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Stereochemical Course of the Reaction Catalyzed by 5′-Nucleotide Phosphodiesterase from Snake Venom†

Floyd R. Bryant and Stephen J. Benkovic*

ABSTRACT: The hydrolysis reaction of ATPαS by snake venom phosphodiesterase is highly specific for the B diastereomer and proceeds with 88% retention of configuration at phosphorus. Since this enzyme also catalyzes the hydrolysis of the *S* enantiomer of *O-p*-nitrophenyl phenylphosphonothioate, the absolute configuration at P_α of ATPαS (B) is assigned as the *R* configuration provided the two substrates are processed identically. A mechanism for the hydrolysis reactions catalyzed by the venom phosphodiesterase involving at least a single covalent phosphoryl-enzyme intermediate is in accord with this result.

The stereochemical course of an enzyme-catalyzed phosphoryl transfer reaction at either the mono- or diester level has been a subject of considerable recent interest (Richard et al., 1978; Orr et al., 1978; Midelfort & Sarton-Miller, 1978; Saenger et al., 1974; Eckstein, 1975; Usher et al., 1972) since its elucidation furnishes information concerning the geometry of the transition state as well as the possible intermediacy of a covalent phosphoryl-enzyme species. The observation of net retention is in accord with a single or odd number of intermediates or lacking such species an adjacent spatial alignment between donor and acceptor substrates during phosphoryl transfer presuming permutational isomerism to be absent. Alternatively, net inversion is most simply interpreted as a direct displacement process through an in-line orientation (Benkovic & Schray, 1973). In view of the predominant observation of inversion stereochemistry at phosphorus for simple nonenzymic nucleophilic displacement processes (Benkovic & Schray, 1978) and, by inference, enzyme-catalyzed transfer processes, perhaps the more important use of this mechanistic probe will be in its detection of transient covalent intermediates in cases not readily amenable to kinetic or isolation methods. We wish to report our findings for such an enzyme, venom phosphodiesterase.

Experimental Procedures

Materials

Phosphodiesterase I (*Crotalus adamanteus venom*), inorganic pyrophosphatase (Bakers yeast), adenylate kinase (rabbit muscle), pyruvate kinase (rabbit muscle), hexokinase (yeast, type F-300), lactate dehydrogenase (rabbit muscle), and glucose-6-phosphate dehydrogenase (yeast) were purchased from Sigma. ATP (Na^+ salt), $NADP^+$, NADH, and phosphoenolpyruvate (monocyclohexylammonium salt) also were from Sigma. Adenosine was purchased from Aldrich. PEI-cellulose plates were obtained from Scientific Products, DEAE-Sephadex A-25 was from Pharmacia, DEAE-cellulose DE-52 was from Whatman, and Dowex 50-X8 was from Bio-Rad. Thiophosphoryl chloride was purchased from Alfa Inorganics and diphenyl phosphorochloridate from Aldrich. All other organic reagents, buffers, and inorganic salts were reagent grade. Doubly deionized distilled water was used throughout. The deuterium oxide (99.7%) was obtained from MSD Isotopes and the $H_2{}^{18}O$ (99%) was from KOR Isotopes.

The syntheses of ADPαS[1] (A + B)[2] and ATPαS (A + B) were accomplished according to the method of Eckstein & Goody (1976) as modified by P. Frey (personal communication). The AMPS was synthesized according to the method of Murray & Atkinson (1968). The purification of approximately 0.5 mmol of crude ATPαS (A + B) (dissolved in 200 mL of water with the pH adjusted to 9 with triethylamine) was by chromatography on a DEAE-Sephadex A-25 column (3.0 × 55 cm) by using a 5-L linear 0.1 M (2.5 L) to 0.75 M (2.5 L) gradient of ammonium bicarbonate. The concentration of the nucleotides was estimated throughout from A_{260} by employing ϵ 15 000 M^{-1} cm^{-1} (Eckstein & Grindl, 1970). The elution resulted in the separation of the A and B diastereomers. There were 350 fractions of approximately 15 mL volume collected. The ATPαS (A) eluted in fractions 280–294, whereas ATPαS (B) eluted in fractions 295–314 monitoring the effluent at A_{260}. The fractions containing the individual diastereomers were pooled separately and evaporated under vacuum to dryness. The residue was dissolved in water

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[1] Abbreviations used: ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); AMPS, adenosine 5′-*O*-thiophosphate; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; PEP, phosphoenolpyruvate; TEA, triethanolamine; Tris, tris(hydroxymethyl)aminomethane.

