(in the UV region, the turbidities are always more serious). Even fluorescent spectra were found to be not free from optical scattering due to the membrane system.

Smaller membrane volumes also simplify the calculation of the potentials to a great extent. For example, the volumes of membrane phase, V_M, are so small in this study that the values of $V_M + V_B$ are considered to be almost the same as the values of V_B.

When the lipid amount is 5–50 mg in 1 mL of aqueous solution, the available volume for a unilamellar liposome, which is composed of about 5000 lipid molecules and has a diameter of 250–300 Å, would be about 10^8–10^9 Å^3. For sodium dodecyl sulfate solutions (Mukerjee & Banerjee, 1964; Fernandez & Fromherz, 1977; Funasaki, 1977), a concentration 10 times the critical micellar concentration results in an available volume per micelle, composed of 60 surfactant molecules, of 10^6 Å^3. These values underline the difficulty of distinguishing the "distinct bulk aqueous phase" where the value of the potential should be taken as zero and also indicate the overlapping of electrical double layers of different liposomes or micelles (Mille & Vanderkooi, 1977; Huisman, 1964; Mukerjee, 1972; Winsor, 1953). The available volume per liposome is at least 3×10^{10} Å^3 in this study. Therefore, the theory of Guoy–Chapman or Donnan could be easily applied and examined. Further work on the theoretical aspects of our findings are in progress and shall be published later.

However, 2×10^{-5} M of MB has been used in this study, although a concentration of 2×10^{-6} M of MB is assumed to be satisfactory. This concentration corresponds to at most one probe for 25 lipid molecules.

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Stereochemical and Kinetic Investigation of ^{32}P-Labeled Inorganic Phosphate Exchange Reaction Catalyzed by Primer-Independent and Primer-Dependent Polynucleotide Phosphorylase from *Micrococcus luteus*†

John F. Marlier, Floyd R. Bryant, and Stephen J. Benkovic*

ABSTRACT: The S_P diastereomer of adenosine 5′-*O*-(1-thiodiphosphate) (ADPαS) is a substrate for the ^{32}P-labeled inorganic phosphate exchange reaction catalyzed by the T and I forms of polynucleotide phosphorylase. The exchange reaction occurs with retention of configuration. This exchange reaction is very slow when only ADPαS(S_P) is present but is greatly activated by dinucleotide primers and ADPαS(R_P), although the latter is not a substrate for the exchange reaction. Ap(S)A(R_P) is an ~50% better activator of the exchange than the S_P diastereomer. Furthermore, high levels of the ADPαS(S_P) eliminate the activation by primers and by ADPαS(R_P). A phosphatase activity is present with the I form of the enzyme which converts ADPαS(R_P) to AMPS. This activity may be responsible for the formation of the 5′-phosphate end group for de novo polymerization or for the processivity of this reaction.

Polynucleotide phosphorylase catalyzes the reversible polymerization of nucleoside diphosphates to polynucleotides and inorganic phosphate. Two forms of this enzyme have been described from the bacterial source *Micrococcus luteus* (Godefroy-Colburn & Grunberg-Manago, 1972). The native, or I form, catalyzes de novo polymerization of nucleoside diphosphates in the 5′ → 3′ direction via a processive mechanism. Only large molecular weight polymers are released from the enzyme (Moses & Singer, 1970), and they are terminated at the 5′ end by a phosphate group (Godefroy-Colburn & Grunberg-Manago, 1972). Polymerization is stimulated by oligonucleotides although they are not incorporated into the 5′ end of the polymer. The trypsinized form of the enzyme, form T, has a reduced de novo polymerization activity but

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readily elongates the 3′ end of an oligonucleotide primer. The elongation is nonprocessive, and only small polymers are formed (Moses & Singer, 1970).

The phosphorolysis reaction, which is assumed to be the one of biological importance in vivo, is similar for both forms of the enzyme. Phosphorolysis is in the 3′ → 5′ direction; the mechanism is processive for polynucleotides (Klee & Singer, 1968) and nonprocessive for oligonucleotides (Chou & Singer, 1970a). The products are nucleoside diphosphates and a dinucleotide, which is resistant to phosphorolysis.

The enzyme also catalyzes the exchange of inorganic [^{32}P]phosphate with the β-phosphate of ADP,[1] presumably an equilibrium between the polymerization and phosphorolysis reactions (Chou & Singer, 1971a). This paper reports the use of diastereomeric thiophosphate analogues of ADP (ADPαS) and of ApA [Ap(S)A] to investigate the stereochemical course and primer requirements, respectively, of this exchange reaction. A phosphatase activity is described which is present in the I form of the enzyme and may be related to the overall mechanism.

Experimental Procedures

Materials and Methods

ApA, ADP, PEP, and Ap_5A were from Sigma Chemical Co. and were used without further purification. ^{32}P-Labeled orthophosphate was from New England Nuclear (5 mCi/mL); water, 99% ^{18}O, was from KOR Isotopes. PEI-F-cellulose TLC plates (20 × 20 cm, 100-μm thickness) from J. T. Baker Chemical Co. were developed in 0.75 M KH_2PO_4, pH 3.5. DEAE-cellulose for column chromatography (Whatman DE-52) was from Reeve Angel; triethylammonium bicarbonate (TEAB) was the eluting buffer in all cases.

Primer-independent (form I) and primer-dependent (form T) polynucleotide phosphorylases were obtained from P-L Biochemicals. Form I enzyme (Lot 4148-2) had a specific activity of 66 polymerization units/mg of protein and contained no appreciable adenylate kinase activity as shown by conversion of ADPαS to AMPS and ATPαS. Form T enzyme (Lot 431-5) had a specific activity of 120 polymerization units/E_{280}, and the ratio of primer-dependent to primer-independent activities was ~15. Myokinase (1290 units/mg of protein), pyruvate kinase (389 units/mg of protein), and hexokinase (210 units/mg of protein) were all from Sigma Chemical Co.

Radioactivity was measured on a Beckman LS-8100 liquid scintillation counter; Aquasol from New England Nuclear was used as the scintillation cocktail. ^{31}P NMR chemical shifts were measured on a Brüker WP-200 NMR spectrometer; UV absorbances were measured on a Gilford UV spectrophotometer; analysis of the ^{18}O content was done on a Finnegan Model 3200 GC-mass spectrometer.

