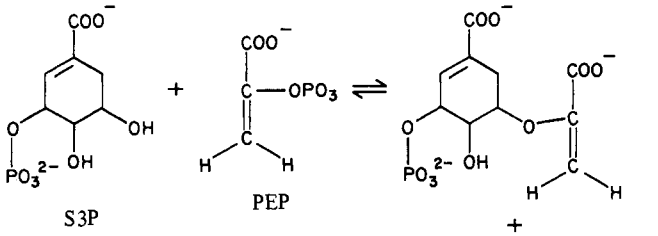
Mechanism of Enolpyruvyl Shikimate-3-phosphate Synthase Exchange of Phosphoenolpyruvate with Solvent Protons†

David L. Anton,‡ Lizbeth Hedstrom, Susan M. Fish, and Robert H. Abeles*

ABSTRACT: Enolpyruvyl shikimate-3-phosphate synthase (EC 2.5.1.19) was purified to near homogeneity (~90% purity) from *Klebsiella pneumoniae*. Glyphosate, a herbicide, competitively inhibits with phosphoenolpyruvate (PEP) with $K_i = 10^{-5}$ M. An analogue of shikimate 3-phosphate, 4,5-dideoxyshikimate 3-phosphate (ddS3P), competitively inhibits with shikimate 3-phosphate with $K_i = 10^{-2}$ M. The enzyme is inactivated by 3-bromopyruvate. $T_{1/2}$ for inactivation is approximately 5 min at 0.15 mM 3-bromopyruvate. PEP protects against inactivation by 3-bromopyruvate, while ddS3P alone has no effect on the rate of inactivation. The protective effect of PEP is enhanced approximately 16-fold in the presence of ddS3P. The enzyme catalyzes exchange of solvent ^3H into PEP in the presence of ddS3P but not in its absence. No exchange is observed between PEP and P_i in the presence of ddS3P, nor does "scrambling" of the bridge oxygen of PEP occur. These results establish that the C-5 OH group of shikimate 3-phosphate is not required to form the complex involved in the exchange of the methylene hydrogens of PEP with solvent protons. The results are consistent with the intermediate formation of an enzyme–PEP complex analogous to that proposed for UDP-GlcNAc-pyruvyl transferase [Zemell, R. I., & Anwar, R. A. (1975) *J. Biol. Chem. 250*, 4959–4964]. It is suggested that glyphosate is a transition-state analogue for the PEP-derived α-carbonium ion in which the positively charged nitrogen corresponds to the carbonium ion.

Enolpyruvyl shikimate-3-phosphate (ES3P)[1] synthase (EC 2.5.1.19) catalyzes the reaction shown in eq 1. A mechanism

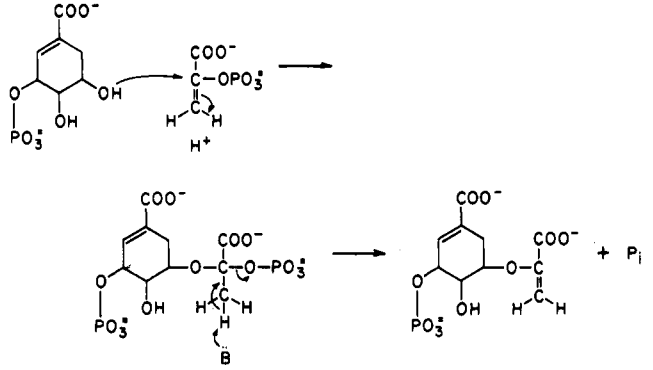


(1)

proposed for this reaction is outlined in Scheme I. According to that mechanism, the OH group of S3P initially adds to the double bond of PEP, and subsequently, P_i is eliminated to give the final reaction product (Levin & Sprinson, 1964). Two experimental observations support this mechanism. First, PEP labeled with ^{18}O in the bridge position produces [^{18}O]phosphate, consistent with C–O bond cleavage (Bondinell et al., 1971; Ife et al., 1976). Second, when the reaction is carried out in $[^3H]H_2O$ or $[^2H]H_2O$, isotopic hydrogen is incorporated into the pyruvyl moiety of the product, 5-(enolpyruvyl)shikimate 3-phosphate, as well as into PEP. When the reaction is carried out with [3-^{2}H]PEP, the pyruvyl moiety of the product contains less ^2H than the substrate. Equal amounts of ^2H are lost from the *E* and *Z* position (Bondinell et al., 1971; Grimshaw et al., 1982). Exchange between the hydrogen of the 3 position of PEP and solvent protons has been observed only in the presence of both substrates, i.e., PEP and S3P.

