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Mechanism of Action of Isopentenyl Pyrophosphate Isomerase: Evidence for a Carbonium Ion Intermediate[†]

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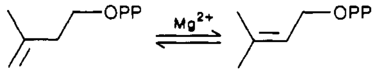
ABSTRACT: Isopentenyl pyrophosphate isomerase catalyzes the interconversion of isopentenyl pyrophosphate and dimethylallyl pyrophosphate. The isomerase from yeast has been purified to near homogeneity (purity >90%). The substrate analogue (*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate reacts at $<1.8 \times 10^{-6}$ times the rate of dimethylallyl pyrophosphate. The enzyme is irreversibly inactivated by 2-(dimethylamino)ethyl pyrophosphate (I). These observations are consistent with a carbonium ion mechanism for the isomerization. Compound I is an analogue of the intermediate carbonium ion and probably acts as a transition state analogue. For I, $k_{on}' = 2.1 \times 10^6$ M^{-1} min^{-1}. No off-rate was detected and, therefore, $K_i < 1.4 \times 10^{-11}$ M. Upon denaturation of the inactivated enzyme, I is released unchanged. 2-(Trimethylammonio)ethyl pyrophosphate also inhibits with $K_i' = 7 \times 10^{-7}$ M, $k_{on}' = 4.4 \times 10^4$ M^{-1} min^{-1}, and $k_{off} = 0.03$ min^{-1}. Substrate analogues without a positively charged nitrogen were relatively poor inhibitors. The best inhibitor of these is ethyl pyrophosphate, $K_i = 10^{-4}$ M. The enzyme is inactivated by sulfhydryl-selective reagents. These reagents also prevent binding of I to the enzyme. The inactivation by iodoacetamide is dependent upon one ionizable group (pK = 9.3). The pH dependence of V and V/K for the isomerase-catalyzed reaction also depends upon a group with pK = 9.3.

Isopentenyl pyrophosphate isomerase (EC 5.3.3.2, isopentenyl-diphosphate Δ-isomerase) catalyzes the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate.



The stereochemistry of the isomerase reaction is consistent with a two-base mechanism (Cornforth et al., 1972). Studies on the isomerase-catalyzed exchange of tritium from ^3H_2O into both isopentenyl pyrophosphate and dimethylallyl pyrophosphate are consistent with either a carbonium ion or a carbanion mechanism (Agranoff et al., 1960; Shah et al., 1965). Evidence for a carbonium ion mechanism has been provided by the inhibition of isopentenyl pyrophosphate isomerase by 2-(dimethylamino)ethyl pyrophosphate (I), the ammonium ion analogue of the proposed carbonium ion in the isomerase-catalyzed reaction (Reardon & Abeles, 1985). Ammonium ion analogues of carbonium ion intermediates are potent inhibitors for several enzymes in the polyisoprene biosynthetic pathway (Sandifer et al., 1982; Narula et al., 1981; Rahier et al., 1985). In this paper, we extend the study of the mechanism of inhibition by I and other structurally related amines.

Additionally, studies were carried out to further characterize the mechanism of action of isopentenyl pyrophosphate isomerase. We have examined the effect of isosteric substitution of a trifluoromethyl group on substrate turnover. Substitution of the electron-withdrawing trifluoromethyl group for the methyl group at C_5 of the substrate would be expected to dramatically increase the energy of the transition state for carbonium ion formation but not for carbanion formation. This approach has been used previously to provide evidence in support of a carbonium ion mechanism in the reaction catalyzed by prenyl transferase (Poulter & Satterwhite, 1977).

MATERIALS AND METHODS

Reagents. Alkaline phosphatase was purchased from Sigma Chemical Co. [4-^{14}C]Isopentenyl pyrophosphate and NaB^3H_4 were purchased from New England Nuclear. Crystalline orthophosphoric acid was purchased from Fluka Chemical Corp. Bis(triethylammonium) phosphate was prepared according to Cornforth and Popjak (1969). Triethylammonium bicarbonate (TEAB)[1] was prepared by passing CO_2 gas, from dry ice, through a gas dispersion frit into a cold-stirred 1 M solution of triethylamine until the pH was 7. 2-(Dimethylamino)ethyl phosphate was prepared according to Ansell and Spanner (1962). 3-Bromo-3-butenol was prepared according to Cousseau (1980). 4-Bromo-3-methyl-3-butenol was prepared according to Cornforth et al. (1966). The product mixture, 90% trans–10% cis, was separated on a reversed-phase semiprep HPLC column in 50% MeOH/H_2O. 2-Cyclopropylethanol was prepared from 3-buten-1-ol by a Simmons–Smith reaction (Winstein & Sonnenberg, 1961). The zinc–copper couple was prepared according to Shank and Schecter (1959): bp 55 °C (20 torr); ^{1}H NMR ($CDCl_3$) δ 0.26 (m, 2 H), 0.63 (m, 2 H), 0.90 (m, 1 H), 1.65 (t, 2 H), 3.16 (s, br, 1 H), 3.86 (t, 2 H).

Synthesis of Trifluoromethyl Substrate Analogues. Both (*E*)- and (*Z*)-3-(trifluoromethyl)-2-butenol were prepared by reduction of the corresponding ethyl esters (Poulter & Satterwhite, 1977). Both isomers were obtained in pure form from a spinning band distillation column. A third compound, which distilled between the *E* and *Z* ethyl esters, was also collected from the spinning band column. The ^{1}H NMR [($CDCl_3$) δ

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; KP_i, potassium phosphate; TEAB, triethylammonium bicarbonate; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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1.26 (t, 3 H), 3.17 (s, br, 2 H), 4.19 (q, 2 H), 5.55 (m, 1 H), 5.83 (m, 1 H)] is consistent with the structure of the [1,3] sigmatropic rearrangement product ethyl 3-(trifluoromethyl)-3-butenoate. The distillation fraction containing this isomer also contained the *E* and *Z* isomers in a 1:1:1 ratio. After lithium aluminum hydride reduction of this mixture, the *E* and *Z* isomers of 3-(trifluoromethyl)-2-butenol were obtained with a third compound [^{1}H NMR ($CDCl_3$) δ 2.45 (t, 2 H), 2.75 (t, br, 1 H), 3.76 (q, 2 H), 5.40 (m, 1 H), 5.73 (m, 1 H)], consistent with the structure of 3-(trifluoromethyl)-3-butenol. This mixture of isomers was used as a carrier for the radiochemical studies described later.