Chemical and Enzymatic Syntheses

ADPαS(S_P) Diastereomer. The S_P diastereomer of ADPαS was prepared enzymatically in the manner described in the literature (Sheu & Frey, 1977), with one exception—a catalytic amount of ADPαS(S_P) was utilized to initiate the phosphorylation instead of ATP. Purification was on DEAE-cellulose (20 × 1.4 cm), eluted with a 0 → 0.4 M linear gradient of TEAB (500 mL each). The ATPαS(S_P) thus obtained was evaporated in vacuo and converted to ADPαS(S_P) by enzymatic degradation with hexokinase (Jaffe & Cohn, 1978). Final purification on DEAE-cellulose was as described above for ATPαS: yield 31.2 μmol (60%). ADPαS(S_P) was evaporated in vacuo and dissolved in 0.75 mL of H_2O to give a 41.7 mM stock solution.

ADPαS(R_P) Diastereomer. ADPαS(R_P) was isolated after phosphorylation of 25 μmol of a chemically synthesized mixture of R_P and S_P ADPαS (Eckstein & Goody, 1976) with phosphoenolpyruvate (PEP) and pyruvate kinase (Sheu & Frey, 1977). The unreacted ADPαS (R_P isomer) was isolated by DEAE-cellulose chromatography (20 × 1.4 cm) eluted with a linear gradient of 0.1 → 0.3 M TEAB (500 mL each). The fractions containing ADPαS were evaporated in vacuo and dissolved in 0.5 mL of water to give a 19.4 mM stock solution (yield, 78%).

Adenylyl (3′→5′)Adenylyl O,O-Phosphorothioate [Ap(S)A], S_P and R_P Diastereomers. The chemical synthesis of these diastereomeric dinucleotide phosphorothioates has been described elsewhere (Marlier & Benkovic, 1980). Final purification and separation of the diastereomers was accomplished on DEAE-cellulose (42 × 2.0 cm), eluted with a linear gradient of 0 → 0.25 M TEAB (750 mL each). ^{31}P NMR showed the R_P diastereomer at 57.5 ppm to be contaminated with <10% of the S_P diastereomer (56.5 ppm). The S_P diastereomer, on the other hand, was essentially pure. These fractions were evaporated in vacuo and dissolved in H_2O to make stock solutions of known concentration as determined by the absorbance at 260 nm.

Kinetic and Stereochemical Assays

TLC Assay for [β-^{32}P]ADPαS and [β-^{32}P]ATPαS. After the appropriate reaction mixtures were cooled to room temperature, a 50-μL aliquot was applied (5-cm spot) to a 7 × 20 cm PEI-cellulose TLC plate. The plate was developed in the solvent system described earlier to a distance of 15 cm from the origin. After being dried, the UV-absorbing spot(s) were visualized under a UV source and noted. Authentic unlabeled AMPS, ADPαS, and ATPαS, which were cochromatographed with the 50-μL aliquots, had R_f's of 0.42, 0.33, and 0.20, respectively. Inorganic [^{32}P]phosphate had an R_f of 0.68 under these conditions and was well separated from ADPαS and ATPαS. The TLC plate was cut into horizontal strips of ~1.5 cm, with some alterations made to avoid cutting through the center of UV-absorbing spots, and the PEI-cellulose was peeled from the plastic backing with a razor. Each strip was placed in a 3-mL scintillation vial to which was added 150 μL of 1 M HCl and 3 mL of Aquasol. The vials were capped, shaken vigorously, and counted in the liquid scintillation counter twice for 10 min. In separate control, as well as the individual experiments, >90% of all the counts applied to the chromatogram could be recovered after TLC.

Stereochemical Experiments. Specific concentrations of substrates and primers as well as the quantities of PNPase used are given in the legends for the tables and figures. In addition to those compounds, all stereochemical experiments contained in a 100-μL total volume, 0.1 M Tris, pH 7.9, 5 mM $MgCl_2$, 0.5 mM DTT, and 6 mM [^{32}P]orthophosphate (specific radioactivity, 200–800 cpm/nmol). Reactions were initiated by the addition of enzyme, and the reaction vials were sealed with a rubber septum and incubated at 37 °C for 20 h. The amount

[1] Abbreviations used: ADPαS(S_P) and ADPαS(R_P), diastereomers of adenosine 5′-*O*-(1-thiodiphosphate); ATPαS(S_P) and ATPαS(R_P), diastereomers of adenosine 5′-*O*-(1-thiotriphosphate); AMPS, adenosine 5′-*O*-thiophosphate; IMPS, inosine 5′-*O*-thiophosphate; PNPase, polynucleotide phosphorylase; Ap_5A, P^1,P^5-di(adenosine-5′)-pentaphosphate; Ap(S)A(R_P) and Ap(S)A(S_P), diastereomers of adenylyl (3′–5′)adenylyl *O,O*-phosphorothioate; TEAB, triethylammonium bicarbonate; PEP, phosphoenolpyruvate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; TLC, thin-layer chromatography; DEAE, diethylaminoethyl; GC, gas chromatograph; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DTT, dithiothreitol; RNA, ribonucleic acid.

Table I: Stereochemistry of Exchange Reaction Catalyzed by Form I and Form T Polynucleotide Phosphorylase[a]

enzyme	substrate(s)	before pyruvate kinase (cpm of ADPαS/cpm of P_i)	after pyruvate kinase: cpm of ADPαS(R_P)/cpm of P_i	after pyruvate kinase: cpm of ATPαS(S_P)/cpm of P_i
I form	ADPαS(S_P and R_P) (1 mM each)	2300/140 000	350/105 000	1800/105 000
I form	ADPαS(S_P) (2 mM)	1700/140 000	170/110 000	1400/110 000
I form	ADPαS(R_P) (1.5 mM)	47/30 000		
I form	ADPαS(S_P) (1 mM), ApA (80 μM)	3000/150 000	171/95 000	2200/95 000
T form	ADPαS(S_P) (1.3 mM), ApA (600 μM)	1900/250 000	240/180 000	1200/180 000
T form	ADPαS(R_P) (1 mM), ApA (200 μM)	60/150 000		
T form	ADPαS(S_P and R_P) (0.64 mM each), ApA (600 μM)	4900/250 000	295/160 000	3600/160 000

[a] Experimental conditions were as described under Experimental Procedures. A total of 1.54 units of form I and 0.24 unit of form T enzyme were used. Incubation was at 37 °C for ~20 h.

