An Apparent Lack of Stereospecificity in the Reaction Catalysed by Deoxyribose 5-Phosphate Aldolase due to Methyl-Group Rotation and Enolization before Product Release

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In the reaction catalysed by deoxyribose 5-phosphate aldolase (2-deoxy-D-ribose 5-phosphate acetaldehyde-lyase, EC 4.1.2.4) from *Salmonella typhimurium*, almost complete equilibration of the methyl-group protons of the product, acetaldehyde, occurs before its release from the enzyme surface. This phenomenon does not allow the stereochemical course of the reaction to be determined by using hydrogen-isotope labelling of the methyl group to generate a chiral centre.

The enzyme deoxyribose 5-phosphate aldolase (2-deoxy-D-ribose 5-phosphate acetaldehyde-lyase, EC 4.1.2.4) from *Salmonella typhimurium* catalyses the reversible cleavage of deoxyribose 5-phosphate to acetaldehyde and glyceraldehyde 3-phosphate (Scheme 1) by a mechanism involving Schiff-base formation (Hoffee, 1968). During the course of this reaction the bond between C-2 and C-3 of the deoxy sugar is broken and is replaced at C-2 by a new C–H bond as a result of proton uptake. It is not known whether this reaction proceeds with retention, as with some other aldolases (Rose & Rieder, 1958; Chiu & Feingold, 1967; Meloche & Mehler, 1972), or with inversion, of configuration at the C-2 position. This problem might be solved by incubating deoxyribose 5-phosphate that has been stereospecifically deuterated in the C-2 position with the aldolase in an ^3H -containing medium, when acetaldehyde with a chiral methyl group should be produced. The absolute configuration of this presumptive chiral acetaldehyde is readily amenable to analysis by the methods developed by Cornforth *et al.* (1970). Comparison of the absolute configuration of C-2 of deoxyribose 5-phosphate and acetaldehyde would establish if the cleavage has proceeded by retention or inversion of configuration at the C-2 position.

Experimental

Materials

Deoxyribose 5-phosphate aldolase was prepared by the method of Hoffee (1968). Pure samples of $[2R\text{-}^2\text{H}]$ - and $[2S\text{-}^2\text{H}]$ -deoxyribose 5-phosphate were provided by Dr. Fraser Reid (Department of Chemistry, University of Waterloo, Waterloo, Ont., Canada) and were prepared by published methods (Radatus *et al.*, 1971). Malate synthase (EC 4.1.3.2) was prepared

from baker's yeast by the method of Dixon & Kornberg (1962). All other enzymes were normal commercial preparations obtained from Calbiochem, Los Angeles, CA, U.S.A., or Sigma Chemical Co., St. Louis, MO, U.S.A.

Methods

Chirality experiments. For the preparation of $[2\text{-}^2\text{H}_1, ^3\text{H}_1]$ acetate, incubations (260 μl) containing triethanolamine/HCl buffer, pH 7.5 (5 μmol), EDTA (0.125 μmol), KCl (30 μmol), β -mercaptoethanol (0.2 μmol), NAD^+ (0.1 μmol), $[2R\text{-}^2\text{H}_1]$ - or $[2S\text{-}^2\text{H}_1]$ -deoxyribose 5-phosphate (50 μmol), yeast aldehyde dehydrogenase (0.04 μkat), deoxyribose 5-phosphate aldolase (0.0016 μkat) and $^3\text{H}_2\text{O}$ (6×10^5 d.p.m./ μg -atom) were incubated at room temperature (22°C). The viability of the reaction was checked by observing the increase in E_{340} , and then triose phosphate isomerase (0.4 μkat) and glycerol phosphate dehydrogenase (0.04 μkat) were added as an NAD^+ -regenerating system. The reaction was continued for 13 h. The incubation mixture was applied to a column (5 cm $\times$ 0.75 cm) of Dowex 1 (Cl^- form), and after elution with 20 ml of water to remove the $^3\text{H}_2\text{O}$, the acetate was eluted with 0.005 M-HCl (12 ml).

The isolated acetates were converted into malate by the method of Cornforth *et al.* (1970). This reaction mixture was then applied to a column (5 cm $\times$ 0.75 cm) of Dowex 1 (Cl^- form), which was eluted successively with water (10 ml), 0.005 M-HCl (15 ml) and finally with 0.01 M-HCl (12 ml) to elute the malate.