General Synthetic Procedures. Alkyl phosphates were prepared according to Cornforth and Popjak (1969). The pyrophosphate monoester was then prepared by using the diphenyl phosphorochloridate coupling method (Michelson, 1964). Allyl pyrophosphates were prepared directly from the alcohol according to Cornforth and Popjak (1969). In all cases, pyrophosphate monoesters were purified by anion-exchange chromatography. Solvent was removed from the reaction mixture (2-mmol scale) by rotary evaporation in vacuo, and the sample was dissolved in 100 mL of H_2O. After the pH was adjusted to 8.0, the sample was applied to a 1.5 × 45 cm QAE-Sephadex A-25 column in the bicarbonate form. The column was washed with one column volume of H_2O and eluted with a 600-mL linear TEAB gradient from 0.20 to 0.60 M. Fractions containing pyrophosphate monoester were pooled and 1 mL of 1 N NaOH was added. The buffer was removed by rotary evaporation in vacuo. Several additions of ethanol were required to remove residual TEAB. The product was precipitated in ethanol and collected by centrifugation. Samples were dissolved in H_2O and stored at −20 °C. Samples for NMR analysis were passed through a small column of Dowex 50, Na^+ form, and lyophilized. The purity of pyrophosphate monoesters was also checked by high-voltage paper electrophoresis in pyridinium acetate, pH 3.5. Phosphate-containing compounds were visualized with ammonium molybdate–perchloric acid spray (Hanes & Isherwood, 1949). The concentration of substrate and inhibitor samples was determined by total phosphate analysis (Ames & Dubin, 1960).

2-(Dimethylamino)ethyl Pyrophosphate (I). To the stirred solution of 6.1 g (0.10 mol) of ethanolamine in 25 mL of dry pyridine was added 8.5 g (0.05 mol) of benzyl chloroformate dropwise over a 2-h period at 4 °C. Stirring was continued for an additional 1 h at room temperature. The reaction mixture was diluted with 150 mL of diethyl ether, washed 4 times with 100-mL portions of 3% H_2SO_4 and 1 time with 100 mL of H_2O, and dried over $MgSO_4$. Solvent was removed by rotary evaporation in vacuo to give 7.1 g (73% yield) of a colorless liquid: ^{1}H NMR [for *N*-carbobenzoxyethanolamine] ($CDCl_3$) δ 3.23 (q, 2 H), 3.56 (t, 2 H), 3.73 (s, br, 1 H), 5.03 (s, 2 H), 5.76 (t, br, 1 H), 7.25 (s, 5 H). 2-(Carbobenzoxyamino)ethyl phosphate was prepared according to Cornforth & Popjak (1969), and the pyrophosphate was prepared by the method of Michelson (1964): ^{1}H NMR (D_2O) δ 3.4 (t, 2 H), 4.0 (m, 2 H), 5.07 (s, 2 H), 7.4 (s, 5 H). Bis(triethylammonium) 2-(*N*-carbobenzoxyamino)ethyl pyrophosphate, 1.2 g (2 mmol), was dissolved in 10 mL of 0.10 M sodium pyrophosphate, pH 7.5. Palladium on carbon (10%), 100 mg, was added, and the sample was stirred at room temperature for 90 min under an atmosphere of hydrogen gas. The catalyst was removed by suction filtration through Celite and washed with 5 mL of H_2O. Formaldehyde (37% w/w), 200 μL (2.3 mmol), was added to the stirred solution at 4 °C. Sodium borohydride (60 μL of a 5 M solution in 0.1 N NaOH) was added, and the pH was maintained between 7 and 8 with 1.0 N HCl. The formaldehyde/$NaBH_4$ additions were repeated 10 times in 2-min intervals. The reaction progress was monitored by using a spectrophotometric assay with ninhydrin. When the additions were complete, the volume was adjusted to 250 mL with H_2O and the pH was adjusted to 8.0. The sample was applied to a 1.5 × 45 cm column of QAE-Sephadex A-25, HCO_3^- form. The column was washed with 40 mL of H_2O and eluted with a 800-mL linear TEAB gradient from 0.0 to 0.45 M. The purified product was concentrated by rotary evaporation in vacuo and precipitated in ethanol as described previously: ^{1}H NMR [for 2-(dimethylamino)ethyl pyrophosphate] (D_2O) δ 2.93 (s, 6 H), 3.43 (t, 2 H), 4.23 (m, 2 H); ^{31}P NMR (D_2O) δ 8.75 (d), 10.28 (d, J_{pp} = 16 Hz).

2-Aminoethyl pyrophosphate (III) was prepared by direct purification of the hydrogenation product on QAE-Sephadex. 2-(Methylamino)ethyl pyrophosphate (IV) was prepared from *N*-methylethanolamine by the above procedure. 2-(Trimethylammonio)ethyl pyrophosphate (II) was prepared from the tosylate salt of choline by the method used for synthesis of dimethylallyl pyrophosphate (Cornforth & Popjak, 1969): ^{1}H NMR (D_2O) δ 3.23 (s, 9 H), 3.65 (t, 2 H), 4.30 (m, 2 H). [*methyl*-^{3}H]-2-(Dimethylamino)ethyl pyrophosphate was prepared by using NaB^3H_4 in the reductive methylation. The radiochemical purity was >98% as determined by high-voltage paper electrophoresis in pyridinium acetate, pH 6.5, and, after treatment with alkaline phosphatase, by chromatography on a PRP polystyrene HPLC column in 15% MeOH/30 mM NaOH.

[1-^{3}H]Dimethylallyl Alcohol. The chromium trioxide–dipyridine complex, 3.1 g (12 mmol), prepared according to Collins et al. (1968), was dissolved in 60 mL of methylene chloride. The solution was stirred in an ice–water bath while 172 mg (2 mmol) of dimethylallyl alcohol in 5 mL of methylene chloride was added dropwise over 2 min. After the solution was stirred for 20 min, the resultant sample was filtered through Celite, washed with 50 mL of 3% HCl, dried over $MgSO_4$, and refiltered through Celite. Solvent was removed by rotary evaporation in vacuo. To the residue was added 50 mL of diethyl ether, and the sample was suction filtered through Florosil. The ether was removed by rotary evaporation in vacuo to give 105 mg (63% yield) of dimethylacrylal: ^{1}H NMR ($CDCl_3$) δ 1.96 (s, 3 H), 2.11 (s, 3 H), 5.85 (dm, 1 H), 9.92 (d, 1 H). Dimethylacrylal (68 mg, 1 mmol) in 0.5 mL of methanol was added to approximately 6 MCi (0.1 mequiv) of NaB^3H_4 in an ice–water bath. After 5 min, 20 mg (0.5 mmol) of $NaBH_4$ in 0.5 mL of MeOH was added and stirring was continued for 20 min; 1.0 mL of 1.0 N HCl was added, and the pH was adjusted to 7. The sample was saturated with NaCl and extracted 4 times with 2 mL of ethyl acetate/hexane (2:1). To the combined extracts was added 10 mL of hexane, and the aqueous layer was removed. The sample was washed with 1 mL of 1 N HCl, dried over $MgSO_4$, and concentrated to 5 mL under a slow stream of nitrogen. To this was added 5 mL of hexane, the sample was again washed with 1 mL of 0.1 N HCl, dried over $MgSO_4$, and concentrated to a colorless liquid under a slow stream of nitrogen: ^{1}H NMR ($CDCl_3$) δ 1.68 (s, 3 H), 1.76 (s, 3 H), 2.15 (s, br, 1 H), 4.10 (d, 2 H), 5.38 (tm, 1 H).

