Hansen, J. N., Dinovo, E. C., & Boyer, P. D. (1969) *J. Biol. Chem. 244*, 6270.
Hanson, K. R., & Havir, E. A. (1972) *Enzymes (3rd Ed.) 7*, 75.
Hill, R. L., & Teipel, J. W. (1971) *Enzymes (3rd Ed.) 6*, 539.
Hsiang, M. W., & Bright, H. J. (1969) *Methods Enzymol. 13*, 347.
Jones, V. T., Lowe, G., & Potter, V. L. (1980) *Eur. J. Biochem. 108*, 433.
Kim, S. C., & Raushel, F. M. (1986a) *Biochemistry 25*, 4744.
Kim, S. C., & Raushel, F. M. (1986b) *J. Biol. Chem. 261*, 8163.
Klinman, J. P. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 456.
Marletta, M. A., Cheung, Y., & Walsh, C. (1982) *Biochemistry 21*, 2637.
Nuiry, I. I., Hermes, J. D., Weiss, P. M., Chen, C., & Cook, P. F. (1984) *Biochemistry 23*, 5168.
Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem. 255*, 4772.
Winkler, M. F., & Williams, V. R. (1967) *Biochim. Biophys. Acta 140*, 284.
Woods, S. A., Miles, J. S., Roberts, R. E., & Guest, J. R. (1986) *Biochem. J. 237*, 547.

Synthesis of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate (AppppA) from Adenosine 5′-Phosphosulfate and Adenosine 5′-Triphosphate Catalyzed by Yeast AppppA Phosphorylase†

Andrzej Guranowski,*,‡ Gerald Just,§ Eggehard Holler,§ and Hieronim Jakubowski*,‡,∥

Katedra Biochemii, Akademia Rolnicza, ul, Wolynska 35, 60-637 Poznan, Poland, Institut für Biophysik und physikalische Biochemie, Universität Regensburg, 8400-Regensburg, FRG, and Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey—New Jersey Medical School, Newark, New Jersey 07103

Received September 8, 1987; Revised Manuscript Received November 18, 1987

ABSTRACT: A novel way of enzymatic synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA), which does not involve aminoacyl-tRNA synthetases, has been discovered. Yeast AppppA α,β-phosphorylase catalyzes irreversible conversion of adenosine 5′-phosphosulfate (APS) and ATP into AppppA according to the equation APS + ATP → AppppA + sulfate. In this reaction, the enzyme exhibits a broad pH optimum (between 6 and 8) and requires Mn^{2+}, Mg^{2+}, or Ca^{2+} ions for activity, with Mn^{2+} being twice as effective as Mg^{2+} or Ca^{2+} at optimal concentration (0.5 mM). The K_m values computed for APS and ATP are 80 μM and 700 μM, respectively. The rate constant for the AppppA synthesis is 3 s^{-1} (pH 8.0, 30 °C, 0.5 mM $MgCl_2$). Some ATP analogues like ppppA, GTP, adenosine 5′-(α,β-methylenetriphosphate), and adenosine 5′-(β,γ-methylenetriphosphate), but not dATP, UTP, or CTP, are also substrates for AppppA phosphorylase and accept adenylate from APS with the formation of AppppppA, AppppG, Appp(CH_2)pA, and App(CH_2)ppA, respectively. Functional versatility of yeast AppppA phosphorylase may provide a link between metabolism of AppppA on one hand and metabolism of APS and phosphate on the other and raises the possibility of participation of AppppA in regulation of metabolism of APS and/or inorganic phosphate in yeast.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA)[1] α,β-phosphorylase discovered in the extracts of yeast *Saccharomyces cerevisiae* (Guranowski & Blanquet, 1985) catalyzes the reaction

$$\text{AppppA} + \text{P*}_i \rightleftarrows \text{pppA} + \text{p*pA} \qquad \text{(I)}$$

