The chemical mechanism of sheep liver 6-phosphogluconate dehydrogenase

A Schiff-base intermediate is not involved

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[2-¹⁸O]Ribulose 5-phosphate was prepared and shown to be converted enzymically by 6-phosphogluconate dehydrogenase from sheep liver into 6-phosphogluconate with complete retention of the heavy isotope. This finding unequivocally excludes the possibility of a Schiff-base mechanism for the enzyme. The involvement of metal ions has already been excluded, and other possible mechanisms are discussed. The enzyme was purified by an improved large-scale procedure, which is briefly described.

INTRODUCTION

6-Phosphogluconate dehydrogenase (EC 1.1.1.44) catalyses the conversion of 6-phosphogluconate into ribulose 5-phosphate and CO₂. The reaction is formally analogous to those catalysed by NADP⁺-linked isocitrate dehydrogenase (EC 1.1.1.42) and malic enzyme (EC 1.1.1.40):

In all these reactions, CO₂, and not HCO₃⁻, is the immediate product (Dalziel & Londesborough, 1968; Villet & Dalziel, 1969).

Isocitrate dehydrogenase and malic enzyme require added bivalent metal ions for activity, and will also catalyse the decarboxylation of oxalosuccinate and oxaloacetate respectively (Siebert *et al.*, 1957; Rutter & Lardy, 1958). These β-keto acids could therefore be intermediates in the oxidative decarboxylations of the β-hydroxy acids. 6-Phosphogluconate dehydrogenase does not require added metal ions for activity (Pontremoli *et al.*, 1961; Villet & Dalziel, 1972), and the enzyme from sheep liver does not contain firmly bound Zn²⁺, Mn²⁺ or Mg²⁺ (Villet & Dalziel, 1972). The putative keto acid intermediate, 3-keto-6-phosphogluconate, has never been

prepared. Like acetoacetate, it is a 3-keto mono-carboxylic acid and would not be thought susceptible to metal-ion-catalysed decarboxylation, a reaction confined to β-keto dicarboxylic acids (Krebs, 1942) such as oxalosuccinate and oxaloacetate. If 3-keto-6-phosphogluconate is an intermediate in the 6-phosphogluconate dehydrogenase reaction, then Schiff-base formation with a lysine residue in the enzyme is a likely step in its decarboxylation, providing an alternative method of stabilization of the carbanion intermediate by enamine formation (Scheme 1), rather than the enolate formation thought to occur with metal-ion-dependent decarboxylases and aldolases. A lysine-dependent mechanism has been established for acetoacetate decarboxylase (Hamilton & Westheimer, 1959), which does not need metal ions for activity, and also for those aldolases, such as muscle fructose biphosphate aldolase, that do not contain Zn²⁺ (Morse & Horecker, 1968). Evidence for enolization of 2-oxoglutarate by isocitrate dehydrogenase, in the presence of NADPH but not CO₂, was obtained by Rose (1960), and it was later shown that the same hydrogen atom was labilized as that which replaces the carboxy group in decarboxylation with retention of configuration (Lienhard & Rose, 1964a). Simultaneous protonation and removal of the carboxy group at C-3 are therefore improbable. With 6-phosphogluconate de-

3-Keto-6-phosphogluconate

Scheme 1.

Abbreviations used: Me₃Si, trimethylsilyl; g.c.-m.s., gas chromatography-mass spectrometry.

hydrogenase, on the other hand, although analogous stereospecific labilization of a tritium atom from C-1 of ribulose 5-phosphate could be demonstrated, it was shown that this hydrogen atom replaces the carboxy group with inversion of configuration (Lienhard & Rose, 1964b). Thus simultaneous protonation and decarboxylation at C-1 without enol intermediate formation could not be ruled out.