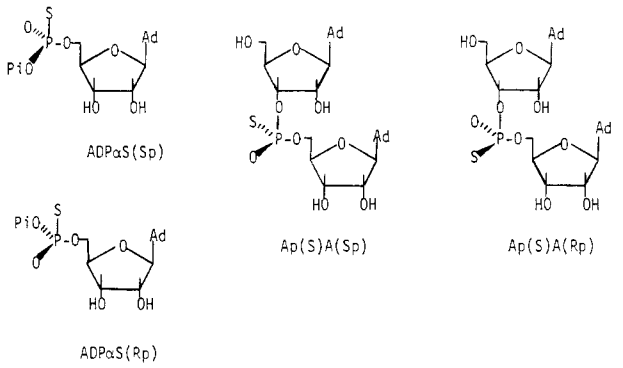
of [β-^{32}P]ADPαS in 50 μL of the reaction mixture was then determined by the TLC assay. To the remaining 50 μL of assay solution was added 10 μL of 40 mM PEP, 2 μL of 4 M KCl, and 2 μL of pyruvate kinase. After a 15-h incubation at room temperature, 50 μL of this reaction solution was subjected to the TLC assay. Since pyruvate kinase will only phosphorylate ADPαS(S_P) to ATPαS(S_P), the counts in the ADPαS spot are due to ADPαS(R_P), and those in the ATPαS spot are due to that derived from ADPαS(S_P) (Sheu & Frey, 1977).[2]

Kinetic Studies. The assay for the amount of [β-^{32}P]-ADPαS produced with time was the TLC system described above. The concentrations of substrate, $MgCl_2$, primers, and enzymes are listed in the figure and table legends. Unless otherwise noted all incubation mixtures contained 0.1 M Tris, pH 7.9, 0.5 mM DTT, and 6 mM [^{32}P]orthophosphate (200–800 cpm/nmol) in 100-μL total volume. The reactions were initiated by the addition of enzyme and incubated at 37 °C. The rate of exchange into ADPαS(S_P) (the only substrate that would exchange) determined by the TLC assay was expressed as nanomoles of ADPαS(S_P) exchanged per hour per polymerization unit of enzyme. This rate was calculated from the cpm in the ADPαS spot and the known specific radioactivity of the orthophosphate. No correction for the decrease in specific radioactivity of the orthophosphate due to release of the unlabeled β-phosphate of ADPαS was necessary because the exchange reaction had proceeded to <10% reaction in all cases.

Phosphatase Activity (Form I)

Product Identification. The 100-μL incubation mixture contained either 2 mM ADPαS(S_P) or 2 mM ADPαS(R_P), 0.1 M Tris, pH 7.9, 5 mM $MgCl_2$, and 1.54 units of form I enzyme. The products were identified three ways. (1) In the TLC method, a small aliquot (~5 μL) of the incubation mixture was developed on the TLC system along with authentic ADPαS and AMPS. Whenever ADPαS(R_P) and form I enzyme were present, a new spot appeared with time at R_f 0.42. This was the same R_f as authentic AMPS, and both gave a yellow color when sprayed with 2,6-dibromobenzoquinone-4-chlorimidate, DBQ (1% in glacial acetic acid), indicating the presence of a thiophosphate (Stenersen, 1968). (2) In the

Scheme I



enzymatic method, 10 μL of the above incubation mixture was added to 180 μL of 0.1 M potassium cacodylate, pH 6.8, in a microcuvette. The optical density at 265 nm was determined, 10 μL (0.55 unit) of 5′-adenylic acid deaminase was added, and the change in optical density at 265 nm was recorded. The concentration of IMPS derived from AMPS was determined from the initial and final optical densities (Murray & Atkinson, 1968). (3) In the inorganic phosphate assay, the concentration of inorganic phosphate in solution was measured from 10-μL aliquots of the above assay solutions exactly as described in the literature (Lanzetta et al., 1979). Reaction mixtures containing a known quantity of inorganic phosphate and either substrate but no enzyme or enzyme but no substrate served as controls for the conditions of these experiments. In each control the total phosphate determined by the assay was equal to the amount known to be present initially.

Determination of $P_β$–O Bond Cleavage. A mixture of chemically synthesized R_P and S_P diastereomers of ADPαS (11.6 μmol) was dissolved in 0.3 mL of 99% $H_2{}^{18}O$, 0.1 mL of 1 M Tris, pH 7.9, 0.1 mL of 0.2 M $MgCl_2$, 0.4 mL of 25 mM inorganic phosphate, 0.1 mL of H_2O, and 0.3 mg of form I enzyme. The reaction mixture was incubated at 37 °C for 60 h. The AMPS produced was purified by DEAE-cellulose chromatography (20 × 1.9 cm) eluted with a linear gradient of 0 → 0.4 M TEAB (500 mL each). AMPS was then analyzed for ^{18}O content by a procedure published earlier (Bryant & Benkovic, 1979). No ^{18}O was incorporated into AMPS.

Results

Stereochemistry of Exchange Reaction. The diastereomers of ADPαS (Scheme I) were tested as substrates for the [^{32}P]P_i exchange reaction. First, ADPαS(S_P) was incubated with form I enzyme, and after 20 h radioactive ADPαS was de-

[2] These experiments were done under conditions of low (<1.5 mM) ADPαS concentrations, essentially as described by Sheu & Frey (1977). Under these conditions, we as well as the authors noted above could detect no ability of pyruvate kinase to accept ADPαS(R_P) as the substrate.

Table II: Relative Rate of Exchange Reaction Catalyzed by I and T Forms of Polynucleotide Phosphorylase in the Presence ApA and Ap(S)A, R_P or S_P[a]

enzyme	primer	relative rate
form I	none	1.0
	ApA	3.0
	Ap(S)A(R_P)	3.2
	Ap(S)A(S_P)	2.1
form T	none	1.0
	ApA	1.9
	Ap(S)A(R_P)	2.1
	ApS(A)(S_P)	1.4

[a] In addition to the reaction components described under Experimental Procedures, experiments with the I form PNPase included 1.54 units of enzyme, 1.0 mM ADPαS(S_P), and 200 μM ApA or Ap(S)A. Experiments with the T form enzyme contained 0.24 unit of enzyme, 1.0 mM ADPαS(S_P), and 400 μM ApA or Ap(S)A.