[2] The A and B designations for the diastereomers differing in configuration at P_α are those suggested by Eckstein (1975).

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and evaporated to dryness several times to remove excess ammonium bicarbonate. The two remaining residues were each dissolved in 25 mL of water. Each solution gave a single spot when chromatographed on PEI-cellulose TLC plates (0.75 M KH_2PO_4, pH 3.5) having a single R_f value identical with that of authentic ATPαS (A + B). Each fraction was assayed for activity with hexokinase by the procedure described below permitting the A or B assignment. ^{31}P NMR analysis was performed on 5 mM solutions by utilizing a Jeol PS-100-FT spectrometer at 40.29 MHz. Chemical shifts are from H_3PO_4.

Methods

Reactivity of ATPαS toward Venom Diesterase. The reaction solution (1 mL) consisted of 1.0 mM ATPαS (A + B), 100 mM Tris–acetate, pH 8.0, 20 mM $MgCl_2$, 0.3 unit/mL of phosphodiesterase, and 3.0 units/mL of inorganic pyrophosphatase incubated at 27 °C. At various times, 0.100-mL aliquots were removed and assayed for inorganic phosphate by the method of Martin & Doty (1949) as modified by Jencks & Gilchrist (1964). In the case of ATPαS (A + B) when the reaction was complete, the enzymes were removed by vortexing 0.5 mL of the reaction solution with 0.25 mL of chloroform.[3] The mixture was then centrifuged and the aqueous layer was removed and assayed for the presence of ATPαS (A isomer) by utilizing hexokinase (Stahl et al., 1974). The assay was initiated by adding 0.1 mL of the hydrolysis solution containing ca. 0.05 μmol of ATPαS to 40 mM TEA, pH 7.6, 40 mM glucose, 8.0 mM $MgCl_2$, 0.91 mM $NADP^+$, 0.056 unit/mL hexokinase, and 0.66 unit/mL glucose-6-phosphate dehydrogenase in a total volume of 1 mL. The NADPH produced was monitored continuously at A_{340}. A control was run omitting the ATPαS in order to correct for dehydrogenase activity at these high levels of glucose. The reactivity of the chromatographically resolved A and B diastereomers of ATPαS was assessed identically.

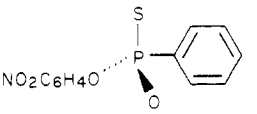
^{18}O Incorporation Experiments. The ^{18}O-labeled AMPS was prepared enzymically from ATPαS (B) by the stereospecific hydrolysis reaction catalyzed by the venom phosphodiesterase. The reaction mixture consisted of 34 mM ATPαS (B), 100 mM Tris–acetate, pH 8.0, 20 mM $MgCl_2$, and 20 mg (2.2 units) of phosphodiesterase in a total volume of 1.1 mL containing 37.5% $H_2{}^{18}O$ (T. Sharp and B. Minard, personal communication). After 40 h,[4] the solution was applied to a column (1.5 × 20 cm) of DEAE-cellulose DE-52 and eluted with a 1000-mL linear 0 (500 mL of water) to 0.4 (500 mL of buffer) M gradient of ammonium bicarbonate. There were 92 fractions of approximately 10 mL volume collected. The AMPS was eluted in fractions 28–34, well separated from AMP in fractions 20–27 and unreacted ATPαS (B) in fractions 35–50. The three fractions were identified by chromatography on PEI-cellulose plates as described above. Fractions containing AMPS (ca. 5 μmol) were pooled and treated with a twofold excess of triethylamine before evaporation. The AMPS was converted to ATPαS (A) by the coupled reactions of adenylate kinase and pyruvate kinase (Rex Sheu & Frey, 1977). The reaction solution consisted of 2.33 mM AMPS, 0.20 mM ATP, 28.8 mM PEP, 25 mM $Mg(NO_3)_2$, 100 mM KCl, 100 mM Hepes, pH 8.0, 500 units of adenylate kinase, and 200 units of pyruvate kinase in a total volume of 1.0 mL. After allowing for a 20-h reaction period, the resultant ^{18}O-labeled ATPαS (A) was purified by DEAE-cellulose chromatography as above followed by degradation and conversion to trimethylphosphorothioate and trimethyl phosphate by the method of Richard et al. (1978). For the analysis of ^{18}O enrichment, the phosphate esters were subjected to gas chromatography–mass spectral analysis by using a Finnigan 3200 system employing a 10% Silan 10-C on 100–120 mesh Gas-Chrom Q (Applied Sciences Laboratories) column.