Many attempts to inactivate 6-phosphogluconate dehydrogenase by borohydride reduction in the presence of ribulose 5-phosphate have been made in this laboratory, without success. However, the question of whether a Schiff-base intermediate is formed can be answered more reliably by determining the fate of the keto oxygen atom of [2- ^{18}O]ribulose 5-phosphate on reductive carboxylation in the presence of the enzyme or by enzymically converting 6-phosphogluconate into ribulose 5-phosphate in an H_2^{18}O -enriched medium. A Schiff-base mechanism would demand the exchange of the ^{18}O label of [2- ^{18}O]ribulose 5-phosphate with the ^{18}O of the water medium in the first case, leading to isotopically normal 6-phosphogluconate, whereas the ribulose 5-phosphate produced by oxidative decarboxylation in an isotopically enriched medium would be expected to contain ^{18}O in excess of natural abundance. On the other hand, if an enolate (enol) intermediate is involved, there would be no obligatory exchange of the keto oxygen atom of [2- ^{18}O]ribulose 5-phosphate, and the C-3 oxygen atom of 6-phosphogluconate would be labelled to the same extent as the substrate for reductive carboxylation. Accordingly, unlabelled ribulose 5-phosphate would be formed from unlabelled 6-phosphogluconate when oxidative decarboxylation is carried out in H_2^{18}O . The present paper reports the results of such experiments with sheep liver 6-phosphogluconate dehydrogenase. Substrates and simply derived related compounds were analysed for their ^{18}O content as their volatile trimethylsilylated derivatives by g.c.-m.s.

MATERIALS AND METHODS

Materials

6-Phosphogluconate (trisodium salt), NADP^+ (disodium salt) and NADPH (tetrasodium salt) were obtained from Boehringer Corp. (Lewes, East Sussex, U.K.). D-Ribulose 5-phosphate (disodium salt), carbazole, L-cysteine hydrochloride, DL-isocitrate (trisodium salt), *threo*-D $_3$ -isocitrate (monopotassium salt), bis(trimethylsilyl)trifluoroacetamide with 1% Me_3SiCl added and 2-mercaptoethanol were from Sigma Chemical Co. (Poole, Dorset, U.K.). H_2^{18}O (98.3 atom%) was obtained from Prochem (British Oxygen Co.) (London S.W.19 U.K.) (now supplied by Amersham International, Amersham, Bucks., U.K.) and agarose-C 8 -(6-amino-hexyl)amino- NADP^+ (agarose- NADP^+ , type 3) from P-L Biochemicals (Milwaukee, WI, U.S.A.). CM-Sephadex C-50, Sephadex G-25 and Sephadex G-100 were from Pharmacia (London W.5, U.K.). All other chemicals were of the highest quality available and were obtained from Fisons Scientific Apparatus (Loughborough, Leics., U.K.) or from Sigma Chemical Co.

Enzyme, coenzyme and substrate assay

6-Phosphogluconate dehydrogenase and bovine heart mitochondrial isocitrate dehydrogenase were assayed spectrophotometrically as described by Silverberg &

Dalziel (1973) and Macfarlane *et al.* (1977) respectively. For use in the isotope experiments, isocitrate dehydrogenase, prepared by the method of Macfarlane *et al.* (1977), was precipitated with $(\text{NH}_4)_2\text{SO}_4$, centrifuged, the pellet resuspended in 50 mM-sodium phosphate buffer, pH 7.4, containing 4.8 mM- MgCl_2 and 33 μM -EDTA, and the suspension finally clarified by centrifugation.

NADPH solutions were assayed by absorption measurements at 340 nm by using a molar absorption coefficient of $\epsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Concentrations of NADP^+ and 6-phosphogluconate were determined enzymically with 6-phosphogluconate dehydrogenase and an excess of the other component of the reaction. Ribulose 5-phosphate was assayed by the cysteine/carbazole method of Dische & Borenfreund (1951), as modified by Ashwell & Hickman (1957).