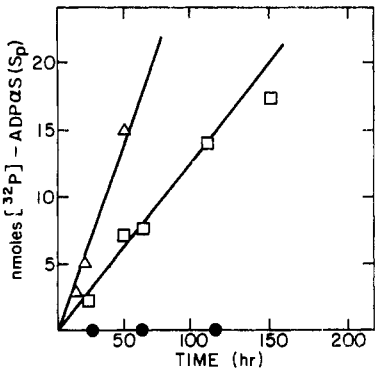


FIGURE 1: Rate of $[^{32}P]P_i \rightleftarrows$ ADPαS(S_P) exchange as a function of enzyme concentration. For these assays ADPαS(S_P) was 1.5 mM, P_i was 3.75 mM (~400 cpm/nmol), and $MgCl_2$ was 5 mM. The reaction was done at 0 (●), 6.6 (□), and (△) 11.5 polymerization units of form I enzyme per total reaction volume (500 μL).

tected by TLC. After specific phosphorylation of ADPαS(S_P) to ATPαS(S_P) by pyruvate kinase (Sheu & Frey, 1977), no ADPαS could be visualized on the TLC plate and essentially all the radioactivity was found in the ATPαS spot (Table I). The small amount of radioactivity still remaining in the region where ADPαS chromatographed was only slightly higher than the background on the TLC plate (50–100 cpm) and could be due to a small amount of ADPαS(S_P) that was not converted to ATPαS. It was concluded that ADPαS(S_P) is a substrate for the exchange reaction which occurs with net retention of configuration.

Secondly, ADPαS(R_P) was tested as a substrate for the form I enzyme. Two UV-absorbing compounds were present after TLC, one at R_f 0.33 (ADPαS) and a new spot at R_f 0.42. The two spots were of roughly equal intensity and neither contained radioactivity. The new product was later identified as AMPS; this reaction will be discussed in detail later. The radioactivity associated with ADPαS(R_P) was not significantly higher than the background on the TLC plate, and, therefore, it was concluded that this diastereomer is not a substrate within our limits of detection.

Similar experiments were carried out either with a 1:1 mixture of R_P and S_P diastereomers of ADPαS or with ADPαS(S_P) in the presence of ApA. In all cases with the form I enzyme the overall stereochemistry of $[^{32}P]P_i$ exchange into ADPαS(S_P) proceeded with net retention of configuration (Table I).

Finally, stereochemical experiments exactly as described for the form I enzyme were carried out on the form T enzyme. In all cases ADPαS(S_P) was the substrate for the exchange reaction and the overall stereochemistry was again retained (Table I). It is interesting that the T form enzyme would not convert ADPαS(R_P) to AMPS as was noted above for the I form enzyme. This was true even when the levels of T form enzyme used would support the exchange of ADPαS(S_P) at a rate equal to or greater than that of levels of the I form used above. At these levels of the I form enzyme considerable quantities (~50%) of AMPS would have been produced in the same reaction time. This will be described more fully later.

Primer Stereochemistry. Dinucleotides are known to activate the exchange reaction with ADP as the substrate, although they do not undergo phosphorolysis (Chou & Singer, 1971a). We have found that ApA activates the exchange of $[^{32}P]P_i$ into ADPαS(S_P) and under these conditions no radioactive ADP (R_f 0.52) could be detected, further confirming the observation that ApA does not undergo phosphorolysis. Quantitative aspects of this activation will be discussed later. The two diastereomers of Ap(S)A (Scheme I) were tested as activators for this exchange reaction. The dinucleotide with the R_P configuration activated the exchange of $[^{32}P]P_i$ into ADPαS(S_P) at the same rate as ApA, whereas the S_P diastereomer of Ap(S)A was only 50–60% as effective at activating this exchange reaction (Table II). Both forms of the enzyme were studied, and the results were essentially the same. Only one concentration of Ap(S)A was tried with each enzyme form; all were at concentrations ~10 times greater than the K_a for ApA.

Table III: Kinetic Constants for $[^{32}P]P_i$–ADPαS(S_P) Exchange[a]

enzyme	variable substrate or activator	apparent[b] K_a or K_m	V_{max}[c]
form I	P_i	2.5 ± 0.4 mM	
	ADPαS(S_P)	1.2 ± 0.2 mM	0.17 ± 0.03
	ApA	33 ± 6 μM	0.47 ± 0.05
form T	ADPαS(S_P)	1.0 ± 0.3 mM	0.50 ± 0.10
	ApA	37 ± 6 μM	0.78 ± 0.10

[a] Experiments were as described under Experimental Procedures. A total of 1.54 units of form I enzyme and 0.24 unit of form T enzyme were used. [b] Measured at one level of the nonvariable substrates; these are given under Experimental Procedures. [c] In nanomoles of ADPαS exchanged·unit^{-1}·hour^{-1}.

Validity of Kinetic Assay. When the S_P diastereomer of ADPαS was incubated with form I enzyme, a linear increase of radioactivity incorporated into ADPαS was observed. This incorporation was linearly dependent on enzyme concentration and did not occur in the absence of enzyme (Figure 1). Similar results were obtained for a mixture of the R_P and S_P diastereomers (results not shown). No lag phases were observed for the exchange reaction in agreement with published results for the exchange reaction with ADP as the substrate (Chou & Singer, 1971a).