Results and Discussion

Incubation of a chemically synthesized mixture of ATPαS (A + B) with snake venom phosphodiesterase results in the formation of 49.4% of the anticipated PP_i measured by coupling the hydrolysis to inorganic pyrophosphatase and quantitating the P_i produced by the method of Martin & Doty (1949). The remaining ATPαS was demonstrated to be 90% isomer A through its reactivity with hexokinase which utilizes preferentially the A diastereomer (Stahl et al., 1974). The quantity of ATPαS (A) remaining was measured through monitoring the formation of NADPH produced by the hexokinase–glucose-6-phosphate dehydrogenase couple.

In addition the chromatographically resolved diastereomers were incubated separately with the venom diesterase with only the B isomer being reactive. One can estimate that the relative reactivity (B/A) ≳ 70. The identity of separated diastereomers was proved by (i) ^{31}P NMR spectra that showed a doublet δ 43.28, 42.59 (J = 27.83 Hz) for the A isomer and δ 43.17, 42.47 (J = 28.32 Hz) for the B isomer[5] (Rex Sheu & Frey, 1977; Jaffe & Cohn, 1978) and (ii) the reactivity of the A isomer but not the B isomer as a substrate toward hexokinase. Finally, the formation of AMPS was demonstrated in runs at initially higher ATPαS (B) concentrations by separation of the reaction components after incubation on DEAE-cellulose followed by their chromatography on PEI-cellulose plates.

Previous work had shown that the S enantiomer of *O-p*-nitrophenyl phenylphosphonothioate (**1**) was more than 50-fold



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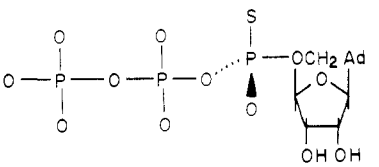
more reactive than the R enantiomer toward the snake venom phosphodiesterase (Dudman & Benkovic, 1977; K. deBruin, personal communication). By presuming then that the venom enzyme utilizes only substrates with the above absolute configuration at phosphorus, the absolute stereochemistry for ATPαS isomers A and B may be assigned by analogy, superimposing PP_i and *p*-nitrophenol as a common leaving group. Thus, isomer B (**2**) has the R configuratin depicted for P_α and



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isomer A has the opposite S configuration. Burgers & Eckstein (1978) independently have arrived at the same absolute assignments. It must be emphasized, however, that

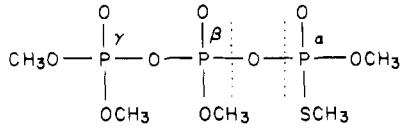
[3] Failure to remove the phosphodiesterase resulted in its interference with the hexokinase assay.

[4] If run for 96 h at an ATPαS (B) concentration of 6 mM, only AMP is recovered.

[5] Owing to the similarity in the chemical shifts for the two isomers, the addition of an authentic sample of the A isomer prepared from AMPS via the adenylate and pyruvate kinase coupled system (Jaffe & Cohn, 1978) to the NMR tube was employed to verify the resonance assignments.

this indirect assignment presumes that the hydrolyses of ATPαS and the phenyl phosphonothioate ester[6] occur at the same venom active site with mechanisms of identical stereochemical consequences.

The overall stereochemical course of the hydrolysis reaction catalyzed by the venom diesterase was elucidated by incubating the B diastereomer of ATPαS with the enzyme in the presence of $H_2{}^{18}O$. The resulting [^{18}O]AMPS was purified by chromatography on DEAE-cellulose and then incubated with ATP and PEP in the presence of adenylate kinase and pyruvate kinase to form stereospecifically the A isomer of [^{18}O]-ATP(αS). The location of the ^{18}O as either a bridged (αβ) or nonbridged (α) ^{18}O was established by degradation of [^{18}O]ATP(αS) (A) through a sequence of reactions that converts it to the pentamethyl polyphosphate (**3**) (Richards



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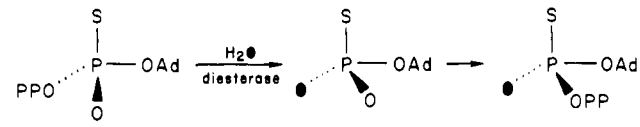
et al., 1978). Hydrolysis of **3** occurs with nearly equal partitioning of P_α and P_β so that the total P–O cleavage process is effectively random (Frey, personal communication; Richards et al., 1978).[7] Consequently a bridged (αβ) ^{18}O would result in ^{18}O enrichment in both the trimethyl phosphate and trimethyl phosphorothioate derived from **3** upon hydrolysis followed by methylation; a nonbridged (α) ^{18}O would lead to ^{18}O enrichment in only the trimethyl phosphorothioate.