(Z)-[1-^{3}H]-3-(Trifluoromethyl)-2-butenol. This analogue was synthesized by the same procedure used for [1-^{3}H]dimethylallyl alcohol on a 50% scale but with 40 MCi of NaB^3H_4. In both cases, the alcohols were pyrophosphorylated according to Cornforth and Popjak (1969). The radiochemical

Table I: Isolation of Isopentenyl Pyrophosphate Isomerase from Yeast

purification step	total units[a]	SA[b]	yield (%)
homogenate supernatent	30	0.4	100
isoelectric precipitation	22.4	1.84	75
$(NH_4)_2PO_4$ precipitation	20.5	2.85	68
DEAE-cellulose	19.8	57.3	66
hydroxylapatite	6.45	170.0	22
gel filtration	5.87	400.0	20
FPLC	2.12	2650.0	

[a] Micromoles per minute. [b] Specific activity in nanomoles per minute per milligram of protein.

purity of both compounds was >99% as judged by high-voltage paper electrophoresis at pH 3.5, and, after treatment with alkaline phosphatase, by chromatography on a μBondapak C_{18} reverse-phase HPLC column in 25% methanol/H_2O.

Purification of Isopentenyl Pyrophosphate Isomerase. All purification steps were performed at 4 °C. All buffers used in the purification contained 1 mM 2-mercaptoethanol. The results are summarized in Table I.

Extraction. Baker's yeast (2 lb) was stirred in 1 L of 100 mM KP_i, pH 7.0, until homogeneous. The cells were broken in 175-mL aliquots with a Biospec Products bead beater, using three 80-s spins with a 1-min cooling period between spins. Cell debris was removed by centrifugation at 15000*g* for 45 min. The supernatant was clarified by stirring while the pH was adjusted to 4.7 with 10% (v/v) acetic acid. Stirring was continued for 10 min. Precipitated protein was removed by centrifugation at 15000*g* for 20 min.

Fractionation with $(NH_4)_2PO_4$. The supernatant was fractionated by slow addition of solid $(NH_4)_2PO_4$ to the stirred solution. The material that precipitated between 0 and 28 g of $(NH_4)_2PO_4$ per 100 mL of solution was removed by centrifugation at 15000*g* for 30 min. The fraction that precipitated between 28 and 45 g of $(NH_4)_2PO_4$ per 100 mL of solution was collected by centrifugation at 15000*g* for 30 min. The pellet was dissolved in 200 mL of H_2O and dialyzed overnight against three changes of 4 L of 120 mM KP_i, pH 7.0.

Anion-Exchange Chromatography. The dialyzed protein was applied to two 2.5 × 15 cm columns of DEAE-cellulose DE-52 equilibrated in 120 mM KP_i, pH 7.0. The columns were each washed with 100 mL of 120 mM KP_i, pH 7.0. The protein was eluted in a band with 600 mM KP_i, pH 7.0. The sample was concentrated to 30 mL by ultrafiltration using an Amicon PM10 membrane at 40 psi. The concentrated protein was dialyzed against 6 L of 2 mM KP_i, pH 7.0.

Absorption Chromatography. The dialyzed protein sample was applied to a 2.5 × 18 cm column of hydroxylapatite equilibrated in 5 mM KP_i, pH 7.0. The column was washed with 30 mL of 5 mM KP_i, pH 7.0, and eluted at 0.5 mL/min with a 1-L linear KP_i gradient from 5 to 200 mM, pH 7.0. Fractions of 10 mL were collected, and those containing isomerase activity (52–65) were pooled and concentrated to 5 mL with an Amicon PM10 membrane at 30 psi.

Gel Permeation Chromatography. The concentrated protein was applied to a 2.5 × 50 cm column of Sephadex G-100 equilibrated in 25 mM KP_i, pH 7.0. The column was eluted with 25 mM KP_i, pH 7.0, at a flow rate of 20 mL/h. Fractions, 5 mL, were collected, and the active fractions (23–31) were pooled and concentrated to 5 mL with an Amicon PM10 membrane at 20 psi. The protein was dialyzed against 2 L of 10 mM KP_i, pH 6.0.

FPLC Anion-Exchange Chromatography. The dialyzed protein was chromatographed on a Pharmacia FPLC system employing a Mono Q anion-exchange column equilibrated in 10 mM KP_i, pH 6.0. Protein was applied in a 1.0-mL aliquot, and the column was washed with 5 mL of 10 mM KP_i, pH 6.0. The column was eluted at 1.0 mL/min by running an 8-mL linear KP_i gradient from 10 to 190 mM, followed by 6 mL isocratic at 190 mM, and then continuing the linear gradient from 190 to 320 mM over 5 mL. The active fractions (22–23) were collected and concentrated to 2 mL with an Amicon PM10 membrane at 10 psi. The sample was diluted to 8.0 mL with H_2O and rechromatographed by using the same elution gradient. The active peak was concentrated to 2 mL with an Amicon PM10 membrane at 10 psi and dialyzed against 1 L of 5 mM KP_i, pH 7.0.

Molecular Weight Estimation. (1) SDS–Polyacrylamide Disc Gel Electrophoresis. The purity and monomer molecular weight of the isomerase were determined by gel electrophoresis. The gel was prepared according to Laemmli (1970). A 5% stacking gel and a 12% running gel were employed, and the gel was stained with Commasie Brilliant Blue. Molecular weight standards were purchased from Bio-Rad Laboratories. The monomer molecular weight of the isomerase is estimated by this method to be 39 000.

(2) HPLC Gel Permeation Chromatography. The native molecular weight of the isomerase was determined by using a Beckman TSK 3000 SW gel permeation column equilibrated in 400 mM KP_i, pH 6.75. Samples were chromatographed at 0.5 mL/min. The only protein peak observed contained isomerase activity. Comparison of V_e/V_0 for the isomerase and a series of molecular weight standards gave an estimated molecular weight of 41 000.

Enzyme Assay. Method 1. This assay method is based on the greater susceptibility of allylic pyrophosphates toward acid hydrolysis. The incubation mixture, 250 μL, contained sodium maleate buffer (0.10 M, pH 6.3), $MgCl_2$ (10 mM), [4-^{14}C]-isopentenyl pyrophosphate (350 μM, 5.0×10^5 cpm/μmol), and enzyme. After 5 min at 37 °C, 5 N HCl (15 μL) was added, and the incubation was continued for 60 min at 37 °C. Then 1.0 N NaOH, 100 μL, and 1.5 mL of H_2O were added, and the sample was applied to a 1.0-mL pipet column of Dowex 1, Cl^- form. The column was washed with 0.7 mL of H_2O, and the eluent was counted in 12 mL of Aquasol liquid scintillation fluid. In control experiments without enzyme, the eluent contained approximately 100 cpm.

Method 2. The assay mixture described above was again incubated for 5 min at 37 °C. Tris, 1 M, pH 9.5 (100 μL), and alkaline phosphatase (50 μL, 1 mg) were added, and the incubation was continued for 45 min at 37 °C. The incubation mixture was saturated with NaCl and extracted 4 times with 2 mL of ether. To the ether extract was added 2 μL of carrier dimethylallyl alcohol and 3-methyl-3-butenol, and the samples were concentrated under a stream of nitrogen. The samples were diluted with 0.5 mL of H_2O and chromatographed on a reverse-phase HPLC column in 20% MeOH. Aliquots from the fraction containing each alcohol were counted in Aquasol liquid scintillation fluid. Both assay methods are based on those described earlier (Banthorpe et al., 1977). Method 1 is limited by the overestimation of isomerase activity in crude enzyme preparations due to the presence of prenyl transferase, which converts isopentenyl pyrophosphate and dimethylallyl pyrophosphate into higher allylic pyrophosphates. Method 1 was, however, used throughout the enzyme purification with no difficulty.