Protein determination

During the protein purification procedure for 6-phosphogluconate dehydrogenase described below, protein was determined in the first step by the method of Warburg & Christian (Layne, 1957), and in the following steps from the absorbance at 280 nm alone, assuming $A_{280}^{1\%} = 10.0$. The concentration of pure enzyme was calculated by using an absorption coefficient of $A_{280}^{1\%} = 11.4$ (Silverberg, 1973; Silverberg & Dalziel, 1973).

Purification of 6-phosphogluconate dehydrogenase

The enzyme was purified from sheep liver by a modification of the method described by Silverberg & Dalziel (1973), in which affinity chromatography replaces the rather variable DEAE-Sephadex chromatography and second $(\text{NH}_4)_2\text{SO}_4$ fractionation (pH 8.0) steps. The procedure was carried out at 0–4 °C, and all buffer solutions contained 1 mM-EDTA.

The initial extraction and $(\text{NH}_4)_2\text{SO}_4$ fractionation (pH 6.0) were performed as described by Silverberg & Dalziel (1973), and the dialysed protein solution was applied to a column of Sephadex G-100 (bed volume 2.0 litres, diameter 5.0 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-2-mercaptoethanol. The column was washed with the same buffer at a flow rate of 30–40 ml/h, and fractions (15–20 ml) containing enzyme were pooled. Protein solutions of up to at least 200 ml could be applied to the column, since, although the specific activity of the pooled enzyme-containing eluent was only about half of that which could be obtained by application of the sample at 5% of the bed volume to a bigger column, it made no difference to the specific activity after affinity chromatography. The pooled enzyme-containing eluent was pumped directly at 30–40 ml/h on to a column (16.0 cm $\times$ 2.0 cm) of agarose- NADP^+ (Lee & Kaplan, 1975) equilibrated with 10 mM-sodium phosphate buffer, pH 7.0, and the column washed with 15 column volumes of the same buffer. Non-specific protein was eluted with 0.135 M-KCl in 10 mM-sodium phosphate buffer, pH 7.0, and the washing was continued until a constant absorbance at 280 nm was reached. No leakage of the enzyme was observed. The enzyme was eluted with 30 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /50 mM- NaH_2PO_4 /NaOH, pH 7.0, pyrophosphate being a competitive inhibitor of 6-phosphogluconate dehydrogenase with respect to both NADP^+ and 6-phosphogluconate (Dyson & D'Orazio, 1973). Fractions (15–20 ml) with specific activities of greater than 8.0 units/mg were

Table 1. Purification of 6-phosphogluconate dehydrogenase from 2.21 kg of sheep liver

For details see the Materials and methods section. One unit of activity is as defined by Silverberg & Dalziel (1973).

Step	Volume (ml)	Concn. of protein (mg/ml)	Total activity (units)	Activity (units/ml)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
1. Extract after acid treatment	3560	32.9	3327	0.93	0.028	100	1.0
2. (NH ₄) ₂ SO ₄ fractionation	193	134.2	2609	13.52	0.101	78	3.6
3. Sephadex G-100	787	25.7	2533	3.22	0.125	76	4.5
4. Affinity chromatography and concentration	36	3.26	1730	48.1	14.7	52	526
5. CM-Sephadex and concentration	14	3.98 (3.50*)	978	69.9	17.5 (20.0*)	29	626

* These values for the pure enzyme are calculated from the true absorption coefficient of $A_{280}^{1\%} = 11.4$ estimated from dry-weight determinations (Silverberg, 1973).