Scheme II shows an alternate mechanism, in which a negatively charged group at the active site stabilizes an intermediate carbonium ion. Possibly, a covalent bond may actually form between the enzyme and PEP, rather than the ion pair shown in Scheme II. According to Scheme II, PEP and enzyme react to form intermediate I in which the β-carbon of PEP is protonated. Scheme II indicates two possible pathways by which intermediate I can react to form products. A mechanism similar to the one shown in Scheme II has been proposed for UDP-GlcNAc-pyruvyl transferase, an enzyme that catalyzes a very similar reaction, the addition of PEP to the OH group of UDP-GlcNAc (Cassidy & Kahan, 1973; Zemell & Anwar, 1975). With the enzyme obtained from *Micrococcus lysodeikticuss*, an adduct of PEP and the transferase has been isolated. This adduct corresponds to structure I (Scheme II) (Cassidy & Kahan, 1973). Others, working with purified enzyme from *Enterobacter cloacae*, have isolated an enzyme–substrate complex that contains the carbon skeleton of PEP but not the phosphate moiety of PEP (Zemell

Scheme I: Mechanism of Action of ES3P Synthase



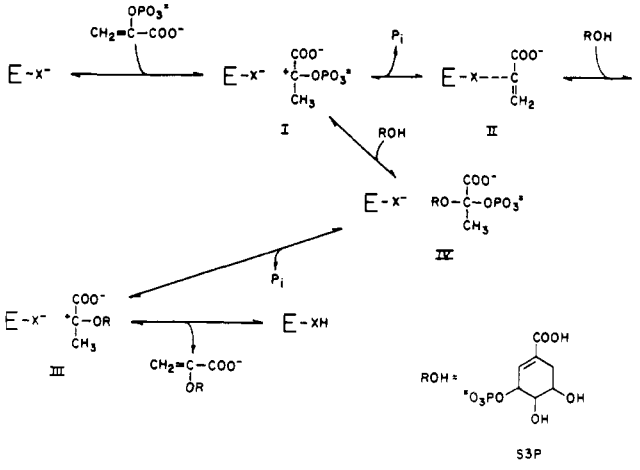
† From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received May 18, 1983.* This is Publication No. 1459 from the Graduate Department of Biochemistry. This work was supported by a research grant to R.H.A. from the National Science Foundation (PCM 80-07670) and by a grant from Shell Development Co. (81-0411) and by a National Institutes of Health Postdoctoral Fellowship to D.L.A. (GM 07517).

‡ Present address: Harvard Medical School, Beth Israel Hospital, Boston, MA 02215.

[1] Abbreviations: ES3P, enolpyruvyl shikimate-3-phosphate; PEP, phosphoenolpyruvate; S3P, shikimate 3-phosphate; ddS3P, 4,5-dideoxyshikimate 3-phosphate (for structure see Materials and Methods); DAHP, 3-deoxy-2-keto-D-*arabino*-heptulosonic acid 7-phosphate; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TCA, trichloroacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/83/0422-5903$01.50/0

Scheme II: Mechanism of Action of ES3P Synthase



& Anwar, 1975). Such an adduct corresponds to II in Scheme II.

In view of the results obtained with UDP-GlcNAc-pyruvyl transferase, it seems likely that the mechanism shown in Scheme II is valid for the reaction catalyzed by ES3P synthase rather than the mechanism in Scheme I. Evidence in support of the mechanism of Scheme II would be provided if it could be established that ES3P synthase catalyzes the exchange of the hydrogen in the 3-position of PEP with solvent protons in the absence of the second substrate, S3P. We have established conditions under which this exchange occurs. These results, as well as some additional properties of ES3P synthase, are reported here.

Materials and Methods

Cyclohexanecarboxylic acid, *N*-bromosuccinamide, and diazald were purchased from Aldrich. Fosfomycin was a gift of Merck and Co., and glyphosate was a gift of Monsanto. [^{18}O]H_2O was purchased from Cambridge Isotope Laboratories. DAHP synthase was a gift from Dr. Klaus Herrmann. Erythrose 4-phosphate was synthesized as previously described (Baxter et al., 1959). Shikimate 3-phosphate was prepared by fermentation with *Aerobacter aerogenes* A170-40 (American Type Culture Collection 25597) as described by Knowles & Sprinson (1970). The barium salt produced by this procedure was converted to the potassium salt by treatment of an aqueous solution with excess Dowex 50W (H^+ form 200–400 mesh) followed by neutralization with potassium hydroxide.

Inorganic phosphate was determined by the procedure of Cassidy & Kahan (1973). Total phosphate was measured by first ashing the sample (Ames & Dubin, 1960) and then determining inorganic phosphate. A Beckman LS-100C liquid scintillation counter was used to determine radioactivity with ACS (Amersham) as scintillation fluid. Proton NMR spectra were obtained on a Brucker FT WH-90 and are reported as δ relative to tetramethylsilane.

Enzyme Assays. ES3P synthase preparations prior to the step of DEAE-cellulose chromatography were assayed by measuring S3P-dependent loss of PEP. The reaction mixtures contained 2 mM PEP, 10 mM Tris-HCl, 10 mM NaF, 3 mM S3P, and enzyme at pH 7.6 in a final volume of 100 μL at 37 °C. Incubations were terminated by dilution with 150 μL of ice-cold Tris-HCl, 10 mM, pH 7.6, prior to heating in a boiling water bath. Aliquots were withdrawn for determination of PEP content (Gollub et al., 1967).

ES3P synthase preparations after DEAE-cellulose chromatography were assayed by inorganic phosphate release (Ames et al., 1960). Reaction conditions were as above. The reaction was stopped after 10 min at 37 °C by the addition of 0.9 mL of the ascorbate molybdate reagent. A unit of activity is defined as the loss of 1 μmol of PEP or the production of 1 μmol of P_i/min at 37 °C. UDP-GlcNAc-pyruvyl transferase was assayed in crude extracts by the procedure of Cassidy & Kahan (1973).