The results of the GC–mass spectrometric analysis on ATPαS (A) corrected for natural abundance (^{18}O, ^{34}S) are a mass % ^{18}O (trimethyl phosphate) = 1.4 ± 0.2 and a mass % ^{18}O (trimethyl phosphorothioate) = 31.7 ± 0.8. The former value is also corrected for the desulfurization arising during the conversion of ATPαS to the trimethyl phosphates.[8] Since the actual analytical scheme employed commences with ATPαS (B) and ends with ATPαS (A) of known opposite relative configuration at P_α, the orientation of the O^{18} in the [O^{18}]AMPS and the pyrophosphate of the starting B isomer can be related without employing the absolute configuration of the diastereomer of ATPαS. The total 34.5% enrichment in the trimethyl phosphorothioate and the trimethyl phosphate corresponds to the enrichment anticipated for incorporation of a single ^{18}O label from 37.5% $H_2{}^{18}O$. Comparison of the analysis of the ^{18}O enrichment in trimethyl phosphorothioate with total ^{18}O enrichment is in accord with the hydrolysis reaction proceeding with 88% stereospecificity.[9] As depicted in Scheme I, the observation of nonbridged (α) ^{18}O requires an overall retention process for the hydrolysis of ATPαS by venom diesterase.

The finding of a stereochemical retention process is in accord with either a mechanism involving a single or odd number of

Scheme I



phosphoryl-enzyme intermediates whose formation and decomposition occur via inversion processes or with a direct transfer process between water and ATPαS possibly involving a pentacovalent species and an associated permutational isomerization if one postulates axial attack on phosphorus with the PP_i leaving group initially equatorial. Retention of configuration at phosphorus has been observed in one other diester type reaction, namely, UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase, a system for which there is considerable evidence for a UMP–enzyme intermediate (P. Frey, personal communication; Wong et al., 1977), whereas other systems such as UDP-glucose pyrophosphorylase (Rex Sheu & Frey, 1978), RNA nucleotidyltransferase (Eckstein et al., 1977), and phosphoribosylpyrophosphate synthetase (Li et al., 1978) catalyze inversion processes at phosphorus. Since there is not available other evidence to corroborate the existence of a venom enzyme–AMPS species, no unequivocal choice between the above alternates can be made at present, although we are inclined to favor the explanation requiring a intermediate phosphoryl-enzyme species. Experiments bearing on this question are in progress.

Added in Proof

In unpublished work (July 1977) D. Usher and D. Yee independently showed that the *Rp* diastereomer of 3′,5′-Up(S)dT was a better substrate than the *Sp* diastereomer for the venom enzyme.

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[6] In the case of the Burgers and Eckstein study, the reference substrate was the UpSA dinucleotide so that similar presumptions are involved.

[7] Degradation under identical conditions of [^{18}O]ATPαS where the P_α moiety contains (α + αβ) [^{18}O]:(αβ) [^{18}O]:(α) [^{18}O] in ratio 8:1:1 yields [^{18}O]trimethyl phosphorothioate with *m/e* 160:158:156 in the ratio 4.04:9.18:1 and trimethyl phosphate with *m/e* 142:140 in the ratio 1:1.85 (corrected for natural abundance). This splitting pattern is consistent with a cleavage of the αβ bridging oxygen bonds in the ratio P_α–O:P_β–O of 1.9:1.

[8] Since the *m/e* 144 can only arise from the loss of sulfur from P_α, the ratio for desulfurization is given directly by *m/e* 144/*m/e* 160 obtained from the [^{18}O]ATPαS degradation discussed in footnote 7.

[9] The calculation is based on the ^{18}O enrichment of trimethyl phosphorothioate adjusting for the partitioning of the αβ oxygen ligand between P_α and P_β by subtracting this value.