pooled and concentrated by ultrafiltration (Amicon 202 cell, PM 30 membrane) to 30–40 ml. The enzyme solution was then centrifuged (40000 *g* for 20 min) to remove denatured protein. The specific activity at this stage (Table 1) represents a 100–130-fold purification for this step, with recoveries in the range 62–70%. The enzyme solution was then desalted by gel filtration on a column (125 cm × 3.0 cm) of Sephadex G-25 equilibrated with 10 mM-sodium phosphate buffer, pH 6.8, containing 0.05 M-NaCl, and applied to a column (42 cm × 2.5 cm) of CM-Sephadex C-50 pre-equilibrated with the same buffer. The column was immediately developed with a linear 0.05–0.20 M-NaCl gradient (total volume 2.0 litres). The flow rate was 27–32 ml/h. Peak fractions (13.5–16.0 ml) with specific activities of not less than 15.8 units/mg (or 18.0 units/mg in terms of the true absorption coefficient of $A_{280}^{1\%} = 11.4$) were pooled and concentrated to about 30 ml by ultrafiltration (Amicon 202 cell, PM 30 membrane). Then 100 ml of 0.125 M-potassium phosphate buffer, pH 7.0, was added, and the volume was finally decreased to 10–16 ml by ultrafiltration. The enzyme solution was centrifuged (40000 *g* for 20 min) and stored at 4 °C.

The specific activity of the final product, 20.0–21.2 units/mg, is similar to that obtained by Silverberg & Dalziel (1973), namely 21.2 units/mg, but is significantly larger than the values reported by either Griffiths *et al.* (1977) or by Carne (1982) when these are corrected appropriately as described by Topham (1984).

The enzyme appeared to be essentially homogeneous as judged by the constant specific activity of the eluates from CM-Sephadex chromatography, and the fact that a single protein band was obtained when samples (25–70 µg) were analysed by 7.5% (w/v)-polyacrylamide-gel electrophoresis in the presence of SDS by the method of Weber *et al.* (1972).

Preparation of [2-¹⁸O]ribulose 5-phosphate

This was prepared by dissolving sufficient ribulose 5-phosphate in 0.2 ml of H₂¹⁸O (98.3 atom%) to give a final concentration of 0.18 M (approx. pH 6.0–6.5), and incubating for 24 h at room temperature in a Teflon-lined screw-top vial.

Reductive carboxylation of [2-¹⁸O]ribulose 5-phosphate

To convert sufficient labelled ribulose 5-phosphate into 6-phosphogluconate for analysis, an NADPH-regene-

rating system utilizing isocitrate dehydrogenase and Mg²⁺-isocitrate was employed, taking advantage of the favourable ratio of the equilibrium constants ([6-phosphogluconate]·[2-oxoglutarate]/[ribulose 5-phosphate]·[isocitrate] ~ 10) for the two reactions (Villet & Dalziel, 1969; Londesborough & Dalziel, 1968). The reaction mixture (4.0 ml) contained: *threo*-D₅-isocitrate, 2.2 mM; MgCl₂, 4.5 mM; NADPH, 0.11 mM; EDTA, 38 µM; 6-phosphogluconate dehydrogenase, 10.5 units; isocitrate dehydrogenase, 22.5 units in a 0.15 M solution of NaHCO₃ saturated with CO₂, pH 6.91; [2-¹⁸O]ribulose 5-phosphate, 2.2 mM. The last-mentioned compound was added last as 50 µl of 0.18 M solution in H₂¹⁸O, the final solution thus being only 1.2 atom% excess with respect to H₂¹⁸O. The mixture was incubated for 7 min at 20 °C, under a small CO₂-containing headspace, at which time a 1 ml sample was removed and acidified with 0.25 ml of 2 M-HCl, vortex-mixed until CO₂ gas ceased to be evolved, and rapidly frozen in a solid-CO₂/ethanol bath. A second sample (2 ml) was removed after 11 min of incubation and treated with 0.25 ml of freshly prepared 1% (w/v) NaBH₄ in water. After 5 min at room temperature, when the remaining [2-¹⁸O]ribulose 5-phosphate had been quantitatively reduced, as indicated by the cysteine/carbazole test in control experiments, the excess borohydride was destroyed by the addition of 0.5 ml of 2 M-HCl, and the solution quickly frozen. The two samples were then freeze-dried in Teflon-lined screw-top test tubes (10 cm × 1.2 cm) suitable for the preparation of derivatives.