Determination of Protein Concentrations. During purification, protein concentrations were determined from absorbance readings at 280 nm. The concentration of pure samples of isomerase was determined according to the method of Bradford (1976). More accurate determination of the concentration of isomerase active sites was achieved by titrating the activity in samples of isomerase with 2-(dimethylamino)ethyl pyrophosphate (I). From the intercept of the linear asymptote of a plot of isomerase activity vs. I concentration, the concentration of isomerase active sites is obtained.

Characterization of the [methyl-^{3}H]-I–Isomerase Complex. The inhibition incubation mixture contained 5 mM KP_i, pH 7, 10 mM $MgCl_2$, 9 mM [*methyl*-^{3}H]-I (sp act. = 2.1×10^4 cpm/nmol), and 300 μg of isomerase in a final volume of 160 μL. The sample was incubated at 25 °C for 60 min and then chromatographed in 30-μL aliquots on a Beckman TSK 3000 SW HPLC gel permeation column equilibrated in 400 mM KP_i, pH 6.75, at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected, and 50-μL aliquots were diluted with 0.5 mL of H_2O and counted in 4 mL of Aquasol liquid scintillation fluid. Fractions containing labeled isomerase were pooled and concentrated by using a Centricon microconcentrator. The denaturation incubation mixture contained 400 mM KP_i, pH 6.75, 6 M urea, 100 mM KCl, and labeled isomerase (250 μg) in a total volume of 300 μL. After 3 h at 37 °C, the sample was chromatographed in 60-μL aliquots on the TSK 3000 SW HPLC column equilibrated in 6 M urea and 10 mM KP_i, pH 6.75, at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected, and 100-μL aliquots were diluted with 1 mL of H_2O and counted in 10 mL of Aquasol liquid scintillation fluid. Fractions containing radioactivity in the small molecule peak were pooled, and 15 mg of I was added. The sample was chromatographed in 1-mL aliquots on a 3 × 200 mm Macroporous AG-1 HPLC ion-exchange column in the $CF_3CO_2^-$ form equilibrated in distilled H_2O at a flow rate of 1.0 mL/min. After a 5-min H_2O wash, the column was eluted with a 20-mL linear trifluoroacetic acid gradient from 0.0 to 75 mM, and the column was washed with 75 mM CF_3CO_2H for 5 min. Fractions of 1.0 mL were collected, and 200-μL aliquots were counted in 4 mL of Aquasol liquid scintillation fluid. The 50-μL aliquots were used for inorganic phosphate and total phosphate analysis. The peak of radioactivity that comigrated with cold I was pooled and concentrated to 3 mL by rotary evaporation in vacuo and lyophilized to dryness. The sample was dissolved in 800 μL of 50 mM K_2CO_3, pH 10.0, and 5 mg of alkaline phosphatase dissolved in 200 μL of 50 mM K_2CO_3, pH 10.0, was added. The sample was incubated at 37 °C for 16 h. To a 500-μL aliquot of the alkaline phosphatase reaction mixture was added 15 μL of 70% $HClO_4$. After 45 min at 4 °C, the sample was centrifuged and 300 μL of the supernatant was mixed with 125 μL of 1.0 N NaOH, 2 μL of *N*-methylethanolamine, and 3 μL of *N,N*-dimethylethanolamine. The sample was chromatographed on a Hamilton PRP-1 polystyrene HPLC column equilibrated in 15% MeOH/30 mM NaOH at a flow rate of 1.5 mL/min. Fractions of 1.5 mL were collected, neutralized with acetic acid, and counted in 12 mL of Aquasol liquid scintillation fluid.

Kinetic Measurements. The inhibition constants for competitive inhibitors were measured as follows. Assay mixtures contained 50 mM buffer, 10 mM $MgCl_2$, 10–100 μM [4-^{14}C]isopentenyl pyrophosphate (sp act. = 5.0×10^5 cpm/μmol), 60 nM isomerase, and several inhibitor concentrations in a total volume of 250 μL. The assays were initiated by addition of enzyme and continued at 37 °C for 5 min. The assay mixtures were quenched, and product formation was determined according to assay method 1.

The pH dependence of the kinetic constants for the isomerase-catalyzed reaction was measured as follows. Incubation mixtures contained 50 mM buffer, 10 mM $MgCl_2$, 10–100 μM [4-^{14}C]isopentenyl pyrophosphate (sp act. = 5.0×10^5 cpm/μmol), and isomerase (0.06–0.60 μM) in a total volume of 250 μL. The assays were initiated by addition of enzyme and continued at 25 °C for 5 min. The assay mixtures were quenched, and product formation was determined according to assay method 1.

The rate of inactivation of isomerase by iodoacetamide was measured as follows. Incubation mixtures contained 50 mM buffer, 0.1–2.0 mM iodoacetamide, and 1.5 μM isomerase in a total volume of 100 μL. Incubations were initiated at 25 °C by addition of enzyme, and 10-μL aliquots were removed at time intervals and assayed for remaining activity according to assay method 1. The same conditions were employed to measure the rate of inactivation by *p*-mercuribenzoate and methyl methanethiosulfonate.

Second-order on-rates for I binding to isomerase were determined as follows. Incubation mixtures contained 50 mM buffer, 10 mM $MgCl_2$, 1.65 μM isomerase, and 2.5 μM I in a total volume of 100 μL. The mixtures were incubated at 25 °C and 10-μL aliquots were removed at time intervals and assayed for remaining enzyme activity according to assay method 1. On-rates for III and IV were measured by using these same experimental conditions. The on-rate for II was measured under pseudo-first-order conditions. Preincubation mixtures contained 50 mM buffer, 10 mM $MgCl_2$, 1.5 μM isomerase, and 17–85 μM II in a total volume of 100 μL. Samples were incubated at 25 °C and periodically assayed for remaining enzyme activity as described above.

The off-rate for II was determined by equilibrium perturbation. Preincubation mixtures contained 50 mM sodium borate, pH 8.7, 10 mM $MgCl_2$, 1.5 μM isomerase, and 6.8 μM II in a total volume of 250 μL. One control incubation contained no inhibitor. The samples were incubated at 25 °C for 45 min and were then diluted 5-fold into either 50 mM sodium maleate, pH 5.3, and 10 mM $MgCl_2$ to give a final pH of 5.5, 50 mM sodium maleate, pH 5.8, and 10 mM $MgCl_2$ to give a final pH of 6.1, or 50 mM sodium maleage, pH 6.3, and 10 mM $MgCl_2$ to gie a final pH of 6.8. A second control sample containing 6.8 μM II was diluted immediately upon addition of enzyme. After dilution, 30-μL aliquots were assayed for recovery of enzyme activity at time intervals according to assay method 1.