The equilibrium of the reaction strongly favors phosphorolysis of AppppA, and by use of sensitive detectors combined with a HPLC system, only traces of enzymatic formation of AppppA could be detected as a result of the reverse reaction (Guranowski & Blanquet, 1985). The equilibrium constant $K = [\text{AppppA}][\text{P}_i]/[\text{ATP}][\text{ADP}]$ is very sensitive to pH and increases 100-fold from $K = 0.0003$ at pH 8.0 to $K = 0.028$ at pH 5.5 (Brevet et al., 1987). The enzyme supports also an exchange between the β-phosphate of nucleoside 5′-diphosphates and inorganic phosphate (Guranowski & Blanquet, 1986a):

$$\text{NDP} + \text{P*}_i \rightleftarrows \text{NDP*} + \text{P}_i \qquad \text{(II)}$$

Among many ADP analogues tested in the exchange reaction,

† This work was supported by the Polish Academy of Sciences within Project CPBR 3.13.4.4.4 to A.G., by Foundation of the University of Medicine and Dentistry of New Jersey Grant 14-86 to H.J., by Deutsche Forschungsgemeinschaft Grant SFB234C7 to G.J. and E.H., and by NIH Grant GM27711 to E. Goldman. A.G. was also the recipient of a short-term EMBO fellowship.

‡ Akademia Rolnicza.
§ Universität Regensburg.
∥ University of Medicine and Dentistry of New Jersey—New Jersey Medical School.

[1] Abbreviations: AppppA, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; HPLC, high-performance liquid chromatography; P_i, inorganic phosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; NDP, unspecified nucleoside 5′-diphosphate; APS, adenosine 5′-phosphosulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; TLC, thin-layer chromatography.

0006-2960/88/0427-2959$01.50/0

adenosine 5′-phosphosulfate (APS) was also a substrate, albeit in this case the reaction was irreversible with ADP and sulfate being the products:

$$APS + P_i \rightarrow ADP + \text{sulfate} \qquad \text{(III)}$$

Presumably, in all three reactions a covalent enzyme–adenylate (or, more generally, an enzyme–nucleotide) complex is formed as an intermediate from which the nucleotidyl moiety is then transferred either onto ATP, as in the reverse of reaction I, or onto P_i, as in reactions II and III. As has been determined earlier (Guranowski & Blanquet, 1986a), the enzyme specificity (k_{cat}/K_m) was 5-fold higher for APS than that for ADP in reactions III and II, respectively. We reasoned that APS could be a better "adenylate donor" in the reverse of reaction I than ADP, the more so as the sulfate that might be liberated during the reaction was inactive as a substrate in reaction I (Guranowski & Blanquet, 1985, 1986b) and therefore would not degrade the AppppA that might be formed.

In this paper we demonstrate that our prediction was correct, and in fact, yeast AppppA phosphorylase becomes an efficient AppppA synthetase when incubated with APS and ATP in the presence of divalent metal cations according to the equation

$$APS + ATP \rightarrow AppppA + \text{sulfate} \qquad \text{(IV)}$$

Kinetic characteristics of this novel synthesis of AppppA are described. We also suggest that the functional versatility of yeast AppppA phosphorylase (synthetase) may provide a direct connection between the metabolism of AppppA on one hand and the metabolism of phosphate and APS on the other.

Materials and Methods

Enzymes. The yeast AppppA α,β-phosphorylase was obtained as described previously (Guranowski & Blanquet, 1985) and kept at −20 °C in 25 mM Hepes/KOH buffer (pH 8.0) containing 10 μM dithiothreitol and 50% glycerol. Lupin asymmetrical AppppA hydrolase was purified as reported earlier (Jakubowski & Guranowski, 1983). Bacterial alkaline phosphatase was purchased from Sigma.