Oxidative decarboxylation of 6-phosphogluconate in H₂¹⁸O-enriched medium

The final reaction mixture (0.5 ml), which was 78.5 atom% excess with respect to H₂¹⁸O, comprised 3.5 units of 6-phosphogluconate dehydrogenase, 5.02 µmol of 6-phosphogluconate and 3.51 µmol of NADP⁺ in 10 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA. After 90 s of incubation at 25 °C, when 29.3% of the 6-phosphogluconate had been converted into ribulose 5-phosphate, 1 ml of freshly prepared 1% NaBH₄ in water was added. The mixture was left for 5 min at room temperature, and the excess borohydride was then destroyed by the addition of 0.4 ml of 2M-HCl, with vigorous vortex-mixing until H₂ evolution ceased. The solution was frozen in a solid CO₂/ethanol bath. The oxidative decarboxylation of

6-phosphogluconate was conducted in the same way in isotopically normal water, and the two frozen samples were freeze-dried in Teflon-lined screw-top test tubes (10 cm $\times$ 1.2 cm).

Preparation of Me₃Si derivatives from reaction mixtures

NaBH₄-treated samples were first extracted two or three times with 1 ml portions of methanol and dried under a stream of N₂, to remove completely the boric acid as volatile methyl borate (Zill *et al.*, 1953). Formation of the Me₃Si ethers required that the phosphate esters be protonated, rather than as the metal salts. Because of difficulties encountered during the earlier stages of this work, some samples were acidified, frozen and sublimed a second time, to obtain increased yields of the Me₃Si derivatives. Accordingly, after methanol extraction, the borohydride-treated sample from the reductive carboxylation experiment was re-acidified with 0.2 ml of 2 M-HCl and those from the oxidative decarboxylation experiment were acidified again with 0.5 ml of 0.25 M-HCl. The sample from the reductive carboxylation reaction that had not been treated with borohydride was not re-acidified. To prepare the Me₃Si derivatives, 200 μ l of anhydrous pyridine (stored over KOH) was added to each freeze-dried residue. After being briefly vortex-mixed and dried under a stream of N₂, 100 μ l of anhydrous pyridine and 200 μ l of bis(trimethylsilyl)trifluoroacetamide containing 1% Me₃SiCl were added, and the mixtures were heated at 100 °C for 1 h. The samples were then dried again under a stream of N₂, and a fresh portion (100 μ l) of bis(trimethylsilyl)trifluoroacetamide containing 1% Me₃SiCl was added before heating at 60 °C for 5 min.

Me₃Si derivatives of alditol 5-phosphates and 6-phosphogluconate

To 0.05 ml of 0.18 M-[2-¹⁸O]ribulose 5-phosphate, prepared as previously described, 0.5 ml of freshly prepared 1% NaBH₄ was added. After 5 min at room temperature, 0.15 ml of 2 M-HCl was added, and the sample trimethylsilylated as described above, except that the second acidification step was omitted. 6-Phosphogluconate (1.7 mg in 0.6 ml of 0.33 M-HCl) was trimethylsilylated in a similar way.

G.c.-m.s.

A VG Micromass 12B mass spectrometer interfaced by a glass jet separator to a Varian 2440 gas chromatograph was used. G.c. was carried out on a 2 m $\times$ 2 mm (internal diam.) glass column packed with 3% SE-30 on Gas-Chrom Q (100–200 mesh) (Applied Science Laboratories, State College, PA, U.S.A.). The He carrier gas flow rate was 30 ml/min. The injector temperature was maintained at 300 °C, and the column was oven-programmed from 200 °C at 4 °C/min. The separator temperature was 320 °C. Mass spectra were scanned at 25 eV ionizing potential, with a trap current of 200 μ A and an accelerating voltage of 2.4 kV. The source temperature was maintained at 260 °C. The scan rate was 3 s per decade exponential down. Spectra were acquired and processed with a VG Data System type 2050.

