n.m.r. tube was assayed at the beginning and at the end of the experiment to ensure that no appreciable enzyme inactivation had occurred during the course of the experiment.

Results and Discussion

The 270MHz spectrum of fructose 1,6-bisphosphate (10mM, $p^2\text{H}$ 7.5) is shown in Fig. 1(a). The resonances at 4.15p.p.m. and 4.06p.p.m. (peaks A and B) are due to the ring protons, and those at 3.93p.p.m. and 3.79p.p.m. (peaks C and D) are due

Fig. 1. ^1H n.m.r. spectra (270 MHz) of 10mM-fructose 1,6-bisphosphate (a) Alone; (b) after incubation for 60min with 2 units of rabbit muscle aldolase/ml; (c) after incubation for 400min with 2 units of *B. stearothermophilus* aldolase/ml. Spectrum (c) was plotted by using a smaller vertical amplification than in spectrum (a).

to the C-6 and C-1 protons respectively (Lowe & Pratt, 1976). It should be noted that the observed peak intensities do not accurately reflect the number of protons contributing to each peak because of the short pulse-to-pulse time (Farrar & Becker, 1971). When rabbit muscle aldolase is added to this fructose 1,6-bisphosphate solution the intensity of the C-1 protons decreases dramatically (Fig. 1*b*), owing to an exchange process with solvent ^2H . A similar C-1 proton exchange was observed on incubating dihydroxyacetone phosphate with rabbit muscle aldolase. ^1H n.m.r. spectra were collected during the course of the exchange reactions, from which the intensity of the C-1 hydrogen resonance at different times was measured. The pseudo-first-order rate constants (Table 1) for the exchange reactions were calculated from these data by using the usual semi-logarithmic plots of intensity against time. Such plots were linear up to 50% product formation.

However, when fructose 1,6-bisphosphate and dihydroxyacetone phosphate were incubated with yeast and *B. stearothermophilus* aldolase between $p^2\text{H}$ 6.4 and $p^2\text{H}$ 7.5, both in the presence and in the absence of 100 mM- K^+ for periods up to 7 h, no C-1 hydrogen exchange was evident (Fig. 1*c*). It was calculated that the maximum rate of any C-1 hydrogen exchange in these cases must be at least two orders of magnitude lower than the rate observed with rabbit muscle aldolase (Table 1). In contrast, under optimal conditions the proton exchange of the *pro-S* hydrogen atom at C-3 in dihydroxyacetone phosphate proceeds at comparable rates with all three enzymes (Richards & Rutter, 1961*b*; H. A. O. Hill & A. Galdes, unpublished work). Hence the ability or lack of ability to catalyse this C-1 hydrogen exchange in fructose 1,6-bisphosphate and dihydroxyacetone phosphate seems to provide another fundamental difference between the class-I and class-II aldolases.

The observed difference in the catalytic activities of rabbit muscle aldolase and yeast and *B. stearothermophilus* aldolases may have its origins in an inherent

distinction between the two classes of enzymes. Rabbit muscle aldolase, as a class-I aldolase, forms a ketimine intermediate with its substrates, whereas in the class-II aldolases the substrates are thought to bind, via the ketone carbonyl group, to the active-site metal [Zn(II) in yeast and *B. stearothermophilus*]. Now the rate observed for the C-1 hydrogen exchange catalysed by rabbit muscle aldolase is comparable with the expected spontaneous rates for a ketimine in solution (Lowe & Pratt, 1976; Bender & Williams, 1966). The spontaneous rate of exchange of a ketone, as opposed to a ketimine, in aqueous solution, however, is extremely slow (Bender & Williams, 1966; Hine *et al.*, 1972). Even after taking into consideration the electron-withdrawing power of the phosphate ester group, the spontaneous rate would only be of the order of $2 \times 10^{-6} \text{ s}^{-1}$ (Bell *et al.*, 1967). The rate increase brought about by Zn(II) catalysis in such a system does not appear to have been investigated, but for acetylacetone, Meany (1969) reported that the exchange rate of the methylene protons is increased approx. 20-fold by the presence of Zn(II) ions. Therefore the spontaneous rate expected in aqueous solution for the Zn(II) -ketone complex would be of the order of $4 \times 10^{-5} \text{ s}^{-1}$, as opposed to 1 s^{-1} for a ketimine intermediate. The failure to observe any C-1 hydrogen exchange with the class-II enzymes as opposed to rabbit muscle aldolase can therefore be explained by assuming that any such exchange is just due to a spontaneous process, the rate of which is dependent on the type of active-site intermediate formed. In such a case the protein part of the enzyme would dictate the stereospecificity, through stereospecific substrate binding, but not the rate of hydrogen exchange. An alternative explanation would involve a different mode of binding of the substrate C-1 phosphate group in the two classes of aldolase. Indeed it has been found that rabbit muscle aldolase differs in its reactions with arginine-selective reagents from yeast and *B. stearothermophilus* aldolases, suggesting a different mode of C-1 phosphate binding

Table 1. Rates of ^2H labelling of substrates by aldolases in $^2\text{H}_2\text{O}$ at 22°C

All the reactions were carried out at a substrate concentration of 10 mM by using 1–3 units of aldolase/ml. The buffer used was 20 mM-sodium cacodylate containing 100 mM- NaCl , except where noted.

Substrate	Aldolase source	$p^2\text{H}$	Exchange rate at C-1 ($\mu\text{mol/min}$ per unit of enzyme)
Fructose 1,6-bisphosphate	Rabbit muscle	7.50	0.115
	<i>B. stearothermophilus</i>	7.40	
	Yeast	6.35	
	Yeast	7.49	
	Yeast, +100 mM- KCl	6.46	
	Yeast, +100 mM- KCl	7.44	<0.001
Dihydroxyacetone phosphate	Rabbit muscle	7.51	0.044
	<i>B. stearothermophilus</i>	7.44	$<5.5 \times 10^{-4}$
	Yeast	7.61	<0.002

(Lobb *et al.*, 1975, 1976; R. R. Lobb, J. F. Riordan & H. A. O. Hill, unpublished work; Lobb, 1976). However, the C-1 phosphate-binding site has not yet been identified in the class-II aldolases, and hence further speculation at this stage is not justified.

On the other hand, the high exchange rate (at least 40s^{-1}) of the C-3 hydrogen in dihydroxyacetone phosphate observed with both classes of aldolase (Richards & Rutter, 1961*b*; Lowe & Pratt, 1976) must be largely due to general base catalysis by a group on the enzyme. Indeed it has long been suggested that an enzymic base is involved in the abstraction of the C-3 proton in both class-I (Hoffee *et al.*, 1967) and class-II (Ingram, 1967) aldolases. This enzymic base in rabbit muscle aldolase is thought to be histidine-359, the penultimate C-terminal residue (Hartman & Welch, 1974; Hartman & Brown, 1976). Interestingly, when this histidine is selectively modified (Hoffee *et al.*, 1967; Hartman *et al.*, 1973), or the C-terminus of the protein is modified by carboxypeptidase treatment (Rose, 1962; Rose *et al.*, 1965), the rate of C-3 hydrogen exchange falls to that of the C-1 hydrogen exchange, supporting the suggestion that the latter is a spontaneous process.

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