Synthesis of DL-*3-Hydroxy-1-cyclohexenecarboxylic Acid Dihydrogen Phosphate (4,5-Dideoxyshikimate Phosphate).* (*A*) *Methyl 1-Cyclohexenecarboxylate.* Cyclohexanecarboxylic acid was converted to Methyl 1-cyclohexenecarboxylate by the procedure of Bailey & Baylouny (1959) except that methyl 1-bromocyclohexanecarboxylate was refluxed overnight in anhydrous pyridine instead of trimethylpyridine: ^{1}H NMR ($CDCl_3$) δ 1.60 (m, 4 H, $-CH_2$), 2.17 (m, 4 H, $-CH_2$), 3.71 (s, 3 H, $-OCH_3$), 6.96 (m, 1 H, H–C).

(*B*) *Methyl Bromo-1-cyclohexenecarboxylate.* Methyl 1-cyclohexenecarboxylate (10 g, 0.072 mol) was heated under reflux with *N*-bromosuccinamide (12 g, 0.068 mol) and benzoyl peroxide (100 mg) in 30 mL of redistilled carbon tetrachloride. After 2.5 h, all the *N*-bromosuccinamide had reacted. The succinamide was filtered off, and the solvent was removed in vacuo. The residue was vacuum distilled. The 3- and 6-bromo isomers distilled at 77–85 °C at 0.4 mmHg: yield 9.7 g, 62%; ^{1}H NMR ($CDCl_3$) mixture of 3- and 6-bromo isomers δ 1.5–2.4 (m, 6 H, $-CH_2-$), 3.72 and 3.75 (2 s, 3 H, $-OCH_3$, isomeric), 4.83 and 5.20 (2 m, 1 H, H–C–Br, isomeric), 6.95 (br m, 1 H, H–C).

Methyl bromo-1-cyclohexenecarboxylate was phosphorylated as follows (Tener et al., 1957). Methyl bromo-1-cyclohexenecarboxylate (3 g, 0.014 mol) was dissolved in 15 mL of anhydrous benzene and dibenzyl phosphate (3.9 g, 0.014 mol) and triethylamine (1.92 mL, 1.39 g, 0.014 mol) in 15 mL of benzene was added. This mixture was stirred overnight at room temperature and then heated under reflux for 3 h. After the mixture cooled on ice, the precipitate that formed was removed by filtration and washed with benzene, and the combined benzene washes were concentrated in vacuo. The residue was taken up in 25 mL of methanol and hydrogenated over 0.6 g of 5% palladium on carbon. The theoretical amount of hydrogen was taken up in 35 min. The catalyst was removed by filtration through celite, and the solvent was removed in vacuo. The residue, dissolved in water and adjusted to pH 11 with 1 N NaOH, was refluxed for 1 h. The pH was maintained at 11 by addition of NaOH. The aqueous solution was adjusted to pH 7.5 with 1 N HCl and loaded on a 2.5 × 40 cm column of Dowex 1-X8 (200–400 mesh), chloride form, equilibrated in water. Fractions (20 mL) were collected. The column was washed with water, 0.015 M HCl (2 L), 0.03 M HCl (1 L), 0.04 M HCl (1 L), and finally with 0.05 M HCl. The phosphorylated compound is eluted with 0.05 M HCl. Fractions that contained organic phosphate (assayed as total phosphate) were pooled, neutralized with NH_4OH, and concentrated in vacuo to 30 mL. Generally, three peaks elute with 0.05 M HCl containing organic phosphate. One of these is benzyl phosphate. The other two are the isomers of dideoxyshikimate phosphate. The desired isomer is identified by NMR. To the fraction containing the desired isomer (30 mL) was added 3 equiv, on the basis of total phosphate, of 1 M barium acetate, followed by 180 mL of 95% ethanol. The precipitated barium salt collected after remaining overnight at 4 °C was washed with 95% ethanol and absolute ethanol and dried in vacuo; yield 1.3 g.

Samples were further purified by chromatography on a 2.5 × 30 cm column of DEAE-cellulose (DE-32 Whatman)

Table I: Carbon-13 NMR Spectrum of Dideoxyshikimate 3-Phosphate[a]

	carbon				
	1	2	3	4, 5, and 6	7
chemical shift[b]	138.85	133.61 (J_{CCOP} = 5.99 Hz)	69.40 (J_{COP} ~ 1 Hz)	29.68, 25.27, 19.25	177.21
splitting in off-resonance-decoupled spectra[c]	s	d	d	t, t, t	s

[a] Carbon-13 NMR spectra were obtained with a Bruker WM-250 spectrometer at 62.9 mHz with D_2O as solvent. [b] Decoupled spectra were obtained with broad-band noise decoupling and a 5-s pulse delay. Chemical shifts are δ relative to tetramethylsilane as external standard. [c] Off-resonance-decoupled spectra were obtained with the decoupler frequency set at 2700 Hz and a 1.5-s pulse delay.

equilibrated in 20 mM ammonium bicarbonate, pH 8.0. The column was washed with the same buffer and eluted with a 1-L gradient (500 mL/flask) from 20 to 300 mM ammonium bicarbonate, pH 8.0. Fractions containing dideoxyshikimate phosphate were lyophilized, resuspended in water, and lyophilized again: NMR (D_2O) δ 6.35 (s, 1 H, H–C), 4.75 (br s, HOD and H–C–OP), 1.5–2.0 (br m, 6 H, $-CH_2-$). ^{13}C NMR data[2] are summarized in Table I.