RESULTS

Isotopic analysis of [2-¹⁸O]ribulose 5-phosphate

This could only be achieved in a reliable manner by quenching the exchange reaction before trimethylsilyla-

tion by reducing the keto sugar phosphate with NaBH₄ to form the two C-2 epimers, arabinitol 5-phosphate and ribitol 5-phosphate. The Me₃Si derivatives of these isomers were partially resolved by g.c., and the ¹⁸O content of either derivative was determined from the shift of the [M–15]⁺ ion at *m/z* 649 to *m/z* 651. This ion is known to arise from the loss of a methyl radical from an Me₃Si group of the molecular ion [M]⁺ (McCloskey *et al.*, 1968). After allowing for the contributions of the natural isotopes of Si and C at *m/z* 651, the ribulose 5-phosphate preparation was found to contain 81 atom% excess ¹⁸O.

Reductive carboxylation of [2-¹⁸O]ribulose 5-phosphate

This was carried out in the presence of CO₂, an NADPH-regenerating system and 6-phosphogluconate dehydrogenase. After a 7 min period of incubation, the reaction mixture was acidified, then frozen, and the phosphate esters of interest were separated by g.c. as their Me₃Si ethers. Four main peaks were seen in the total ion-current chromatogram, and these were identified by means of their mass spectra. The first two peaks corresponded to the Me₃Si derivatives of ribulose 5-phosphate and the enol form of ribulose 5-phosphate. In accord with earlier observations during trial experiments, neither derivative contained ¹⁸O in excess of natural abundance, the label presumably having been lost by back exchange during the work-up procedure, and the retention of ¹⁸O in the substrate was established by taking a second sample from the reaction mixture after 11 min incubation and treating it with borohydride before trimethylsilylation and analysis: 79 atom% excess ¹⁸O was found in the Me₃Si-alditol 5-phosphate with the greater retention time.

The 6-phosphogluconate product of the reductive carboxylation of [2-¹⁸O]ribulose 5-phosphate when trimethylsilylated also ran as two peaks in the gas chromatogram. The first, much larger, peak was identified as the fully trimethylsilylated derivative of a lactone form of 6-phosphogluconate, and the second as Me₃Si-6-phosphogluconate itself. Both these derivatives were found to contain the same level of ¹⁸O enrichment as the

Fig. 1. Portion of the mass spectrum of Me₃Si-[3-¹⁸O]6-phosphogluconolactone showing the shift of *m/z* 603 ([M–15]⁺) to *m/z* 605

Fig. 2. Portion of the mass spectrum of $\text{Me}_3\text{Si}-[3\text{-}^{18}\text{O}]\text{6-phosphogluconate}$ showing the shift of m/z 471 to m/z 473

keto sugar phosphate substrate. Theoretically the lactone could be either 6-phosphoglucono-1,4-lactone or 6-phosphoglucono-1,5-lactone, these being the only forms possessing a stable five- or six-membered ring structure. It is probably the 1,4-lactone that Bauer *et al.* (1983) have prepared in an apparently specific manner from 6-phosphogluconate by allowing the intramolecular esterification to occur under acidic conditions, similar to those that would prevail during the slight melting of the frozen acidified mixture that took place during freeze-drying. Indeed, when the isotopically normal 6-phosphogluconate standard was trimethylsilylated, only the Me_3Si -lactone was observed in the gas chromatogram. The mass spectrum of the Me_3Si -lactone from the enzyme reaction mixture, recorded with a high-speed recorder on light-sensitive chart paper, showed that the $[M-15]^+$ ion at m/z 603 had been shifted by 2 mass units (Fig. 1), indicating the presence of 80 atom% excess ^{18}O and representing 99% retention of keto oxygen atom of ribulose 5-phosphate. The calculated contributions of the natural isotopes of Si and C at m/z 605 were allowed for in this estimation. It was also apparent that ions at m/z 157, 204, 217, 230, 243, 289 and 333 largely retained the O-3 atom. The $[M-105]^+$ ion at m/z 513, which is generally recognized to arise from the loss of the neutral fragments ($\text{CH}_3^+ + \text{Me}_3\text{SiOH}$) from the molecular ion, did not contain ^{18}O , and this is attributed to the specific loss of an Me_3SiOH molecule from the C-3 position.