Exchange Rate vs. Inorganic Phosphate and ADPαS(S_P) Concentration. The rate of $[^{32}P]P_i$ exchange was measured at several P_i concentrations and at a constant 1.5 mM ADPαS(S_P). An apparent K_M for P_i was obtained from a double-reciprocal plot (not shown) and is reported in Table III. Conversely, the rate of $[^{32}P]P_i$ exchange was measured at several ADPαS(S_P) concentrations and at constant 6.0 mM P_i. In these experiments a constant high level of Mg^{2+} (the Mg^{2+} is saturating) was used, since the substrate is the uncomplexed nucleotide (Chou & Singer, 1971b). Under these conditions the [ADPαS] and [ADPαS·Mg] increase in a nearly linear fashion with $[ADP\alpha S]_{total}$, and we derived all of our kinetic constants by plotting the total ADPαS concentration. From reciprocal plots of the initial velocity of exchange vs. total ADPαS(S_P) concentration, the kinetic pa-

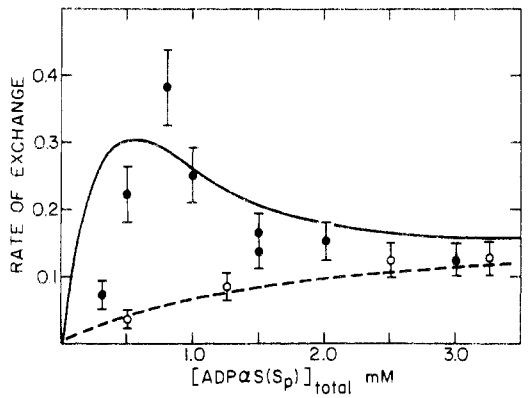


FIGURE 2: Rate of $[^{32}P]P_i \rightleftarrows ADP\alpha S(S_P)$ exchange in the presence of 80 μM ApA (●) and in the absence of ApA (○). The total ADPαS concentration ($ADPS_{free}$ + ADPS·Mg) is plotted on the ordinate at 13 mM $MgCl_2$ and 5 mM P_i (~700 cpm/nmol). The solid and dashed lines were generated from eq 1 with the following values of the kinetic parameters: K_P = 50 μM; K_{AAA} = 1200 μM; $K_A = K_{AA}$ = 400 μM; K_{PA} = 1200 μM; k_1 = 0.17 h^{-1} $unit^{-1}$; k_2 = 3.0 h^{-1} $unit^{-1}$.

rameters listed in Table III were obtained. Identical experiments with the form T enzyme gave kinetic constants similar to those for form I.

Activation of Exchange by ApA. The rate of $[^{32}P]P_i$ exchange into $ADP\alpha S(S_P)$ is greatly enhanced by the presence of ApA. This has also been reported for the exchange reactions of ADP and dADP (Chou & Singer, 1971a). Normal hyperbolic activation is observed at constant levels of $ADP\alpha S(S_P)$ for both forms of the enzyme. Double-reciprocal plots yield the apparent steady-state kinetic parameters given in Table III for the I and T forms of the enzyme at 1 mM total $ADP\alpha S(S_P)$.

The rate of $[^{32}P]P_i$ exchange was determined as a function of total $ADP\alpha S(S_P)$ concentration in the presence of constant 80 μM ApA. These data are plotted in Figure 2 for form I enzyme along with rate data obtained in the absence of ApA. There are two distinct regions to this plot in the presence of ApA. First, at low $ADP\alpha S(S_P)$ concentrations the rate of exchange in the presence of ApA increases more rapidly with increasing $ADP\alpha S(S_P)$ concentration than that of the de novo reaction. At higher concentrations of $ADP\alpha S(S_P)$ in the presence of ApA, the rate falls off with increasing $ADP\alpha S(S_P)$, eventually approaching the rate obtained in the absence of ApA. This second region represents inhibition by $ADP\alpha S(S_P)$ of the activation by ApA. Essentially identical results were obtained for the form T enzyme (data not shown).

Exchange into $ADP\alpha S(S_P)$ is accelerated by ADP. The rate of exchange into $ADP\alpha S(S_P)$ as a function of its concentration at a constant ADP concentration (160 μM) shows behavior that is strikingly similar to that obtained at a constant level of ApA, i.e., inhibition of the activated exchange by high levels of $ADP\alpha S(S_P)$.

Activation of Exchange by $ADP\alpha S(R_P)$. The R_P diastereomer, even though it is not a substrate for the exchange reaction, was found to activate exchange into $ADP\alpha S(S_P)$. Data for this activation are given in Table IV for both forms of the enzyme. During the time course of exchange, $ADP\alpha S(R_P)$ is being converted to AMPS by the I form enzyme. To determine if the $ADP\alpha S(R_P)$ or the product, AMPS, was responsible for the activation, we incubated $ADP\alpha S(S_P)$ in the presence of various levels of AMPS (Table IV). No activation or inhibition was observed for concentrations of AMPS up to 1.5 mM. A reliable apparent K_a for $ADP\alpha S(R_P)$ could not be determined because the concentration of $ADP\alpha S(R_P)$ is substantially changed at the first assay point. In the case of the T form, $ADP\alpha S(R_P)$ is not being hydrolyzed and, as anticipated, exhibits apparent substrate inhibition of the activation at high levels of $ADP\alpha S(R_P)$. For the form I enzyme, inhibition of the activated exchange can be observed at high $ADP\alpha S(S_P)$ concentrations (similar to that observed for ApA) if the initial concentration of $ADP\alpha S(R_P)$ is at least 800 μM.

Table IV: Activation of ADPαS-$[^{32}P]P_i$ Exchange by $ADP\alpha S(R_P)$[a]

enzyme	nucleotide concn (mM) $ADP\alpha S(S_P)$	$ADP\alpha S(R_P)$[b]	AMPS	rate of exchange[c]
form I	0.7			0.072
	0.7	0.4		0.269
	0.7	0.8		0.357
	0.7	1.6		0.400
	1.5		1.5	0.085
	1.5			0.091
form T	0.7			0.260
	0.7	0.41		1.461
	0.7	0.82		1.720
	0.7	1.65		1.150

[a] Rate determined by the TLC assay described under Experimental Procedures. [b] For form I enzyme, the $ADP\alpha S(R_P)$ concentration is the initial concentration; the final concentration is lower due to hydrolysis. [c] In nanomoles of ADPαS exchanged·$unit^{-1}$·$hour^{-1}$.