The rate of product formation over time in the presence of II was measured as follows. Incubation mixtures contained 50 mM sodium maleate, pH 6.8, 10 mM $MgCl_2$, 30 nM isomerase, 350 μM [4-^{14}C]isopentenyl pyrophosphate (5×10^5 cpm/μmol), and 0–400 μM II in a total volume of 1.5 mL. Assays were initiated by addition of enzyme, 125-μL aliquots were removed at time intervals and quenched with 15 μL of 2.5 N HCl, and product formation was determined by using assay method 1 at half-scale.

The buffers used for kinetic measurements were sodium maleate (pH 5.3–6.8), HEPES (pH 6.8–8.2), sodium borate (pH 8.2–9.7), and sodium carbonate (pH 9.7–10.4). The magnesium concentration used in all kinetic studies (10 mM) was saturating over the pH range investigated.

In order to determine whether (*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate was a substrate for the isomerase, (*Z*)-[1-^{3}H]-3-(trifluoromethyl)-2-butenyl pyrophosphate and [1-^{3}H]-3,3-dimethylallyl pyrophosphate were prepared, and the turnover of both compounds was examined. The initial rate for isomerization of dimethylallyl pyrophosphate was measured as follows. The incubation mixtures contained 100 mM sodium maleate, pH 6.3, 10 mM $MgCl_2$, 2 mM [1-

^{3}H]dimethylallyl pyrophosphate (2.6×10^3 cpm/nmol), and 2 μg of isomerase in a total volume of 250 μL at 25 °C. The incubations were initiated by addition of enzyme and were quenched after 5, 9, and 13 min by addition of 100 μL of 1 N Na_2CO_3, pH 10.4, containing 2 mg of alkaline phosphatase. The samples were incubated for an additional 60 min at 37 °C. Two control incubations with no enzyme added were also quenched after 13 min. MeOH (1.0 mL) was added, the samples were left at 4 °C for 60 min and then centrifuged, and the supernatant was mixed with 4 mL of H_2O, carrier dimethylallyl alcohol, and 3-methyl-3-butenol. Aliquots, 2.0 mL, were chromatographed on a reverse-phase μBondapak C_{18} column equilibrated in 15% MeOH at a flow rate of 2 mL/min. Fractions of 2.0 mL were collected and counted in Aquasol LSF. The rate of isomerization of dimethylallyl pyrophosphate was 0.47 μmol min^{-1} mg^{-1}. The duplicate incubation mixtures for measuring the turnover of the trifluoromethyl analogue contained 100 mM sodium maleate, pH 6.3, 10 mM $MgCl_2$, 4 mM (*Z*)-[1-^{3}H]-3-(trifluoromethyl)-2-butenyl pyrophosphate (2.4×10^4 cpm/nmol), andd50 μg of isomerase. Duplicate control samples without enzyme were also prepared. A third control sample without substrate was used to measure loss of enzyme activity during the incubation. The incubations were initiated by addition of enzyme and left at 25 °C for 5.0 h. The samples were quenched and prepared for reverse-phase HPLC chromatography as described for dimethylallyl pyrophosphate incubations, using the carrier mixture containing both (*E*)- and (*Z*)-3-(trifluoromethyl)-2-butenol and 3-(trifluoromethyl)-3-butenol. The samples were chromatographed in 25% MeOH. There was no [1-^{3}H]-3-(trifluoromethyl)-3-butenol detected in the elution profile. The rate of isomerization of (*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate is $<8 \times 10^{-7}$ μmol min^{-1} mg^{-1}.

Data Analysis. Inhibition constants for competitive inhibitors were determined by using eq 1, where A is the concentration of substrate and I is the concentration of inhibitor.

$$v = VA/[K(1 + I/K_i) + A] \qquad (1)$$

pH-dependent values of V and V/K were fit to eq 2 and 3, respectively, where H is the hydrogen ion concentration.

$$V = V'/[1 + K/H] \qquad (2)$$

$$V/K = (V/K')/(1 + H/K_1 + K_2/H) \qquad (3)$$

Second-order on-rate constants for inhibition by I, III, and IV were obtained by plotting experimental data according to

$$\frac{1}{[\mathrm{I}]_0 - [\mathrm{E}]_0} \ln \frac{[\mathrm{E}]_0([\mathrm{I}]_0 - [\mathrm{EI}])}{[\mathrm{I}]_0([\mathrm{E}]_0 - [\mathrm{EI}])} = kt \qquad (4)$$

The pH-dependent values of k_{on} for I and II were fit to eq 5 and 6, respectively. Similarly, the inhibition constant for II

$$k_{on} = k_{on}'/(1 + H/K_1 + K_2/H) \qquad (5)$$

$$k_{on} = k_{on}'/(1 + H/K) \qquad (6)$$

was obtained by fitting the observed values to

$$K_i = K_i'/(1 + H/K) \qquad (7)$$

The rate of product formation over time in the presence of II was fit to eq 8 (Williams & Morrison, 1979), where P is the net product formed, v_0 is the initial velocity, v_s is the final steady-state velocity, and k is the first-order rate constant describing the change from v_0 to v_s. The values of k_{on} and k_{off} were obtained from least-squares analysis of a plot of k vs. inhibitor concentration

$$P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \qquad (8)$$

$$k = k_{off} + k_{on}I/(1 + A/K_m) \qquad (9)$$

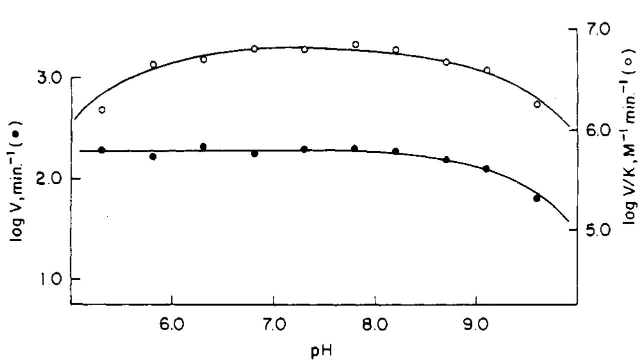

FIGURE 1: pH dependence of V (●) and V/K (○) for isopentenyl pyrophosphate isomerase. Assay conditions are described under Materials and Methods. The line drawn for the V/K data is the best fit of the data to eq 3, where pK_1 and pK_2 = 5.5 and 9.4, respectively. The line drawn for the V data is the best fit of the data to eq 2, where pK_1 = 9.4.

The pH-dependent rate of inactivation by iodoacetamide was fit to eq 10.

$$k = \mathrm{K}_1'/(1 + H/K_1) + k_2'/(1 + H/K_2) \qquad (10)$$

RESULTS

Purification and Properties of Isopentenyl Pyrophosphate. Isopentenyl pyrophosphate isomerase has been purified to near homogeneity from pig liver (Banthorpe et al., 1977) and has been obtained in less pure form from a variety of sources (Satterwhite, 1985). The procedure reported here for purification of isomerase from Baker's yeast, to >90% purity as judged by gel electrophoresis, is summarized in Table I. The specific activity of the purified enzyme, 2.65 μmol min^{-1} mg^{-1} (Table I), is close to the value of 6.7 μmol min^{-1} mg^{-1} for the enzyme purified from pig liver (Banthorpe et al., 1977). The purified enzyme is a monomer with an estimated molecular weight of 40 000. This is in contrast to the reported dimeric structure for the isomerase from pig liver (Banthorpe et al., 1977). The preparation is free of phosphatase and prenyl transferase activities. The enzyme displays a broad pH activity profile (Figure 1), consistent with earlier results (Agranoff et al., 1960). The value of K for isopentenyl pyrophosphate, 35 μM, is identical with the value reported previously (Agranoff et al., 1960).