The equilibration of the malate with fumarate hydratase from pig heart was carried out in 0.1 M-imidazole/HCl buffer, pH 7.0 (0.55 ml), containing the malate sample (0.01–0.1 μmol) and fumarate hydratase (0.16 μkat) for 1 h at room temperature. At

the end of the reaction the sample was freeze-dried and the radioactivity in the residue and water was compared in an ethanol/toluene counting fluid with an Intertechnique SL30 scintillation spectrometer.

The lability of the radioactivity in the malate samples was checked by incubating samples of malate (0.01–0.1 μmol) in 0.2M-glycine/NaOH buffer, pH9.5 (0.5ml), with acetylpyridine–adenine dinucleotide (0.3 μmol) and malate dehydrogenase (0.16 μkat) for 1½h at room temperature. At the end of the reaction the sample was freeze-dried and the radioactivity in the residue and water was compared.

Mass-spectral analysis experiments. Deuterated acetate was produced from unlabelled deoxyribose 5-phosphate under conditions described above for [2- $^2\text{H}_1$, $^3\text{H}_1$]acetate, except that the reaction was carried out in 100% $^2\text{H}_2\text{O}$. The isolated acetate was converted into its benzyl ester (Corina & Dunstan, 1973) and was analysed by g.l.c.–mass spectroscopy by using a column (2.75cm $\times$ 0.3cm) of 3% OV1 operated at 110°C linked to an AEI MS 30 mass spectrometer.

For the preparation of [^2H]ethanol, incubations (1ml) contained triethanolamine/HCl buffer, pH7.5 (40 μmol), EDTA (1 μmol), deoxyribose 5-phosphate (13 μmol), NADH (11 μmol), yeast alcohol dehydrogenase (1.6 μkat) and deoxyribose 5-phosphate aldolase (0.0024 μkat) in $^2\text{H}_2\text{O}$ (approx. 90–95%). Samples (50 μl) were removed after 2h and injected directly into the g.l.c.–mass-spectroscopy instrument and separated on a column (210cm $\times$ 0.3cm) of 10% Carbowax 1540 on Teflon 6 operated at 90°C. The water, which had a longer retention time than the ethanol, was 'dumped' before it reached the molecular separator. Separate analysis of NADH oxidation indicated that 2.9 μmol of ethanol had been produced while the pH had changed from 7.5 to 7.7. The pH values were measured with a type CMAT 72 electrode

from Russel pH Ltd., Auchtermuchty, Fife, Scotland, U.K. The values for pD are those of the measured 'pH' and are uncorrected.

Results and Discussion

A chiral methyl group should be produced as a result of the cleavage of [2R- ^2H]- or [2S- ^2H]deoxyribose 5-phosphate by the enzyme deoxyribose 5-phosphate aldolase in a tritiated medium provided that the enzyme is stereospecific. However, an inspection of the enzyme mechanism shown in Scheme 1 indicates that if k_{-3} is larger than k_{+4} , then a rotation of the resulting enzyme-bound methyl group in the product followed by enolization will result in a loss of chirality in the acetaldehyde. As a check that such an equilibration was not occurring, the reaction was carried out in $^3\text{H}_2\text{O}$ and the specific radioactivity of the product was determined. A reasonably low ^3H incorporation (0.5g-atom/mol) provided encouragement to pursue the problem. The back reaction (k_{-4}) was eliminated by coupling the system to a large excess of either yeast aldehyde dehydrogenase or alcohol dehydrogenase so that the acetaldehyde was immediately metabolized as soon as it was released from the enzyme surface.

Incubation of stereospecifically deuterated [2R- ^2H]- or [2S- ^2H]-deoxyribose 5-phosphate with the aldolase in the presence of $^3\text{H}_2\text{O}$ and with an excess of yeast aldehyde dehydrogenase gave, after isolation and purification, radioactive acetates. To determine if these biosynthesized acetates possessed chiral methyl groups they were analysed by the procedure developed by Cornforth *et al.* (1970), in which the acetate is first converted into malate and then the ^3H distribution between [3R- ^3H]- and [3S- ^3H]-malate is determined by dehydration by using the fumarate dehydratase reaction, which

Scheme 1.