Table V: Product Distribution of Reaction Catalyzed by Phosphase Activity Associated with Form I Enzyme

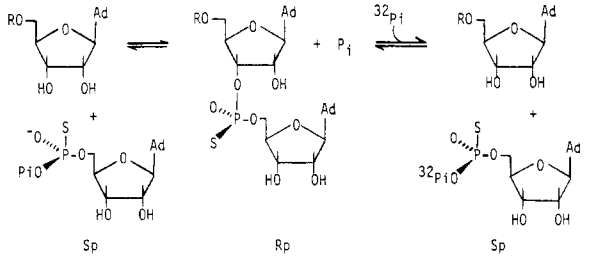
expt	time (h)	product concn[a] (mM) AMPS (%)	P_i (%)	treatment with alkaline phosphatase[b] P_i (%)
control (no enzyme)	17	0.33 (16)	0.30 (15)	2.04 (94)
	41	0.38 (19)	0.40 (18)	
form I	17	1.47 (68)	0.25 (12)	2.37 (110)
	41	1.65 (76)	0.40 (18)	
form T	21	0.14 (13)		

[a] Initial $ADP\alpha S(R_P)$ concentrations were determined spectrophotometrically at 260 nm and were 2.16 mM for the control and form I enzyme and 1.04 mM for form T. P_i and AMPS concentrations were measured as described under Experimental Procedures. [b] 0.01 unit of alkaline phosphatase was added to 20 μL of reaction mixture and the phosphate assay done as described. For the form I enzyme a slow linear release of P_i was observed, but only the point after 24 h (the longest time) is given. Under these conditions, <8% hydrolysis of AMPS occurs, as measured by adenosine deaminase.

Phosphatase Activity (Form I). The R_P diastereomer of ADPαS was converted to AMPS in the presence of the form I enzyme by an activity which cleaves the P_β–O bond. This was demonstrated by running the reaction in 33% $H_2{}^{18}O$ and isolating the AMPS produced. The isolated AMPS was phosphorylated enzymatically to ATPαS, and then the triphosphate group was eliminated from the ribose and converted to thiophosphate and inorganic phosphate (Bryant & Benkovic, 1979). After methylation, the thiophosphate and inorganic phosphate were demonstrated by GC–mass spectrometry to have only natural abundance levels of ^{18}O.

The production of both products, AMPS and inorganic phosphate, was monitored simultaneously. A much larger amount of AMPS than of inorganic phosphate was produced after 20 h of incubation (Table V). A phosphorylated form of the enzyme is insufficient to account for the quantity of missing P_i. No other products were detectable by TLC. Subsequent addition of alkaline phosphatase slowly liberated the missing inorganic phosphate (Table V). It is apparent that the phosphatase activity in the form I enzyme can phospho-

Scheme II[a]



[a] R = pp(S)Ap(S) (de novo) or Ap (ApA activation).

rylate some other species, possibly Tris, even more efficiently than alkaline phosphatase, presumably through a phosphorylated enzyme (Reid & Wilson, 1971). Unlike alkaline phosphatase from bovine intestine (F. R. Bryant, unpublished results), the discovered phosphatase will accept only one diastereomer of ADPαS, the one with the R_P configuration.

Discussion

Polynucleotide phosphorylase from *M. luteus* catalyzes an exchange reaction between the β-phosphate of ADP and $[^{32}P]P_i$ from solution (Chou & Singer, 1971a). Stereochemical experiments reported in this paper employing ADPαS demonstrate that both the I and T forms of polynucleotide phosphorylase will accept only the S_P diastereomer for this exchange reaction. The radioactive label is incorporated into ADPαS which also has the S_P configuration, leading to the conclusion that the mechanism is one of retention of configuration.

Retention mechanisms for phosphoryl transfer reactions of this nature arise from an even number of displacements and are usually diagnostic of enzyme–AMP intermediates, but all reported attempts to detect such intermediates with this enzyme have failed (Chou & Singer, 1971a). Recently, Eckstein has reported that ADPαS(S_P) is the substrate for the polymerization reaction of polynucleotide phosphorylase. The polymeric product was shown to contain internucleotide phosphodiester bonds with the R_P configuration, and therefore the mechanism was one of inversion (Burgers & Eckstein, 1979).

In view of these results, the observed retention of configuration is best explained by a dual inversion process yielding net retention, involving an oligonucleotide intermediate. Since neither dinucleotides nor dinucleotide 5′-phosphates are substrates for the reverse phosphorolysis reaction, it is most likely that in the de novo exchange reaction a dinucleotide is first synthesized, which in turn supports exchange at the diester bond formed with a third molecule of ADPαS(S_P). Under our conditions small amounts of such dinucleotides, if present off the enzyme, would not be detectable.[3] This overall mechanism is depicted in Scheme II.

We have also demonstrated that certain activator molecules, such as ApA, Ap(S)A, ADP, or ADPαS(R_P), will stimulate $[^{32}P]P_i$ exchange into ADPαS(S_P) for both the I and T forms of the enzyme. None of the activators was found to alter the observed stereochemistry of exchange into ADPαS(S_P), and with the exception of ADP, none participated as substrates for the exchange reaction. This is in agreement with reports that dinucleotides activate $[^{32}P]P_i$ exchange into ADP or dADP and that ADP activates $[^{32}P]P_i$ exchange into dADP for both forms of the enzyme (Chou & Singer, 1970a). The activation by dinucleotides is also consistent with the hypothesis stated above, namely, that exchange occurs via a trinucleotide. Activation of exchange would then be due, at least in part, to the fact that the dinucleotide need not be synthesized de novo. However, the discovery that ADPαS(R_P) would activate exchange into ADPαS(S_P), without undergoing exchange itself, was unique. This means that polynucleotide phosphorylase can utilize both diastereomers of ADPαS, although each diastereomer has a different role in the overall reaction. This is discussed more fully later.

Utilizing the diastereomeric dinucleotide, Ap(S)A, we have demonstrated that both forms of the enzyme prefer the R_P stereochemistry of dinucleotide activator molecules. This preference, however, is not absolute, since Ap(S)A(S_P) can function as an activator at approximately half the effectiveness of Ap(S)A(R_P), which is equivalent to ApA. As mentioned above ADPαS(R_P) is also an activator of the exchange; thus, it is plausible on the basis of their identical configuration at phosphorus that the dinucleotide primers and ADPαS(R_P) share a common site on the enzyme.