Zinbo & Sherman (1970) first described the 70 eV mass spectrum of completely trimethylsilylated 6-phosphogluconate, and gave details of some of the fragmentation processes that occur. Comparison of this spectrum with that obtained from the enzyme reaction mixture aided not only the identification of the Me_3Si derivative, but also helped to establish the positions of several of the lower-mass ions (m/z 157, 204, 230 and 333) carrying a significant proportion of the ^{18}O isotope. A separate spectrum of the higher-mass ions was recorded on light-sensitive chart paper, from which it was possible to estimate the extent of ^{18}O incorporation, the most useful ion being m/z 471, shifted to m/z 473 (Fig. 2) and indicating 84 atom% excess ^{18}O . This ion is formed by the direct loss of an Me_3SiOH molecule from m/z 561, as evidenced by the presence of a metastable ion at m/z 395.6 in the 70 eV mass spectrum (Zinbo & Sherman, 1970). Given that the fragment ion at m/z 561 would arise as a result of chain cleavage at the C-2-C-3 bond, the elemental composition of the ion at m/z 471 can be deduced to be $\text{C}_{16}\text{H}_{40}\text{O}_6\text{PSi}_4$. The reported ratio of ion intensities at m/z 471 and m/z 473 in the 70 eV mass spectrum of isotopically normal 6-phosphogluconate (1:0.23) (see Stenhagen *et al.*, 1970-1972) is in close agreement with that predicted solely on the basis of the

contributions of the natural isotopes of Si and C at m/z 473 from the m/z 471 ion (1:0.16), and so it is clear (at least in the 70 eV mass spectrum) that no other ion contributes at m/z 473. The use of the shift of m/z 471 to m/z 473 to determine the level of ^{18}O enrichment in Me_3Si -6-phosphogluconate from the trimethylsilylated reaction mixture (84 atom% excess ^{18}O) is therefore justified. The result is in good agreement with the value obtained for the Me_3Si -lactone, and confirms that reductive carboxylation occurs with complete retention of the C-2 oxygen atom of ribulose 5-phosphate. The presence of label was also apparent in ions at m/z 561 and 675 ($[M-105]^+$), but the peaks were not sufficiently well defined for isotopic analysis.

Oxidative decarboxylation of 6-phosphogluconate in H_2^{18}O

This reaction was conducted at pH 7.0 at 25 °C in the presence of 78.5 atom% excess H_2^{18}O . Mass-spectral scans were recorded on light-sensitive chart paper as the partially resolved mixture of Me_3Si -alditol 5-phosphates entered the mass spectrometer. Neither of the isomers exhibited a mass-spectral pattern characteristic of a labelled molecule. Measurement of the proportions of ion intensities at m/z 649, 650 and 651 gave 1:0.50:0.31 for the Me_3Si -alditol 5-phosphate with the greater retention times, compared with 1:0.64:0.36 obtained for the corresponding derivative prepared from an identical reaction mixture but containing isotopically normal water.

DISCUSSION

The experiments show that $[2\text{-}^{18}\text{O}]\text{ribulose 5-phosphate}$ (81 atom% excess ^{18}O), prepared by exchange of the keto oxygen atom with H_2^{18}O , is converted enzymically into 6-phosphogluconate with complete retention of the heavy isotope, 80 atom% excess ^{18}O being found in a completely trimethylsilylated lactone form of the phosphate ester and 84 atom% excess ^{18}O in the Me_3Si derivative of 6-phosphogluconate itself. This finding was confirmed by conducting the oxidative decarboxylation of isotopically normal 6-phosphogluconate in ^{18}O -enriched aqueous medium (78.5 atom% excess ^{18}O), and showing that the keto sugar phosphate product was devoid of label.