Purification of ES3P Synthase. *Klebsiella pneumoniae* (ATCC 8724) were grown aerobically to mid log phase in a 120-L fermentor as described by Morgan et al. (1963). The cell paste was collected by continuous centrifugation and stored frozen until use. Cell paste, 130 g, was thawed in 700 mL of Tris–succinate, 10 mM, and dithioerythritol, 0.1 mM, pH 6.8 (enzyme buffer). This suspension was sonicated at full power on a Branson sonifier for 30 min in three pulses. The temperature was kept below 10 °C. Cell debris was removed by centrifugation at 27200*g* for 15 min. To this crude extract was added 1.0 mL of 2% protamine sulfate per 100 mg of protein while the pH was maintained at 6.8 by the addition of 1 M Tris base. After a 1-h stirring at 4 °C, the precipitate was removed by centrifugation at 17700*g* for 15 min.

To the protamine sulfate supernate was added solid ammonium sulfate to a final concentration of 35% saturation, and the pH was maintained at 6.8 by the addition of Tris base. After 30 min, the precipitate was removed by centrifugation at 17700*g* for 15 min. The supernate solution was adjusted to 70% saturation with ammonium sulfate as above and the precipitate collected after 1 h by centrifugation at 17700*g* for 30 min. The 35–70% ammonium sulfate fraction was suspended in 100 mL of enzyme buffer and dialyzed overnight against 20 volumes of enzyme buffer containing 1 mM potassium ethylenediaminetetraacetic acid with several changes.

The dialyzed ammonium sulfate fraction was added to a 2.5 × 37 cm column of DE-32 DEAE-cellulose equilibrated with enzyme buffer. The column was washed with 300 mL of the buffer and eluted with a 4-L (2 L per chamber) linear gradient going from enzyme buffer to 100 mM Tris–succinate–0.1 mM dithioerythritol, pH 6.8. The fractions containing peak enzyme activity were pooled and concentrated by the addition of ammonium sulfate to 90% saturation, and this was resuspended in 2 mL of enzyme buffer. This was loaded on a 1.7 × 75 cm column of Sephadex G-150 equilibrated in enzyme buffer. Fractions with peak enzyme activity were pooled and concentrated by adsorbing activity on 0.5 × 3 cm column of DEAE-cellulose DE-32 equilibrated in enzyme buffer and eluting with 100 mM Tris–succinate–0.1 mM dithioerythritol, pH 6.8. This enzyme was stored at −20 °C.

Tritium Exchange Experiments. The composition of the reaction mixture is described in Table III. The reaction was terminated by bulb to bulb distillation in a sealed glass apparatus to remove water. The residue was dissolved in the original volume of water and distilled again. Carrier PEP (25 μmol) was added and the reaction mixture chromatographed on 1.5 × 20 cm column of Dowex 1-Cl (X8) according to the procedure of Dinovo & Boyer (1971).

Table II: Purification of Enolpyruvyl Shikimate-3-phosphate Synthase

	total vol (mL)	protein (mg/mL)	total units	sp act.
sonicate	740	18	799	0.06
redissolved dialyzed $(NH_4)_2SO_4$ precipitate	169	27.4	788	0.17
DEAE pool	175	0.55	422	4.4
concentrated G-150 eluate				
pool I	3.7	0.43	58	36
pool II	4.6	0.37	78	45

In some experiments, the PEP recovered from Dowex 1 chromatography was enzymatically converted to lactate with a mixture of pyruvate kinase and lactate dehydrogenase, ADP, and NADH. The lactate produced was subjected to high-pressure liquid chromatography (HPLC) analysis on a Bio-Rad organic acid column eluted with 5 mM H_2SO_4. Lactate was detected on a differential refractometer (Waters).

^{18}O Scrambling. Synthesis of [^{18}O]PEP. PEP with the enolic oxygen specifically labeled with ^{18}O was prepared by a modification of published procedures (Bondinell et al., 1971). Bromopyruvate (0.5g, 3.5 mmol) was incubated with 98% [^{18}O]H_2O (1.0 g) overnight. The solution was lyophilized and the bromopyruvate treated with trimethyl phosphite (Stubbe & Kenyon, 1972). The PEP thus prepared contained ^{18}O in the phosphoryl oxygens as well as the enolic positions. The ^{18}O in the phosphoryl oxygens was removed by treating the PEP (570 μmol) with 1 N HCl (10 mL) at 100 °C for 5 min (O'Neal et al., 1983).

After incubation with the enzyme and [^{18}O]PEP, the reaction was quenched with 10% trichloroacetic acid and centrifuged, and the supernate was applied to a Dowex 1-Cl column as described for the tritium exchange experiment. PEP was eluted and concentrated to dryness. The residue was dissolved in water and brought to pH 10.5 with KOH. PEP was hydroyzed by the addition of alkaline phosphatase or with DAHP synthase. The inorganic phosphate produced was isolated as $MgNH_4PO_4$ precipitate (Boyer & Bryan, 1967) and converted to trimethyl phosphate as described by Middlefort & Rose (1976). The content of trimethyl [^{18}O]-phosphate was determined from the ratio of *m/e* 112 vs. 110 by mass spectroscopic analysis with a Hewlett-Packer 5985B spectrometer.