Table 1. Analysis of presumptive $[3\text{-}^2\text{H}_1, ^3\text{H}_1]\text{malates}$ by fumarate hydratase equilibration

For details see the Experimental section. In all cases treatment of a portion of the malate with malate dehydrogenase at pH 9.5 resulted in a labilization of over 95% of the ^3H . 1 and 2 are different experiments under identical conditions.

Substrate	Radioactivity in fumarate + malate (d.p.m.)	Radioactivity in the medium (d.p.m.)	^3H labilized (%)
Commercial $[3\text{-}^3\text{H}]\text{malate}$	32100	32400	50.2
Malate derived from commercial $[2\text{-}^3\text{H}]\text{acetate}$	27600	28400	50.7
Malate derived from 1	3150	3300	51.2
$[2R\text{-}^2\text{H}_1]\text{deoxyribose 5-phosphate}$ 2	1530	1710	52.7
Malate derived from 1	1230	1250	50.6
$[2S\text{-}^2\text{H}_1]\text{deoxyribose 5-phosphate}$ 2	1215	1290	51.4

removes the 3-pro-*R* hydrogen. An unequal distribution of ^3H between the medium and the products is a measure of the chirality in the original acetate. The results are shown in Table 1 and clearly indicate a complete lack of chirality, not only in the non-specifically labelled acetates, as expected, but also in those derived from the aldolase reaction.

Because it is extremely unlikely that the aldolase reaction is non-stereospecific with regard to proton uptake, the most likely explanation for the lack of chirality was that, in fact, considerable equilibration of the methyl group must have occurred on the enzyme surface. To clarify this point, the reaction catalysed by the aldolase was carried out with unlabelled substrate in 100% $^2\text{H}_2\text{O}$, and the ^2H content of the product determined by mass spectrometry. If considerable methyl-group rotation and enolization had occurred before product release ($k_{-3} > k_{+4}$), then the product would contain more than one ^2H atom. Therefore in two separate experiments the acetaldehyde was converted into acetate or ethanol by using an excess of yeast aldehyde or alcohol dehydrogenase respectively. For the acetate experiment the isolated product was converted into its benzyl ester and analysed by g.l.c.-mass spectroscopy. This benzyl acetate gave predominantly the trideuterated species (85%) with some dideuterated material (15%) and no monodeuterated product. Analysis of the ethanol, although less accurate, confirmed this distribution, giving also the trideuterated ion as the major species. Further, the sampling of this reaction mixture at various times between 15 min and 7 h gave increasing amounts of ethanol, but with a constant deuterium distribution, a result consistent with the deuterium equilibration at the time of product formation. The fact that both the acetate and the ethanol existed primarily as the trideuterated species established a very high degree of equilibration during the aldolase reaction and explains the complete loss of chirality demonstrated in Table 1.

The low incorporation of ^3H (0.5 g-atom/mol)

initially observed must therefore reflect a fairly large ^3H -product-isotope effect in the C-H bond-forming events, since there are in reality three hydrogen atoms to be exchanged per molecule of product. In an attempt to increase the rate of product release (k_{+4}) relative to the enolization step (k_{-3}), the reaction was also performed at pH 6.5 and 8.5 in 100% $^2\text{H}_2\text{O}$. However, in both cases the trideuterated ethanol was the major product.

With pyruvate kinase, a small but significant loss of ^3H from phosphoenolpyruvate is observed during the course of the normal reaction, owing to enolization of enzyme-bound pyruvate. In this instance the rate of enolization could be greatly enhanced relative to the rate of dissociation of pyruvate by various changes in the incubation conditions, including pH and cofactors (Robinson & Rose, 1972). It is hoped that with a more detailed study of the aldolase reaction in $^2\text{H}_2\text{O}$, conditions might be found where the incorporation of ^2H is decreased to one atom per molecule of product, so that the stereochemical course of the reaction can be determined.

In summary, deoxyribose 5-phosphate aldolase from *Salmonella typhimurium* is an example of an enzyme where product release is a slow step relative to the actual chemical transformation. The presence of an enzyme-bound chiral methyl intermediate provides a sensitive probe for identifying the phenomenon in this instance, because the resulting methyl-group rotation and enolization give rise to both a loss of chirality and the incorporation of more than one ^2H atom.

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