The activation kinetics for ApA were remarkably similar for both forms of the enzyme with nearly identical apparent activation constants determined at constant ADPαS(S_P) concentrations (Table III). When the ApA level was held constant and ADPαS(S_P) was varied, high levels of ADPαS(S_P) were found to inhibit this activation by ApA. In all of these experiments a constant high level of Mg^{2+} was maintained to assure that free ADPαS(S_P) (and consequently the Mg·ADPαS complex) varied in a nearly linear fashion with total ADPαS(S_P). Thus, no choice can be made as to the identity of the substrate or inhibitory ADPαS species, nor was it our intent. However, the free ADP species has been demonstrated to be the substrate for the polymerization (Chou & Singer, 1971b).[4]

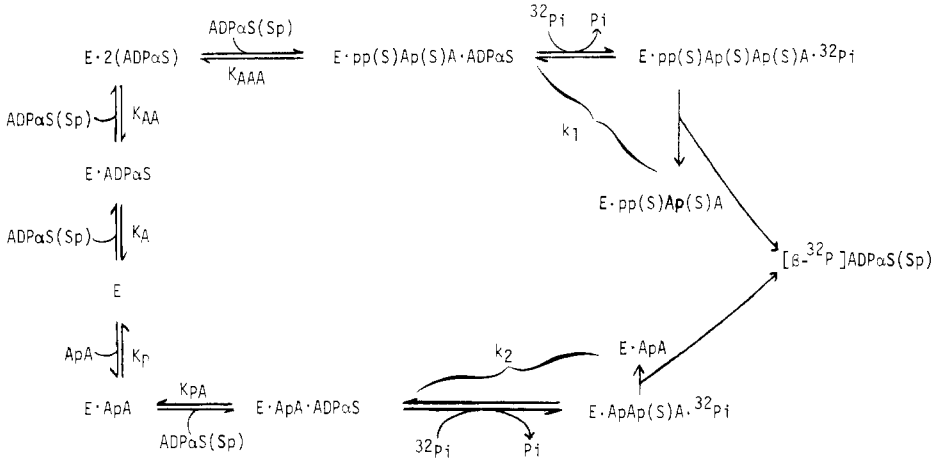
Within the framework of the stereochemical results discussed earlier and outlined in Scheme II, a mechanism can be proposed which explains the activation by ApA and its analogues (Scheme III).[5] This "donor" mechanism, requires at least two binding sites on the enzyme. The first site accommodates activator molecules such as ApA with preference for the R_P stereochemistry of thiophosphoryl dinucleotides. This site may be related to the oligonucleotide binding site for phosphorolysis which shows identical K_m values for both forms of the enzyme (Chou & Singer, 1970a). The second site mainly binds ADPαS(S_P) and P_i and is the actual exchange site. This site prefers the S_P stereochemistry since activation by ADPαS(R_P), which is not a substrate for exchange, is self-inhibited at higher levels. This type of mechanism is consistent with the known ability of the T form enzyme to utilize the 3′ terminus of a primer in the polymerization process (Moses & Singer, 1970). In the absence of ApA, ADPαS(S_P) fits into both the activator and the exchange sites. This is supported by the observation that high levels of ADPαS(S_P) effectively inhibit activation by dinucleotide primers. The slower rate of de novo exchange with ADPαS(S_P) alone could

[3] It has never been demonstrated that dinucleotide 5′-pyrophosphates do not support the exchange reaction, although this is clearly not expected. If such compounds were substrates, then exchange might occur at the dinucleotide level.

[4] NMR measurements indicate that complex formation involving ADPαS and ADP with Mn^{2+} is similar. Assuming this relationship holds for Mg^{2+}, dissociation constants of 1000–3000 M^{-1} for Mg·ADPS would apply to the pH and ionic strength used in this study.

[5] This scheme does not intend to imply an order of addition of substrate or activator to the enzyme, since the best available data indicates an overall rapid equilibrium random mechanism (Chou & Singer, 1970b).

Scheme III



simply result from its nonproductive binding or slower synthesis to generate the trinucleotide.

To ascertain whether the reaction model proposed is reasonable, we fit the kinetic data for activation by ApA to a theoretical curve generated from eq 1 by assuming rapid

$$\frac{v}{E_t} = \frac{\dfrac{k_1A^3}{K_AK_{AA}K_{AAA}} + \dfrac{k_2PA}{K_PK_{PA}}}{1 + \dfrac{A}{K_A} + \dfrac{P}{K_P} + \dfrac{AP}{K_PK_{PA}} + \dfrac{A^2}{K_AK_{AA}} + \dfrac{A^3}{K_AK_{AA}K_{AAA}}} \quad (1)$$

equilibrium within the scheme (Chou & Singer, 1970b). The appropriate kinetic and dissociation constants are given in Scheme III; A and P designate ADPαS(S_P) and primer (i.e., ApA) concentrations, respectively. Values for the kinetic parameters were estimated from the data in Table III or found by iteration and are given in the legend to Figure 2. The derived curve describes both the increase in rate of exchange with increasing ADPαS(S_P) concentration at low levels (0–500 μM) and the inhibition of this activation at high concentrations of ADPαS(S_P). The concentration-dependent lag observed in the data at lower ADPαS(S_P) concentrations might be due to the synthesis by polynucleotide phosphorylase of higher homologues than the dinucleotide, which are expected to be better activators (Chou & Singer, 1971a).

The kinetics of activation by ADPαS(R_P) were qualitatively very similar to those for ApA. The activation was a hyperbolic function of ADPαS(R_P) concentration at a constant level of ADPαS(S_P) for both forms of the enzyme. At a constant high level of ADPαS(R_P), inhibition of activation was again demonstrated with increasing ADPαS(S_P) by experiments analogous to those for ApA. On the basis of the similar kinetic behavior for ADPαS(R_P) and ApA and the preference of both enzyme forms for the dinucleotide with the R_P stereochemistry, it is possible to hypothesize a similar role for them. The activation by the R_P isomer might then be due to its increased affinity for the primer site and/or a more rapid formation of the prerequisite dinucleotide. It remains to be demonstrated whether the R_P stereoisomer of ADPαS is incorporated into the 5′ terminus of an RNA chain in order to eliminate the alternative hypothesis that it, as well as other primers, simply is an allosteric activator.