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Purification and Characterization of Human Liver Glycolate Oxidase. Molecular Weight, Subunit, and Kinetic Properties†

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ABSTRACT: Glycolic acid oxidase (EC 1.1.3.1, glycolate:oxygen oxidoreductase) has been purified 1000-fold from human liver to apparent homogeneity by gel electrophoresis. The molecular weight of the enzyme, determined by sedimentation equilibrium, by Sephadex gel filtration, and by dodecyl sulfate gel electrophoresis of the enzyme cross-linked with dimethyl suberimidate, was found to range between 160 000 and 179 000. A subunit molecular weight ranging from 40 000 to 43 000 was found by sodium dodecyl sulfate gel electrophoresis and sedimentation equilibrium in 5 M guanidine hydrochloride. A tetrameric structure for the active enzyme has been established by the molecular weight studies and enzyme cross-linking experiments with dimethyl suberimidate. The enzyme, shown to contain the FMN coenzyme by spectroscopic analysis, was found to be rapidly and irreversibly inactivated by the acetylenic substrate DL-2-hydroxy-3-butynoate. Enzyme turnover studies with the acetylenic substrate have shown that inactivation occurs after 130 catalytic events. In contrast, two 4-substituted 2-hydroxy-3-butynoates, DL-2-hydroxy-4-phenyl-3-butynoate, and DL-2-hydroxy-6-phenyl-3-hexynoate were shown not to be inhibitors of the oxidase. *N*-Octyloxamate was found to be a potent, novel type of inhibitor of the enzyme, but in contrast to hydroxybutynoate inhibition, this inhibitor is of the reversible, noncompetitive type with a K_I of 3.2×10^{-6} M. Both *N*-octyloxamate and DL-β-phenyllactate, also shown to be a reversible noncompetitive inhibitor of the enzyme, were shown to reduce the rate of irreversible inhibition of the enzyme by hydroxybutynoate.

Seventy years ago, Dakin (1907) demonstrated that dogs possess an activity capable of converting glycolate or glyoxylate to oxalate. The site(s) of the metabolic conversion was not known until Dohan (1940) noted that rabbit and rat liver exhibited an oxidase activity, independent of added cofactors, that metabolized glycolate. Kun (1952), twelve years later, showed that a supernatant from homogenized rat livers contained the glycolate oxidizing activity and molecular oxygen participated in the process. Zelitch & Ochoa (1953) and Kun et al. (1954), extending earlier work, achieved partial purifications of the rat liver glycolate oxidizing activity and presented evidence suggesting the oxidase activity to be a flavoprotein. Robinson & Sizer (1959), Keay et al. (1960), and Robinson et al. (1962) in a series of papers described the isolation and properties of a pig kidney cortex enzyme with FMN as the prosthetic group that catalyzed the oxidation of glycolate, other short chain L-α-hydroxy acids, and glyoxylate. Schuman & Massey (1971a,b) purified and characterized glycolic acid oxidase from pig liver. Their preparation was shown to contain 1 mol of FMN per 51 000 daltons. Holmes & Duley (1975) probed the physical properties of the purified rat liver enzyme and reported the holoenzyme to be composed of a set of four 43 000 molecular weight subunits. In this paper we report the isolation and purification of human liver glycolate oxidase and evaluate its structural, kinetic, and subunit properties.

Experimental Procedure

All experimental details are given in the supplementary material (see paragraph concerning supplementary material at the end of this paper).

Results

Table I shows an overall purification of about 400-fold when the 65% ammonium sulfate precipitate is taken as the starting point. Starting from the homogenization step, the purification achieved is greater, but since the dye reduction assay at this initial stage of the isolation scheme undergoes a spurious nonenzymatic reduction, the 65% ammonium sulfate precipitate step was chosen as the starting point for yield and purity calculations.

Lactate dehydrogenase, which can also oxidize glyoxylate to oxalate, was assayed throughout the purification scheme by the procedure of Gibbs & Watts (1973).

The enzyme was found to be homogeneous on 5, 6, 7, and 8% analytical disc gels stained for protein or enzyme activity, and on $NaDodSO_4$ gels only a single subunit band was detected.

Molecular Weight. The molecular weight of human glycolate oxidase was investigated by the gel filtration method utilizing Sephadex G-200 as the gel and xanthine oxidase, γ-globulin, lactic acid dehydrogenase, and bovine serum albumin as the protein standards. Human liver glycolate oxidase eluted from the column as a single, symmetrical peak corresponding to a molecular weight of 160 000.

When native human liver glycolate oxidase was treated with sodium dodecyl sulfate–mercaptoethanol and subjected to

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