Results

Enzyme Purification. ES3P synthase has been purified more than 700-fold by the described procedure. This preparation is at least 95% homogeneous as judged by SDS–polyacrylamide disc gel electrophresis. The results of a typical purification procedure are shown in Table II.

[2] We thank G. R. Bratt, Department of Biochemistry at the University of Minneapolis, for carrying out the ^{13}C NMR determinations.

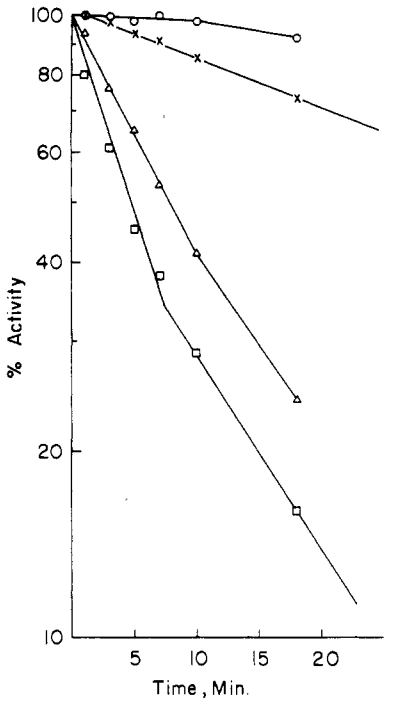


FIGURE 1: Inactivation of ES3P synthase by bromopyruvate. Reaction conditions are as in Table III with addition and modifications as indicated: PEP, 5 mM (○); bromopyruvate, 0.15 mM (□); bromopyruvate, 0.15 mM, + PEP, 5 mM (△); bromopyruvate, 0.15 mM, + PEP, 5 mM, + ddS3P, 20 mM (×). Reaction was at 37 °C; 0.065 unit of enzyme was used. At indicated times, 5-μL aliquots were withdrawn and added to standard assay mixture.

Inhibitors and Inactivators of ES3P Synthase. The effect of a number of compounds on the reaction catalyzed by ES3P synthase was examined. Glyphosate, a herbicide, inhibits ES3P synthase from plants (Steinrucken & Amrhein, 1980; Amrhein et al., 1980). The compound also inhibits the bacterial enzyme. The inhibition is competitive with PEP with K_i = 0.01 mM (S3P at 6 mM) as determined from reciprocal plots. Under these conditions K_m = 0.5 mM for PEP. Glyphosate[3] at 5 mM showed no detectable inhibition of UDP-GlcNAc-pyruvyl transferase. That enzyme catalyzes a reaction similar to ES3P synthase in that the pyruvyl moiety of PEP is transferred to a –OH.

4,5-Dideoxyshikimate phosphate, an analogue of S3P that does not have the OH group that reacts with PEP inhibits ES3P synthase and is competitive with S3P with K_i = 10 mM (PEP at 5 mM). Under these conditions, K_m for S3P is 3 mM. The compound is racemic, and presumably, only one of the two isomers is inhibitory.

Time-dependent inactivation of ES3P synthase occurs with bromopyruvate. The data are shown in Figure 1. The rate of inactivation is not affected by ddS3P (data not shown) in the absence of PEP and is decreased by PEP in the absence of ddS3P. The protective effect of PEP is greatly enhanced by ddS3P. The data show that PEP binds to the enzyme in the absence of the second substrate. Under these conditions, K_d for PEP is approximately 16 mM, as compared to K_m for PEP of 0.5 mM obtained from initial velocity data. The binding of PEP is enhances in the presence of 20 mM ddS3P; K_d is approximately 1.0 mM.

No inhibition was observed with 3.0 mM iodoacetate or with 3.0 mM iodoacetamide in the absence or presence of 3.0 mM S3P. Other compounds tested were phospholactate (35 mM), 4-phosphonobutyrate, 3-phosphonopropionate (30 mM), and phospho-PEP (15 mM). These compounds showed no effect on the reaction.

[3] Glyphosate, $^{2-}O_3PCH_2N^+H_2CH_2COO^-$.

Table III: Exchange of ^3H into PEP[a]

additions	reaction time (h)	total cpm of ^3H in PEP ($\times 10^{-3}$)
PEP, 1 mM	3	0
PEP, 5 mM	3	<1.5
PEP, 1 mM, and ddS3P, 20 mM	3	76.4
PEP, 5 mM, and ddS3P, 20 mM	3	185
PEP, 1 mM, and ddS3P, 20 mM	1	28.7
PEP, 1 mM, ddS3P, 20 mM, and glyphosate, 5 mM	3	54.5[b]

[a] The reaction contained in addition to the above compounds the following: NaF, 10 mM, Tris-Cl, pH 7.6, 100 mM, in a total volume of 0.25 mL, $[^3H]H_2O$, 3 × 10^{11} cpm/mL, and enzyme, 0.29 unit (sp act. 21), 37 °C. PEP was isolated and ^3H content determined as described under Materials and Methods. [b] The inhibition by glyphosate is less than would be expected on the basis of the K_i for glyphosate determined kinetically, i.e., in the presence of S3P. We have established, with experiments other than the ones cited in Table III, that glyphosphate binds to enzyme-ddS3P much less tightly than to enzyme-S3P. Binding of PEP, on the other hand, is affected to a lesser extent (R. H. Abeles and S. M. Fish, unpublished results).