The ability of (*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate to serve as a substrate for the isomerase was examined. Because of the expected unreactive nature of the trifluoromethyl analogue, the enzyme concentration was increased 25-fold and the reaction time was increased 60-fold compared to the conditions used for the nonfluorinated substrate. In addition, the specific activity of the fluoro analogue was almost 10-fold greater than that of the [1-^{3}H]dimethylallyl pyrophosphate used. Because of the relatively long incubation time, the loss of enzyme activity was monitored in a control incubation. After 5 h the enzyme had 70% of its original activity. The ratio of turnover numbers for the fluorinated substrate compared to dimethylallyl pyrophosphate, V_F/V_H, was $< 1.8 \times 10^{-6}$.

Competitive Inhibitors. A series of substrate/product analogues were prepared (Table II), and their ability to inhibit the isomerase-catalyzed reaction was examined. In all cases, inhibition was competitive and rapidly reversible. The K_i

Table II: Inhibitors of Isopentenyl Pyrophosphate Isomerase

compound	K_i (M)
inorganic phosphate	$>5.0 \times 10^{-2}$
inorganic pyrophosphate	$>1.0 \times 10^{-3}$
methyl pyrophosphate	1.0×10^{-4}
ethyl pyrophosphate	8.0×10^{-5}
n-propyl pyrophosphate	5.6×10^{-4}
n-butyl pyrophosphate	6.0×10^{-5}
3-butenyl pyrophosphate	8.5×10^{-5}
isoamyl pyrophosphate	3.0×10^{-4}
isopentenyl pyrophosphate	3.5×10^{-5} (K_m)
3,3-dimethylbutyl pyrophosphate	4.0×10^{-4}
(*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate	4.0×10^{-4}
(*E*)-3-(trifluoromethyl)-2-butenyl pyrophosphate	4.0×10^{-5}
3-bromo-3-butenyl pyrophosphate	6.0×10^{-5}
4-bromo-3-methyl-3-butenyl pyrophosphate	4.0×10^{-5}
2-cyclopropylethyl pyrophosphate	2.7×10^{-4}
cyclopropylmethyl pyrophosphate	2.1×10^{-3}
2-(dimethylamino)ethyl phosphate	2.0×10^{-3}

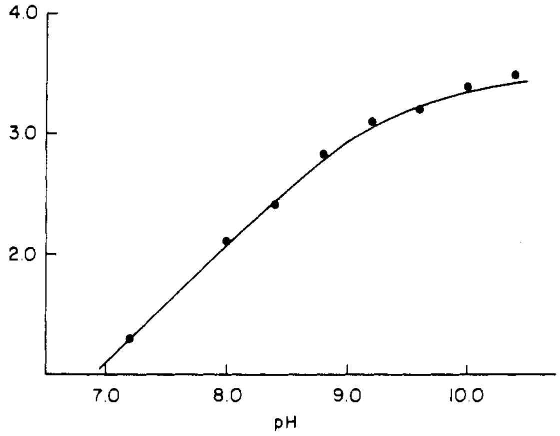

FIGURE 2: pH dependence of the inactivation of isopentenyl pyrophosphate isomerase by iodoacetamide. Assay conditions are described under Materials and Methods. The line drawn is the best fit of the data to eq 11, where $pK_1 = 10.3$.

values for ethyl pyrophosphate, isoamyl pyrophosphate, 3,3-dimethylbutyl pyrophosphate, and 2-(dimethylamino)ethyl phosphate were independent of pH between 6.5 and 9.7 as is K for isopentenyl pyrophosphate.

Inactivation by Sulfhydryl Reagents. The rate of inactivation of isomerase by iodoacetamide was pH dependent (Figure 2). The best fit of the data to eq 10 gave a pK of 9.3. The inactivation could be completely prevented by saturating concentrations of isopentenyl pyrophosphate. In contrast, saturating concentrations of methyl pyrophosphate do not protect against the inactivation. The same results were obtained with methyl methanethiosulfonate (Smith et al., 1975) as the inactivating reagent. Inactivation of the isomerase by reaction with methyl methanethiosulfonate prevented binding of [*methyl*-^{3}H]-I.

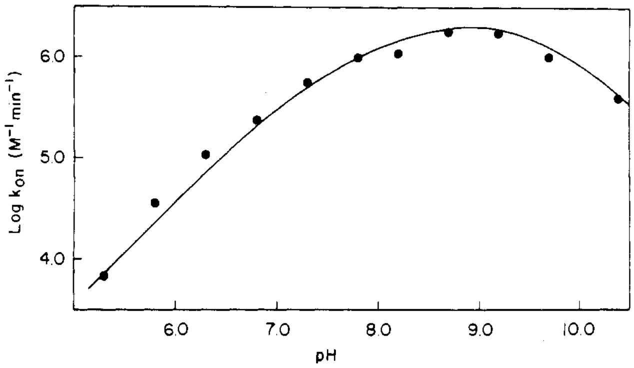

FIGURE 3: Variation of k_{on} with pH for 2-(dimethylamino)ethyl pyrophosphate (I). The values of k_{on} were determined as described under Materials and Methods. The line drawn is the best fit of the data to eq 5. The values of pK_1 and pK_2 = 7.8 and 9.8, respectively.

Inhibition by 2-(Dimethylamino)ethyl Pyrophosphate (I). It was previously shown (Reardon & Abeles, 1985) that I inactivates isomerase. The inactivation appeared to be irreversible and was time dependent. We have now further investigated the kinetics of inhibition by I. The rate constant for the inhibition of isomerase by I was measured under second-order experimental conditions. The value of k_{on} is pH dependent as shown in Figure 3. The best fit of the data to eq 5 gave $k_{on}' = 2.1 \times 10^6$ M^{-1} min^{-1} and pK values of 7.8 and 9.8. To determine the rate of release of I from the enzyme, isomerase was inhibited with [*methyl*-^{3}H]-I. Excess free inhibitor was removed by either dialysis or HPLC gel permeation chromatography, and the isomerase–[*methyl*-^{3}H]-I complex was incubated with excess unlabeled I and 10 mM $MgCl_2$ at 25 °C. The rate of release of radioactivity from the enzyme was determined by chromatographing aliquots on an HPLC gel permeation column followed by liquid scintillation counting of the column fractions. No change in the specific radioactivity of the protein peak was detected over 48 h. From these data, $k_{off} < 3 \times 10^{-5}$ min^{-1} and $K_i < 1.4 \times 10^{-11}$ M were calculated.