Chemicals. [U-^{14}C]ATP (18.5 GBq/mmol), [2,8-^{3}H]ATP (555 GBq/mmol), and [2,8-^{3}H]AppppA (322 GBq/mmol) were purchased from Amersham. Appp(CH_2)pA and App(CH_2)ppA (Guranowski et al., 1987) were synthesized in Prof. R. Khomutov's laboratory in the Institute of Molecular Biology, Academy of Sciences, USSR, Moscow. All other nucleotides were from Sigma. Other reagents and thin-layer chromatography plates were from Merck.

Chromatographic Systems. System I was poly(ethylenimine)–cellulose (with fluorescent indicator) plastic sheets developed first for 30 min in 75% methanol and then for 90 min in 0.9 M LiCl. Mobilities of ATP, ADP, AppppA, and APS in system I are shown in Figure 1. System II was silica gel (containing fluorescent indicator) aluminum sheets, developed for 90 min in dioxane/ammonia/water (6:1:4 v/v). R_f values for ATP, AppppA, and APS in system II were 0.19, 0.60, and 0.80, respectively. High-performance liquid chromatography (HPLC) was performed on a Waters μBondapak C_{18} column (3.9 mm × 30 cm) equipped with a guard column of the same (10 μm) material. Two buffer solutions, A (0.1 M $NH_4H_2PO_4$, pH 5.8) and B (buffer A plus 30% methanol), were used according to the following time schedule: 5 min 2% B, 5 min 2–20% B, and 30 min 20–33% B in A, at the flow rate of 1 mL/min. The column was attached to a LKB machine combined with a low-pressure mixing 1-pump system and variable wavelength monitor set at 254 nm. The injector (Reodyne) had a 2-mL sample loop; the delay between mixer and injector was 1.4 mL, and the column void volume was 2.8 mL. Processing of the chromatographs was performed on the Shimadzu CR 3A integrator device with constant memory for reprocessing and on the IBM/XT personal computer with integrator softward coupled to a digitalizer.

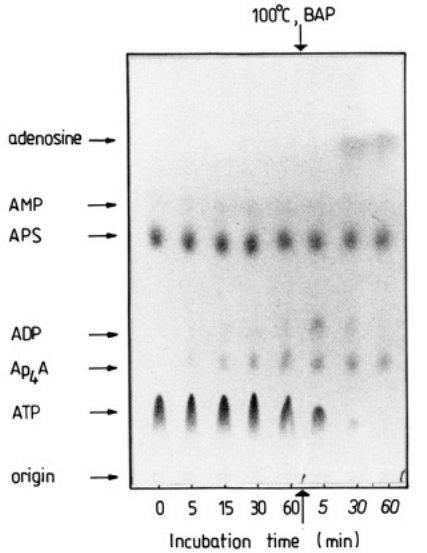

FIGURE 1: Demonstration by thin-layer chromatography that yeast AppppA phosphorylase catalyzes effective synthesis of AppppA from APS and ATP. The assay mixture, 50 μL final volume, contained 50 mM Hepes/KOH (pH 8.0), 0.5 mM $MgCl_2$, 0.1 mM dithiothreitol, 2 mM ATP, 2 mM APS, and yeast AppppA phosphorylase (1 μM final concentration) added last. Incubation was carried out at 30 °C. At indicated times, 3-μL aliquots were spotted on poly(ethylenimine)–cellulose sheets. After 60 min, the remaining mixture was heated for 3 min in a 100 °C bath to inactivate AppppA phosphorylase, and then, in an ice bath, 10 μL of bacterial alkaline phosphatase [about 0.5 unit in 25 mM Hepes buffer (pH 8.0) containing 10% glycerol, 10 μM dithiothreitol, and 1 mg/mL bovine serum albumin] was added. The second incubation was carried out at 30 °C, and 5-μL aliquots of the mixture were withdrawn for TLC analysis after 5, 30, and 60 min. The chromatogram was developed in system I. The picture was taken under shortwave ultraviolet light.