Exchange Reactions. Incorporation of solvent H^+ into the β-position of PEP, catalyzed by ES3P synthase, would provide evidence for the formation of an enzyme–substrate adduct. When the enzyme was allowed to react with PEP in $[^3H]H_2O$, no ^3H incorporation was observed (Table II). Possibly, the presence of the second substrate (S3P), or an analogue of the second substrate, is required for the formation of such an adduct. We therefore carried out the exchange reaction in the presence of ddS3P. Under these conditions, ^3H was incorporated into PEP (Table III). The rate of ^3H incorporation was reduced by glyphosate, an inhibitor competitive with PEP.

The identity of $[^3H]$PEP, formed in the exchange reaction, was confirmed by converting the compound to lactate with pyruvate kinase, lactic dehydrogenase, NADH, and ADP. The resulting $[^3H]$lactate cochromotographed with authentic lactate on HPLC.

The exchange reaction described above is consistent with the formation of an enzyme–substrate complex. If the complex corresponds to structure II, Scheme II, then exchange should occur between PEP and P_i. This point was tested. An incubation was carried out in the presence of ddS3P under conditions of Table III except that 3.3 μmol of $[^{32}P]P_i$ (1.1×10^7 cpm/μmol) was added, PEP was 1 mM, and the total volume was 650 μL. After 2 h, the reaction was terminated, and PEP was reisolated. PEP contained no ^{32}P; i.e., the specific activity of PEP was <200 cpm/μmol. Therefore, the enzyme does not catalyze exchange between P_i and PEP the absence of S3P.

Possibly, P_i is eliminated (or displaced) from PEP but not released from the enzyme. This possibility was examined with the oxygen scrambling technique developed by Rose (Middlefort & Rose, 1976). PEP containing approximately 62% ^{18}O in the bridge position was allowed to react with ES3P synthase in the presence of ddS3P (20 mM) under conditions of Table IV. At the end of the reaction, PEP was isolated as described under Materials and Methods, and the amount of ^{18}O in the nonbridging position was determined. The results are summarized in Table IV. Two kinds of experiments were done: (1) P_i from $[^{18}O]$PEP was obtained by reaction with DAHP synthase, and its ^{18}O content was determined. This gives the total ^{18}O content in the phosphate moiety of PEP including the bridge oxygen (experiment 1, Table IV). (2)

Table IV: ^{18}O Content of P_i Derived from PEP Containing ^{18}O in the Bridge Position

expt	sample	% ^{18}O enrichment[a]
1	P_i from [^{18}O]PEP obtained with DAHP synthase[b]	61.5 ± 0.1
2	P_i from [^{18}O]PEP treated with ES3P[c]	6.0 ± 0.1
3	P_i from [^{18}O]PEP control[d]	5.1 ± 0.1

[a] The ^{18}O enrichment was determined by mass spectral analysis of the trimethyl phosphate derivatives. The ^{18}O content was calculated from the *m/e* 110 and 112 peaks because of the greater absorbance of these peaks relative to the molecular ion peaks at *m/e* 140 and 142. [b] A solution (50 mL) containing [^{18}O]PEP (1.7 mM), erythrose 4-phosphate (0.85 mM), BSA (0.6 mg/mL), Hepes (0.1 M, pH 7.0), and DAHP synthase was incubated 2 h at 37 °C. The protein was removed by TCA precipitation and the phosphate isolated as a magnesium-ammonium precipitate. [c] A solution containing ES3P synthase (0.81 mg, 22 units), ddS3P (20 mM), [^{18}O]PEP (5 mM), NaF (10 mM), and Tris (0.1 M, pH 7.6) was incubated at 37 °C for 24 h. The protein was removed by TCA precipitation and the PEP purified by Dowex 1-Cl chromatography. The PEP was hydrolyzed by treatment with alkaline phosphatase and phosphate isolated as a magnesium-ammonium precipitate. [d] A solution (2.0 mL) containing only [^{18}O]PEP (5 mM) and Tris (0.1 M, pH 7.6) was incubated at 37 °C for 24 h and treated identically with that described in footnote *b*.

PEP was hydrolyzed with alkaline phosphatase. This reaction proceeds with P–O cleavage; therefore, the resulting P_i will not contain the bridge oxygen of PEP. If scrambling occurs, ^{18}O from the bridge oxygen should appear in the P_i obtained through alkaline phosphatase treatment. If cleavage of the C–O bond of PEP (scrambling) had occurred at 0.001 μmol min^{-1} (mg of enzyme)$^{-1}$, 4×10^5 slower than the normal reaction, the enrichment observed would have been 10%, i.e., 5% above the control experiment (experiment 2). The value actually observed was 6.0% (experiment 3). Therefore, no significant scrambling of the oxygens of the phosphate moiety occurred.