To determine whether the apparent irreversible inhibition of isomerase by I was due to covalent modification, isomerase was inhibited with [*methyl*-^{3}H]-I and the inactive isomerase was isolated. Upon rechromatography of the purified complex, a 3% contamination of ^{3}H small molecules remained. The purified complex was incubated in 6 M urea. After 3 h at 37 °C, 95% of the radioactivity was released from the denatured enzyme as determined by gel permeation chromatography. The radioactive small molecule released upon denaturation of the enzyme was identified as [*methyl*-^{3}H]-I by comigration of the radioactivity with carrier I on a Macroporous Ag-1 HPLC ion-exchange column and, following treatment with alkaline phosphatase, comigration of the radioactivity with carrier 2-(dimethylamino)ethanol on a Beckman PRP-1 polystyrene HPLC column. These results show that inactivation of isomerase by I does not involve irreversible covalent modification of the enzyme and most probably does not involve a chemical reaction between enzyme and inhibitor.

Inhibition by 2-(Trimethylammonio)ethyl Pyrophosphate (II). The inhibition by I is dependent upon two groups with pK = 7.8 and 9.8. The higher pK is close to the pK (10.0) of the amino group of I as determined by titration and could therefore be due to ionization of the inhibitor rather than the enzyme. We, therefore, examined the inhibition by II, which contains a quaternary ammonium group and has a positive charge over the entire pH range.

The inhibition constant, K_i, for II was determined directly by measuring the activity remaining in incubation mixtures of isomerase and II after equilibrium had been established. The K_i for II was pH dependent (Figure 4) and reached a limiting value of 1.0×10^{-6} M. The value of k_{on} was also pH dependent (Figure 4) and reached a limiting value of 4.4×10^4 M^{-1} min^{-1}. K_i for II as well as the on-rate is dependent on a group with pK = 7.8. The pH dependence of 9.8 observed for I is, therefore, due to the ionization of the inhibitor. The first-order rate constant for reactivation of inhibited enzyme was also determined. The value of k_{off}, 0.03 ± 0.01 min^{-1}, was pH independent over the range studied (pH 5.5–6.8). The value of K_i measured directly, 1.0 μM, is in good agreement with the value of 0.7 μM calculated from the measured values of k_{on} and k_{off}.

When an incubation mixture contining II was initiated with isomerase, a decrease in the rate of product formation over

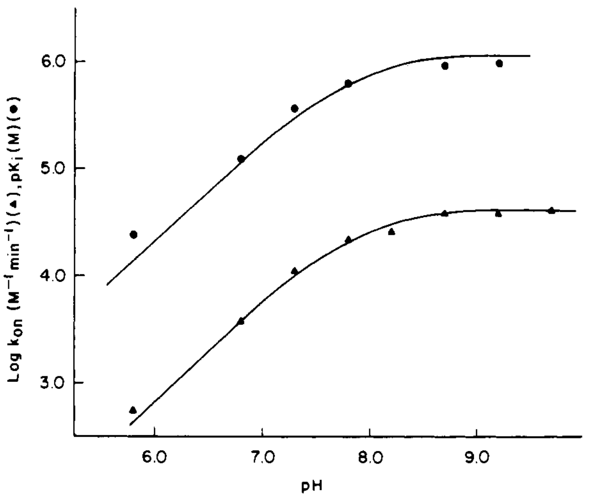

FIGURE 4: Variation of pK_i (●) and log k_{on} (▲) with pH for (trimethylammonio)ethyl pyrophosphate (II). The values of K_i and k_{on} were determined as described under Materials and Methods. The lines are the best fit of the data to eq 6 and 7. The values of pK_1 = 7.8.

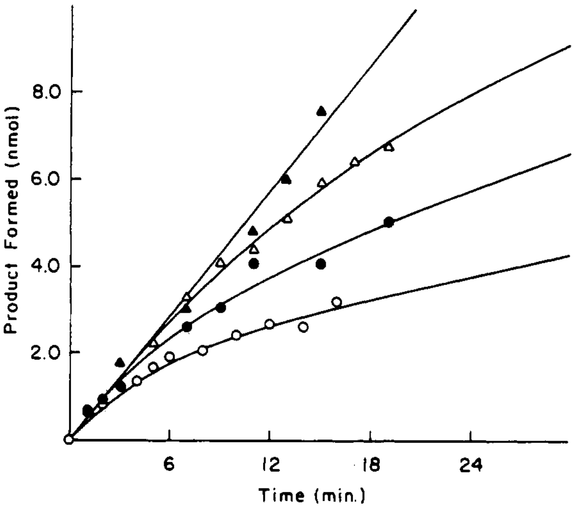

FIGURE 5: Slow-binding inhibition by 2-(trimethylammonio)ethyl pyrophosphate. Assay conditions are given under Materials and Methods. The concentrations of inhibitor are (▲) 0, (△) 0.10, (●) 0.3, and (○) 0.4 mM. The lines drawn are the best fit of the data to eq 8.

time is observed as shown in Figure 5. The rate constants for the change in velocity as a function of inhibitor concentration were obtained by fitting the time courses to eq 8. From least-squares analysis of a plot of k vs. [I], eq 9, values for k_{on}' and k_{off} of 5.8 × 10^4 M^{-1} min^{-1} and 0.023 min^{-1}, respectively, were obtained. From these results a value of 0.40 μM is obtained for the inhibition constant.

The rate constants for inhibition by 2-aminoethyl pyrophosphate (III) and 2-(methylamino)ethyl pyrophosphate (IV) were also measured under second-order experimental conditions. Table III summarizes the kinetic constants for the inhibition of isomerase by I–IV.

DISCUSSION

The observation that (*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate reacts more than 10^6 times slower than the normal substrate is consistent with a carbonium ion mechanism. Electron withdrawal by the trifluoromethyl group is expected to greatly destabilize carbonium ion formation. In a similar study on prenyl transferase, substitution of the trifluoromethyl group for a methyl group in dimethylallyl pyrophosphate resulted in a 10^7-fold decrease in V (Poulter & Satterwhite, 1977). This decrease was attributed to decreased rate of formation of the allylic carbonium ion intermediate. In nonenzymatic model reactions, substitution of the trifluoromethyl group in dimethylallyl methanesulfonate resulted in a 10^7-fold decrease in the rate of S_N1 solvolysis, in agreement with the enzymatic studies (Poulter & Satterwhite, 1977).

Table III: Kinetic Constants for Tight-Binding Inhibitors of Isopentenyl Pyrophosphate Isomerase

compound	k_{on} (M^{-1} min^{-1})[a]	k_{off} (min^{-1})	K_i (M)
2-aminoethyl pyrophosphate (III)	5.2 × 10^6 [b]	nd[i]	nd[i]
2-(methylamino)ethyl pyrophosphate (IV)	1.9 × 10^6 [b]	nd[i]	nd[i]
2-(dimethylamino)ethyl pyrophosphate (I)	2.1 × 10^6 [b]	3.0 × 10^{-5} [d]	1.4 × 10^{-11} [f]
2-(trimethylammonio)ethyl pyrophosphate (II)	4.4 × 10^4 [c]	0.030[e]	6.8 × 10^{-7} [f]
	5.8 × 10^4 [g]	0.023[g]	4.0 × 10^{-7} [g] 9.0 × 10^{-7} [h]

[a] $k_{on} = k_{obsd}/(1 + H/K_1)(1 + K_2/h)$. [b] Rate of loss of enzyme activity measured under second-order experimental conditions. [c] Rate of loss of enzyme activity measured under pseudo-first-order experimental conditions. [d] First-order rate of release of ^3H-labeled inhibitor from the enzyme–^3H-labeled inhibitor complex in the presence of excess unlabeled inhibitor. [e] First-order rate of recovery of enzyme activity after dilution–pH jump (equilibrium perturbation). [f] Calculated from the independently measured values of k_{on} and k_{off}. [g] Measured from product formation progress curves in the presence of inhibitor (Williams & Morrison, 1979. [h] Measured from residual enzyme activity after equilibration of inhibitor binding. [i] nd = not determined.