Enzyme Assays. Synthesis of AppppA from APS and ATP was routinely assayed at 30 °C. The standard incubation mixture in a final volume of 50 μL contained 50 mM Hepes/KOH buffer (pH 8.0), 0.5 mM $MgCl_2$, 0.5 mM APS, 100 μM dithiothreitol, and 2 mM [U-^{14}C]ATP (1 pmol = 4 cpm) and rate-limiting amounts of the enzyme diluted in 25 mM Hepes/KOH buffer (pH 8.0) containing 0.1 mM dithiothreitol, 10% glycerol, and 1 mg/mL bovine serum albumin. For initial velocity measurements, 5-μL aliquots were transferred onto silica gel sheets after 3, 6, and 12 min of reaction. A standard of AppppA was added and the chromatogram developed for 90 min in system II. Spots of AppppA were cut out, immersed in 5 mL of scintillation mixture, and counted for radioactivity.

Results

Yeast AppppA Phosphorylase Catalyzes Synthesis of AppppA from APS and ATP. Yeast AppppA phosphorylase (1 μM) was incubated with APS and ATP (2 mM each) at 30 °C in 50 mM Hepes/KOH (pH 8.0), 0.5 mM $MgCl_2$, and 0.1 mM dithiothreitol. The reaction mixture was analyzed by thin-layer chromatography (TLC) in system I. As shown in Figure 1, a new compound, well separated from ATP and APS and comigrating with an AppppA standard, appeared in the reaction mixture. The compound was already visible on the chromatograms after 5 min of reaction, and its amount continued to increase up to the end of the experiment at 1 h. About 30% of ATP and APS were consumed under these conditions. There was no reaction when the enzyme was

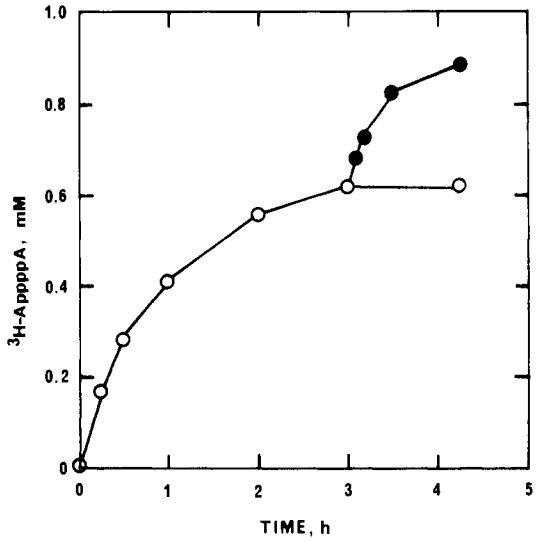

FIGURE 2: Time course of enzymatic synthesis of AppppA from APS and ATP. The assay mixture contained 50 mM Hepes/KOH (pH 8.0), 0.5 mM $MgCl_2$, 0.1 mM dithiothreitol, 2 mM [^{3}H]ATP (1 pmol = 4 cpm), 1 mM APS, and 1 μM yeast AppppA phosphorylase. Incubation was carried out at 30 °C. After 3 h the reaction mixture was divided into two parts, to one an additional amount of AppppA phosphorylase (1 μM) was added, and the two parts were incubated further for an additional 75 min. At indicated times, 3-μL aliquots of the reaction mixture were spotted on a poly(ethylenimine)–cellulose sheet. The chromatogram was developed in system I. Spots of [^{3}H]AppppA were cut out and quantitated by scintillation counting. Formation of [^{3}H]AppppA is plotted as a function of time in the presence of 1 μM enzyme (○) and in the presence of an additional 1 μM enzyme added at 3 h (●).