Discussion

ES3P synthase catalyzes the exchange of the methylene hydrogens of PEP with solvent protons in the presence of an analogue of S3P that does not have the OH group that interacts with PEP. This observation establishes that addition of the hydroxyl group of S3P to PEP is not required for the exchange reaction to occur, and therefore, the mechanism shown in Scheme I does not apply. This result is consistent with the mechanism shown in Scheme II, i.e., a mechanism involving an enzyme–substrate complex. The lack of exchange between P_i and PEP and the failure to observe scrambling of the bridge oxygen indicate that the PEP–enzyme complex formed under these conditions is complex I (Scheme II). This does not exclude the involvement of complex II in the catalytic process since the formation of that complex may not occur in the absence of the normal substrate. The mechanism of action of ES3P synthase is, therefore, probably very similar to that proposed for UDP-GlcNAc-pyruvyl transferase, an enzyme that catalyzes an analogous reaction.

The rate of exchange of the methylene hydrogens in the presence of ddS3P proceeds 900-fold slower than the V_{max} in the forward direction for the normal reactions. In the normal catalytic process, the rate of ^3H incorporation into reaction product from [^3H]H_2O is 4-fold slower than product formation. ^3H incorporation into unreacted PEP is 10-fold slower than into the reaction product (Grimshaw et al., 1982). The tritium incorporation rate observed in these experiments is, therefore, 225-fold slower than the fastest incorporation rate observed in the normal catalytic process.

An alternate explanation for the ^3H incorporation into PEP reported here is that H_2O adds to pyruvate in place of S3P. We consider this extremely unlikely. The adduct resulting from the addition of H_2O to PEP is, on chemical grounds, very unstable and would form pyruvate. Furthermore, the catalytic action of the enzyme should also convert this adduct to pyruvate. Experiments were carried out to establish whether ES3P synthase catalyzes pyruvate formation from PEP in the presence of ddS3P. No pyruvate was detected.[4]

The data suggest that there are two modes in which PEP binds to the enzyme. PEP protects the enzyme against inactivation by bromopyruvate and therefore can bind to the enzyme in the absence of the second substrate. The K_d for this enzyme–PEP complex is approximately 16 mM. However, little or no exchange of the PEP methylene protons is observed. The exchange is maximally 1% that observed in the presence of 20 mM ddS3P. Thus, there is a binding mode, in the absence of the second substrate, in which the methylene hydrogens of PEP are not subject to exchange. A second binding mode occurs in the ternary complex. In the presence of 20 mM ddS3P, binding of PEP is increased approximately 16-fold and the exchange reaction at least 150-fold. Thus, only in the presence of ddS3P, or presumably also in the presence of S3P, the normal substrate, is the covalent complex formed from which exchange can occur.

ES3P synthase is inactivated by 3-bromopyruvate but not by iodoacetate or iodoacetamide. This suggests that it does not simply act as a nonspecific alkylating agent but is an active site directed inactivator. UDP-GlcNAc-pyruvyl transferase is also inactivated by 3-bromopyruvate (Anwar & Vlaovic, 1980). This susceptibility to the same active site directed reagent further illustrates the similarity of the two enzymes. Glyphosate inhibits ES3P synthase competitively with PEP, with K_i = 0.01 mM. A possible reason for this tight binding may be that glyphosate is a transition-state analogue in which the positively charged nitrogen mimics the carbonium ion. Another example of a transition-state analogue of a carbonium ion has recently been reported in which a nitrogen replaces the carbonium ion (Sandifer et al., 1982). If glyphosate is indeed a transition-state analogue, it is surprising that this compound does not inhibit UDP-GlcNAc-pyruvyl transferase. Possibly in that reaction the transition state has less carbonium ion character; i.e., the reaction is more concerted.

Registry No. ES3P synthase, 9068-73-9; S3P, 63959-45-5; PEP, 138-08-9; ddS3P, 87518-54-5; glyphosate, 1071-83-6; 3-bromopyruvate, 1113-59-3; methyl 1-cyclohexenecarboxylate, 18448-47-0; methyl 3-bromo-1-cyclohexenecarboxylate, 87518-55-6; methyl 6-bromo-1-cyclohexenecarboxylate, 87518-56-7.

References

Ames, B. N., & Dubin, A. T. (1960) *J. Biol. Chem. 235*, 769–775.

Amrhein, N., Schab, J., & Steinrueken, H. C. (1980) *Naturwissenschaften 67*, 356–357.

Anwar, R. A., & Vlaovic, M. (1980) *Biochim. Biophys. Acta 616*, 389–394.

Bailey, W. J., & Baylouny, R. A. (1959) *J. Am. Chem. Soc. 81*, 2126–2129.

Baxter, J. N., Perlin, A. S., & Simpson, F. J. (1959) *Can. J. Biochem. Physiol. 37*, 199–209.

Bondinell, W. E., Vnek, J., Knowles, P. F., Sprecher, M., & Sprinson, D. B. (1971) *J. Biol. Chem. 246*, 6191–6196.

[4] R. H. Abeles and S. M. Fish, unpublished results.