The inhibition of the reaction catalyzed by isopentenyl pyrophosphate isomerase by I provides additional support for a carbonium ion mechanism. The inactivation of isomerase by I does not involve covalent interaction. Binding of the inhibitor is prevented by substrate as well as by sulfhydryl reagents, which also abolish catalytic activity. Therefore, I, most probably, binds at the active site. Binding of I to isomerase is extremely tight (K_i <10^{-11} M). A major contributing factor to this tight binding must be the interaction of the positive charge of I with a negative charge on the enzyme, since K_i for inhibitors that do not have a positive charge is at least 10^6-fold greater. The tight binding of I can best be rationalized if it is assumed that I is a transition-state analogue (Wolfenden, 1976) in which the positively charged ammonium ion mimics the carbonium ion. It is likely that the positive center of I interacts with the same negative site on the enzyme that stabilizes the carbonium ion in the catalytic reaction.

The pH dependence of k_{on} for I and of k_{on} and K_i for II shows that the positively charged form of I (pK = 10.0) combines with a deprotonated form of the enzyme (pK = 7.8). For II, a quaternary amine, k_{on} and K_i depend on only one ionizable group (pK = 7.8). V/K for the isomerization of isopentenyl pyrophosphate (Figure 1) depends on a pK of 9.4 and V on a pK of 9.4 and a pK of 5.6. The latter is most probably the third pK of the Mg^{2+} isopentenyl pyrophosphate complex, and it is probably not a group involved in catalysis. The group with pK = 7.8, involved in the binding of inhibitors I and II, is not involved in the binding of the substrate nor is it apparent in the catalytically active complex, i.e., in V. The substrate, unlike inhibitors I and II, is not charged. It is, therefore, not surprising that the ionization state of a group at the active site has no apparent influence on substrate binding. Whether the group with pK = 7.8 is involved in catalysis cannot be determined. It is possible that, in the enzyme–substrate complex, that group is shielded from solvent or its p*K* so altered that it is not detectable in the pH range under investigation.

It has previously been reported that the enzyme activity is sensitive to sulfhydryl-selective reagents (Agranoff et al., 1960), and it has been proposed that a sulfhydryl group is located at the active site (Shah et al., 1965). We have confirmed this observation and have also shown that a "small" sulfhydryl reagent (Smith et al., 1975) also leads to complete inactivation. Furthermore, substrate, but not the competitive inhibitor methyl pyrophosphate, protects completely against inactivation by sulfhydryl reagents. These results are consistent with the proposed location of the sulfhydryl group in the active site, most likely, in the binding domain for the isopentene portion of the substrate. The sulfhydryl group could, therefore, directly participate in catalysis. Evidence that the sulfhydryl group is involved in catalysis is provided by the pH dependence for the inactivation of isomerase by iodoacetamide. This reaction depends on a p*K* of 9.3, which is in close agreement with the p*K* of 9.4 for V/K and V. Possibly the sulfhydryl group provides the proton required for carbonium ion formation.

The binding of inhibitors I and II is strongly dependent on the presence of the pyrophosphate moiety. Conversion of the pyrophosphate moiety to phosphate leads to marginal inhibitors ($K_i > 10^{-3}$ M). Similarly, monophosphates show no substrate activity. Substituents on the nitrogen of I also strongly affect K_i. Conversion of I to II, i.e., addition of the methyl group to the nitrogen of I, decreases K_i minimally 10^4-fold. Analogous structural modifications do not significantly affect K_i of rapidly equilibrating competitive inhibitors. 3,3-Dimethylbutyl pyrophosphate and isoamyl pyrophosphate, carbon analogues of I and II, have nearly identical K_i values. These results indicate a qualitative difference in the interaction between isomerase and the two types of inhibitors. These results suggest that interactions between the carbon portion of the substrate and the enzyme are of relatively little importance in formation of the initial Michaelis complex but become increasingly important as the structure of the enzyme–substrate complex approaches the transition-state structure.

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REFERENCES

Agranoff, B. W., Eggerer, H., Henning, U., & Oynen, F. (1960) *J. Biol. Chem. 235*, 326.

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

Ansell, G. B., & Spanner, S. (1962) *J. Neurochem. 9*, 253.

Banthorpe, D. V., Doonan, S., & Gutowski, J. A. (1977) *Arch. Biochem. Biophys. 184*, 381.

Bradford, M. (1976) *Anal. Biochem. 72*, 248.

Collins, J. C., Shess, W. W., & Frank, F. J. (1968) *Tetrahedron Lett. 30*, 3363.

Cornforth, J. W., & Popjak, G. (1969) *Methods Enzymol. 15*, 359.

Cornforth, J. W., Cornforth, R. H., Popjak, G., & Yengoyan, L. (1966) *J. Biol. Chem. 241*, 3970.

Cornforth, J. W., Clifford, J., Mallaby, R., & Phillips, G. T. (1972) *Proc. R. Soc. London, B 182*, 277.

Cousseau, J. (1980) *Synthesis*, 805.

Hanes, C. S., & Isherwood, F. A. (1949) *Nature (London) 64*, 1107.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Michelson, A. M. (1964) *Biochim. Biophys. Acta 91*, 1.

Narula, A. S., Rahier, A., Benveniste, P., & Schuber, F. (1981) *J. Am. Chem. Soc. 103*, 2408.

Poulter, C. D., & Satterwhite, D. M. (1977) *Biochemistry 16*, 5470.

Rahier, A., Taton, M., Schmitt, P., Benveniste, P., Place, P., & Anding, C. (1985) *Phytochemistry 24*, 1223.

Reardon, J. E., & Abeles, R. H. (1985) *J. Am. Chem. Soc. 107*, 4078.

Sandifer, R. M., Thompson, M. D., Gaughan, R. G., & Poulter, C. D. (1982) *J. Am. Chem. Soc. 104*, 7376.

Satterwhite, D. M. (1985) *Methods Enzymol. 110*, 92.

Shah, D. H., Cleland, W. W., & Porter, J. W. (1965) *J. Biol. Chem. 240*, 1946.

Shank, R. S., & Shechter, H. (1959) *J. Org. Chem. 24*, 1825.

Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766.

Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.

Winstein, S., & Sonnenberg, J. (1961) *J. Am. Chem. Soc. 83*, 3235.

Wolfenden, R. (1976) *Annu. Rev. Biochem. 45*, 271.