omitted from the reaction mixture (not shown). In order to confirm the identity of the product, several other tests were performed. First, the product comigrated with AppppA also in other chromatographic systems: TLC system II and HPLC. Second, the product was resistant to bacterial alkaline phosphatase (Figure 1). Third, it was susceptible to phosphorolysis by yeast AppppA phosphorylase and disappeared immediately when either 1 mM P_i was added into the reaction mixture or AppppA phosphorylase in the reaction mixture was not inactivated before the addition of bacterial alkaline phosphatase. Finally, the product was degraded to ATP and AMP by lupin asymmetrical AppppA hydrolase. Additional controls in which either AppppA phosphorylase, APS, or ATP was omitted and the reaction mixtures were analyzed by HPLC established that the reaction is enzyme-dependent and requires both APS and ATP. Therefore, we conclude that APS is an adenylate donor in the synthesis of AppppA catalyzed by yeast AppppA phosphorylase.

Is the AppppA Synthesis from APS and ATP Reversible? As shown in Figure 1, only about 30% of APS was converted into AppppA during the reaction. In an attempt to push the reaction to completion, we subsequently used less APS, extended incubation time, and added more enzyme after the reaction reached a plateau level. We incubated yeast AppppA phosphorylase (1 μM) with 1 mM APS and 2 mM [^{3}H]ATP for up to 4 h and 15 min at 30 °C in 50 mM Hepes/KOH (pH 8.0), 0.5 mM $MgCl_2$, and 0.1 mM dithiothreitol. The progress of the reaction was monitored by TLC in system I, spots of AppppA were cut out from the chromatogram, and [^{3}H]AppppA formed during the reaction was quantitated by scintillation counting. Under these conditions, the reaction reached a plateau level after 3 h with 62% APS being converted into [^{3}H]AppppA (Figure 2). Addition of more enzyme at 3 h and further incubation for an additional 75 min led to further conversion of APS into [^{3}H]AppppA, the maximum level of the reaction being 88%. At this point the reaction was complete; all APS was consumed. The remaining 12% of APS was converted to ADP, which can be seen on the chromatograms at the end of the reaction. This ADP was not ^{3}H labeled; therefore, it was not derived from [^{3}H]ATP but was formed by phosphorolysis of APS due to inorganic phosphate contamination present in commercial preparations of ATP. Direct assay for inorganic phosphate (Fiske & Subbarow, 1925) indicated that the commercial ATP preparation contains 10% (mol/mol) P_i and the commercial APS contains 0.4% (mol/mol) P_i.

In order to determine whether the AppppA synthesis from APS and ATP is reversible, yeast AppppA phosphorylase was incubated at 30 °C with 0.5 mM [^{3}H]AppppA (1 pmol = 35 cpm) and 5 mM Na_2SO_4 in a medium containing Hepes/KOH (pH 8.0), 0.5 mM $MgCl_2$, and 0.1 mM dithiothreitol. At time intervals up to 2 h, the reaction mixture was analyzed by TLC in system I (unlabeled APS was used as standard). Spots of APS and AppppA were cut out, and their radioactivity was determined by scintillation counting. No degradation of [^{3}H]AppppA was observed, and there was also no [^{3}H]APS formed under these conditions throughout the 2-h reaction period. This result confirms previous observations that the sulfate anion is inactive as a substrate for AppppA phosphorylase both in AppppA decomposition (Guranowski & Blanquet, 1985) and in exchange of the β-phosphate of NDPs with some anions (Guranowski & Blanquet, 1986b).

Metal Ion Requirements. Divalent cations (Mn^{2+}, Mg^{2+}, or Ca^{2+}) are absolutely required for the AppppA synthesis. No activity was observed in the presence of 1 mM EDTA. All the cations used as chlorides exhibited the highest efect at 0.5 mM concentration. At 0.5 mM $MnCl_2$, the velocity of AppppA synthesis was about 50% higher than in the presence of either $MgCl_2$ or $CaCl_2$. Zinc ions (at concentrations up to 0.5 mM) do not affect the reaction.