These observations unequivocally exclude the possibility of a Schiff-base intermediate in the overall enzyme-catalysed reaction, as this would demand exchange of the C-2 oxygen atom of ribulose 5-phosphate with the water of the medium. The untenability of a mechanism involving the formation of a Schiff base between the enzyme and keto substrate also provides an explanation of the failure to inactivate the enzyme specifically by borohydride reduction in the presence of ribulose 5-phosphate (Silverberg, 1973). Mechanistically, these results are somewhat surprising, in view of the recognition of both lysine and metal-ion-dependent decarboxylases and aldolases, but are not unprecedented. The decarboxylase involved in the C-4 demethylation step of cholesterol biosynthesis also catalyses the reaction without Schiff-base formation, and like 6-phosphogluconate dehydrogenase does not require metal ions (Wilton & Akhtar, 1975).

A reasonable mechanism for the enzyme-catalysed oxidative decarboxylation of 6-phosphogluconate should provide for the stabilization of the transition state formed

Scheme 2.

during decarboxylation. This may be effected by enzyme-mediated protonation of the carbonyl group of the putative intermediate, 3-keto-6-phosphogluconate, to give an enol rather than a less-stable enolate intermediate (Scheme 2). The X-ray-crystallographic studies of the enzyme at 0.26 nm (2.6 Å) resolution by Adams *et al.* (1983) show that there are several amino acid residues capable of acting as a proton donor and acceptor in the active-site region. Glutamate-195 and glutamate-222 as well as tyrosine-196 are present in a pocket thought to be the substrate-binding site. Aspartate-188 is also close to the pocket. However, the histidine residue that, from chemical-modification studies in this laboratory, seems to be essential for activity (Topham, 1984) might be particularly suited to such a role. Although this histidine residue, tentatively identified as histidine-242, points away from the pocket and is 0.8 nm (8 Å) distant from it, it is tempting to speculate that a substrate-induced conformational change allows the closer approach of histidine-242 to the carbonyl oxygen atom of the postulated intermediate. Such an interpretation requires confirmation by further X-ray-crystallographic studies.

Alternatively, it is possible that oxidative decarboxylation may occur without the existence of a distinct β -keto acid intermediate by concerted oxidation and decarboxylation through a single transition state. This type of mechanism was first suggested for malic enzyme and isocitrate dehydrogenase by Boyer (1960). Siebert *et al.* (1957) had previously found only minor incorporation of label from $^{14}\text{CO}_2$ or $[^{14}\text{C}]$ isocitrate into a pool of oxalosuccinate in the presence of heart muscle isocitrate dehydrogenase and coenzyme, and it was concluded that the β -keto acid must be firmly bound to the enzyme. In the concerted mechanism (Scheme 3), decarboxylation is facilitated by concomitant electron withdrawal from the β -carbon atom owing to the transfer of a hydride ion to NADP^+ , leading to the direct production of an enol. In both these mechanisms, however, ketonization of the enol might still have to occur via an unstable enolate ion, but Bender & Breslow (1962) have suggested that enol-ketone conversions may be concerted processes involving

Scheme 3.

acid-base catalysis, and such mechanisms are clearly suited to reactions occurring on an enzyme surface.

To distinguish experimentally between a concerted mechanism and one in which dehydrogenation precedes decarboxylation is a challenging problem. One worthwhile approach makes use of observed multiple isotope effects on kinetic parameters, and has been used to demonstrate that the oxidative decarboxylation of malate catalysed by chicken liver malic enzyme is a stepwise reaction with hydride transfer preceding decarboxylation (Hermes *et al.*, 1982).

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