Behavior of the I and T forms of the enzyme toward ADPαS(R_P) differs in one important respect. The I form, but not the T form, converts ADPαS(R_P) to AMPS. Further characterization of the phosphatase showed the following. (1) There was no ^{18}O incorporation into AMPS; therefore, the P_β–O bond is cleaved. (2) Transphosphorylation of Tris buffer, or some other species, might possibly occur through a phosphoryl enzyme intermediate. We estimate that the phosphatase activity will hydrolyze 10 ADPαS(R_P) molecules for every 1 ADPαS(S_P) that undergoes [^{32}P]P_i exchange.

Although we have not yet been able to determine whether or not this activity is an integral part of the form I enzyme, it is interesting to speculate on a possible role. For example, phosphorylation of the enzyme or retention of the 5′-pyrophosphate linkage might be responsible for the processive de novo polymerization ability of the form I enzyme. Dephosphorylation of the enzyme or hydrolysis of the pyrophosphate might be the signal for chain termination, both leading to the observed formation of a 5′-phosphate end group in the polymerization. The ability to detect such an activity derives from the unique situation that the phosphatase and polymerase reaction prefer substrates of opposite chirality at phosphorus, thus permitting their detection through a kinetic discrimination that would not exist for isotopically generated chiral phosphorus centers.

Added in Proof

While this paper was in press, we determined that the missing P_i, that should have been produced by hydrolysis of ADPαS(R_P) by the phosphatase activity, was present in solution as polyinorganic phosphate. Therefore the phosphatase activity is in reality a polyphosphate kinase activity. These results will be published later.

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Interaction of Sugar Phosphates with the Catalytic Site of Ribulose-1,5-bisphosphate Carboxylase†

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ABSTRACT: The activated and catalytically competent form of ribulose-1,5-bisphosphate carboxylase is a ternary complex of enzyme·activator CO_2·Mg. The effectors NADPH and 6-phosphogluconate promoted activation by formation of a rapid equilibrium quaternary complex of enzyme·activator CO_2·Mg·effector; i.e., the effectors did not activate the enzyme per se but promoted the basic activation process by stabilizing the activated enzyme·activator CO_2·Mg complex. Kinetic and gel filtration studies showed that the effectors stabilized the binding of the activator CO_2 and Mg^{2+} (or Mn^{2+}), thereby decreasing the rate of deactivation. Binding studies indicated the presence of one 6-phosphogluconate binding site per protomer. The binding of 6-phosphogluconate and NADPH to the enzyme·activator CO_2·Mg complex was (a) completely prevented when the catalytic site for ribulose bisphosphate was occupied by the transition-state analogue, 2-carboxyarabinitol 1,5-bisphosphate, and (b) competitively diminished in the presence of 3-phosphoglycerate, the product of the carboxylation reaction. NADPH, 6-phosphogluconate, and 3-phosphoglycerate acted as linear competitive inhibitors of carboxylation with respect to ribulose bisphosphate. These results demonstrate that the effectors elicit their response through interaction at the catalytic site for ribulose bisphosphate and that their effect is secondary to the basic CO_2-Mg^{2+}-dependent activation reaction. An enzyme molecule cannot be simultaneously catalytically competent (capable of binding and carboxylating ribulose bisphosphate) and activated by an effector, since the latter involves occupancy of the ribulose bisphosphate binding site.

Previous studies (Lorimer et al., 1976, 1977; Miziorko & Mildvan, 1974) established that the activation of ribulose 1,5-bisphosphate (RuBP)[1] carboxylase involves the ordered addition of CO_2 and Mg^{2+}, with the addition of CO_2 being the rate-determining step (eq 1). Kinetic turnover (Lorimer,

$$\underset{\text{inactive}}{E\text{–}NH_2} + {}^{A}CO_2 \underset{\pm H^+}{\overset{\text{slow}}{\rightleftharpoons}} E\text{–}NH\text{–}{}^{A}COO^- + Mg^{2+} \overset{\text{fast}}{\rightleftharpoons} \underset{\text{active}}{E\text{–}NH\text{–}{}^{A}COO^{-}\cdots Mg^{2+}} \quad (1)$$

1979) and CO_2 binding (Miziorko, 1979) experiments demonstrate that the activator carbon dioxide ($^{A}CO_2$)[2] is distinct from the substrate carbon dioxide ($^{S}CO_2$) which becomes fixed during carboxylation. $^{A}CO_2$ is bound to the enzyme in the form of a carbamate on the ϵ-amino group of a lysine residue located within the large (catalytic) subunit (Lorimer et al., 1976; O'Leary et al., 1979; Lorimer & Miziorko, 1980).

A number of investigators reported the stimulatory effects of a variety of sugar phosphates upon the activation process (Buchanan & Schürmann, 1973; Chu & Bassham, 1975; Ryan & Tolbert, 1975; Chollet & Anderson, 1976; Lendzian, 1978; Vater & Salnikow, 1979; Whitman et al., 1979). Two compounds in particular, 6-phosphogluconate and NADPH, stand out as being especially effective, and physiological significance has been attributed to the effects they elicit (Buchanan & Schürmann, 1973; Chu & Bassham, 1975). Various models have been advanced to explain these effects (Chu & Bassham, 1975; Vater & Salnikow, 1979). A common feature is the involvement of allosterism, the binding of the effector at one or more sites distinct from the catalytic site. In this report, we present kinetic and physical evidence that the effects of 6-phosphogluconate and NADPH are exerted through interaction at the catalytic site to which RuBP binds. A model which accounts for many of the rather diverse observations concerning the effects of various sugar phosphates on the activation of RuBP carboxylase is presented.

Experimental Procedures

Materials

RuBP carboxylase from spinach leaves was purified, activated, and assayed as previously described (Lorimer et al., 1976, 1977). Care was taken to ensure that contaminating enzymes such as phosphatases or NADPH oxidase were absent from the preparations of carboxylase used in this study. Enzyme protomer was determined at 280 nm by using an extinction coefficient of 1.15×10^5 M^{-1} cm^{-1} (Paulsen & Lane, 1966). Biochemicals were obtained from either Sigma

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[1] Abbreviations used: RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxyarabinitol 1,5-bisphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)-glycine.

[2] The superscripts $^{A}CO_2$ and $^{S}CO_2$ are used to distinguish between the molecule of CO_2 involved in activation and that used as substrate, respectively.

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