Effect of pH. The dependence of the initial velocity of AppppA synthesis on pH was examined in the following 50 mM buffers: sodium acetate (pH 4.7), 2-(*N*-morpholino)-ethanesulfonic acid/KOH (Mes/KOH, pH 5.3, 6.0, and 6.5), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid/KOH (Hepes/KOH, pH 7.0, 7.5, and 8.0), *N*,*N*-bis(2-hydroxyethyl)glycine/KOH (Bicine/KOH, pH 8.0 and 8.35), and 2-(cyclohexylamino)ethanesulfonic acid/KOH (Ches/KOH, pH 8.7, 9.05, and 9.5). It exhibited a regular bell shape (not shown). A broad pH optimum was observed between pH 6 and pH 8 with half of the maximum velocity at pH 5 and 8.7.

Michaelis Constants. The synthesis of AppppA from APS and ATP catalyzed by the yeast AppppA phosphorylase followed Michaelis–Menten kinetics. The K_m value for APS was measured at a fixed concentration of ATP (2 mM), and the concentration of APS was varied from 50 to 300 μM. The K_m value for ATP was measured at a fixed APS concentration (0.5 mM), and the concentration of ATP was varied within the 0.25–2 mM range. The K_m values for APS and ATP computed from Eadie–Hofstee plots (v versus v/[S]) were 80 μM and 700 μM, respectively.

Comparison of the Rate of AppppA Synthesis with That of AppppA Phosphorolysis. In order to assess the rates of AppppA synthesis (from APS and ATP) in relation to the rate of AppppA pyrophosphorolysis, both reactions were carried out in parallel with different concentrations of yeast AppppA phosphorylase from the same enzyme batch and with optimal conditions for each reaction. AppppA phosphorolysis was measured in a reaction mixture containing 50 mM Hepes (pH 8.0), 20 μM dithiothreitol, 0.5 mM [^{3}H]AppppA, 5 mM

K_2HPO_4, 5 mM $MgCl_2$, and the enzyme. Under these conditions, the rate constant for AppppA phosphorolysis was 36 s^{-1} (Guranowski & Blanquet, 1985). The rate of AppppA synthesis from APS and ATP measured in standard reaction mixture containing 0.5 mM $MgCl_2$ (see Materials and Methods) was 12-fold slower than the rate of AppppA phosphorolysis determined in the parallel experiment. The rate constant for AppppA synthesis was therefore determined to be 3 s^{-1}. In the presence of $MnCl_2$ instead of $MgCl_2$, the rate constant increased to 4.5 s^{-1}.

Inhibition Studies. The effect of Na_2SO_4, AppppA, ApppA, ADP, and AMP on the rate of AppppA synthesis was determined. The rates of AppppA synthesis were estimated in standard incubation mixtures containing 0.5 mM APS, 2 mM ATP, and varying concentrations (between 0.5 and 2 mM) of each of the tested compounds. In no case was any inhibition observed. Also no inhibition of AppppA synthesis was observed when both reaction products, AppppA and sulfate, 1 mM each, were present in the incubation mixture.