Boyer, P. D., & Bryan, D. M. (1967) *Methods Enzymol. 10*, 60-71.
Cassidy, P. J., & Kahan, F. M. (1973) *Biochemistry 12*, 1364-1374.
Deleo, A. B., & Sprinson, D. B. (1968) *Biochem. Biophys. Res. Commun. 32*, 873-877.
Dinovo, E. C., & Boyer, P. D. (1971) *J. Biol. Chem. 246*, 4586-4593.
Furniss, B. S., Ed. (1978) *Vogel's Textbook of Practical Organic Chemistry*, Longman Group Ltd., London.
Gollub, E., Zalkin, H., & Sprinson, D. B. (1967) *J. Biol. Chem. 242*, 5323-5328.
Grimshaw, C. E., Sogo, S. G., & Knowles, J. R. (1982) *J. Biol. Chem. 257*, 596-598.
Gunetileke, K. G., & Anwar, R. A. (1968) *J. Biol. Chem. 243*, 5770-5778.
Ife, R. J., Ball, L. F., Lowe, P., & Haslam, E. (1976) *J. Chem. Soc., Perkin Trans. 1*, 1776-1783.
Knowles, P. F., & Sprinson, D. B. (1970) *Methods Enzymol. 17A*, 351-352.
Levin, J. G., & Sprinson, D. B. (1964) *J. Biol. Chem. 239*, 1142-1150.
McConnell, R. L., & Coover, H. W., Jr. (1956) *J. Am. Chem. Soc. 78*, 4453-4455.
Middlefort, C. F., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5881-5887.
Millican, R. C. (1970) *Methods Enzymol. 17A*, 352-354.
Morgan, P. N., Gibson, M. I., & Gibson, F. (1963) *Biochem. J. 89*, 229-239.
O'Leary, M. H., DeGooyer, W. J., Dougherty, T. M., & Anderson, V. (1981) *Biochem. Biophys. Res. Commun. 100*, 1320-1325.
O'Neal, C. C., Jr., Bild, G. S., & Smith, L. T. (1983) *Biochemistry 22*, 611-617.
Sandifer, R. M., Thompson, M. D., Gaughan, R. G., & Poulter, C. D. (1982) *J. Am. Chem. Soc. 104*, 7376-6366.
Steinrucken, H. C., & Amrhein, N. (1980) *Biochem. Biophys. Res. Commun. 94*, 1207-1212.
Stubbe, J. A., & Kenyon, G. L. (1971) *Biochemistry 10*, 2669-2677.
Stubbe, J. A., & Kenyon, G. L. (1972) *Biochemistry 11*, 338-345.
Stubbe, J. A., & Ables, R. H., (1980) *Biochemistry 19*, 5505-5512.
Tener, G. M., Wright, R. S., & Khorana, H. G. (1957) *J. Am. Chem. Soc. 79*, 441-443.
Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384-421.
Wauchope, D. (1976) *J. Agric. Food Chem. 24*, 717-721.
Zemell, R. I., & Anwar, R. A. (1975) *J. Biol. Chem. 250*, 4959-4964.

Rabbit Muscle Phosphofructokinase. 1. Activation by Affinity Labeling Approximately Two Adenosine Cyclic 3′,5′-Monophosphate Binding Sites per Tetramer†

James W. Ogilvie

ABSTRACT: The smallest enzymatically active form of rabbit muscle phosphofructokinase consists of four identical or nearly identical subunits, and each subunit contains one binding site specific for the activating adenine nucleotides cAMP, AMP, and ADP. These activator binding sites on the enzyme have been covalently labeled to various degrees, ranging from an average value of less than one label/tetramer to four labels/tetramer, with the affinity label 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine, and the kinetic and regulatory properties of these modified phosphofructokinase preparations have been investigated. The kinetic and regulatory properties of the affinity-labeled phosphofructokinase are essentially identical with those of native enzyme activated by cAMP, and a near maximum activation of the affinity-labeled enzyme is observed in those preparations modified to the extent of two or more groups/tetramer, suggesting that the covalent attachment of approximately two affinity labels/tetramer is necessary and sufficient for full activation of the enzyme. This requirement for approximately two groups/tetramer for full activation of the enzyme has been substantiated by dissociating a solution containing both native enzyme and affinity-labeled enzyme modified to the extent of approximately four groups/tetramer and then allowing the resulting solution of labeled and unlabeled monomers to reassemble into active tetramers and observing that the extent of activation in the solution of reassembled enzyme is greater than that initially observed in the enzyme solution before dissociation.

Rabbit skeletal muscle phosphofructokinase (EC 2.7.1.11) is comprised of identical or nearly identical subunits of M_r 80 000, and the smallest enzymatically active form of the enzyme is a tetramer (Paetkau & Lardy, 1967; Pavelich & Hammes, 1973; Aaronson & Frieden, 1972; Lad et al., 1973). Below pH 7.5, phosphofructokinase is activated and inhibited by a number of metabolites, enabling the enzyme to play a key role in the regulation of the glycolytic pathway. Among the metabolites that influence the kinetic properties of phosphofructokinase are the adenine nucleotides ATP, ADP, AMP, and cAMP.[1] Not only is ATP a substrate for the enzyme but

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received February 24, 1983.* This work was supported by a grant from the National Institutes of Health (GM 27840).

[1] Abbreviations: 5′-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine; SO_2BzAdo, covalently bound 5′-(*p*-sulfonylbenzoyl)adenosine group; cAMP, adenosine cyclic 3′,5′-monophosphate; PFK, phosphofructokinase; F-6-P, fructose 6-phosphate; F-1,6-P_2, fructose 1,6-diphosphate; Gdn·HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-5908$01.50/0