Substrate Specificity. The following nucleotides structurally related to ATP were examined as potential acceptors of adenylate from APS: ADP, ppppA, GTP, UTP, CTP, dATP, adenosine 5′-(α,β-methylenetriphosphate), and adenosine 5′-(β,γ-methylenetriphosphate). Reaction mixtures containing 1 μM yeast AppppA phosphorylase, 2 mM APS, and 2 mM nucleotide to be tested in 50 mM Hepes/KOH (pH 8.0), 0.5 mM $MgCl_2$, and 0.1 mM dithiothreitol were incubated at 30 °C. Aliquots of the reaction mixtures were analyzed by TLC and/or HPLC. It was clearly seen on TLC and/or HPLC that the enzyme catalyzed transfer of adenylate from APS onto ppppA, GTP, adenosine 5′-(α,β-methylenetriphosphate) and, poorly, adenosine 5′-(β,γ-methylenetriphosphate) with the formation of AppppppA, AppppG, Appp(CH_2)pA, and App(CH_2)ppA, respectively, as products. Even after prolonged, 16-h incubation, no ApppA, Apppp(dA), AppppU, or AppppC was detected in reaction mixtures containing ADP, dATP, UTP, and CTP, respectively. Figures 3 and 4 illustrate synthesis of AppppppA (AppppA) and AppppG (AppppppG), respectively. As shown in Figure 3, synthesis of AppppppA from APS and ppppA is accompanied by formation of AppppA, which is due to contamination of commercial preparations of ppppA with ATP. Other minor contaminants of commercial ppppA, i.e., ADP and possibly pppppA, do not react with APS. There was no formation of AppppppA or AppppA when APS was omitted from the reaction mixtures. Figure 4 presents the results of HPLC analysis of the reaction mixtures containing APS, GTP, and yeast AppppA phosphorylase. A new peak, which was identified as AppppG, appeared after only 10 min of incubation, increased considerably after 40 min, and disappeared after addition of lupin AppppA hydrolase (Figure 4, panels C and D). Two other compounds present in the reaction mixtures are also sensitive to lupin AppppA hydrolase. One, eluting before GTP plus GDP but used up during reaction with APS (Figure 4, panels A–C), is most likely ppppG, which is a known contaminant of commercial preparations of GTP (Vallejo et al., 1974). The second compound, eluting before APS, was formed during incubation with yeast AppppA phosphorylase and is most likely the product of reaction of ppppG with APS, i.e., AppppppG. Both of the peaks ascribed to AppppG and AppppppG, but not the one ascribed to ppppG, disappeared when 1 mM P_i was added to the reaction mixtures, thus confirming the identities of the compounds formed during incubation. Although only qualitative studies on ATP analogues were performed, comparison of the time–course analysis of the incubation mixtures containing the nucleotides examined

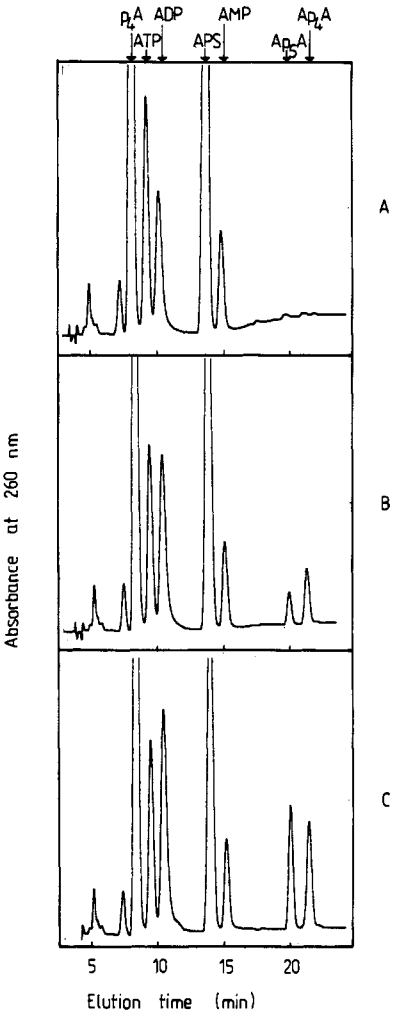

FIGURE 3: Demonstration by high-performance liquid chromatography that yeast AppppA phosphorylase catalyzes synthesis of AppppppA from APS and adenosine 5′-tetraphosphate (ppppA). The assay mixture (150-μL final volume) was the same as described in the legend to Figure 1 except that ppppA was used instead of ATP. At appropriate times, 25-μL aliquots were heated for 3 min in boiling water, chilled, and centrifuged, and 5-μL portions of the supernatant were analyzed on the reverse-phase column as described under Materials and Methods. Panels A, B, and C depict elution profiles of the mixture after 0-, 10-, and 60-min incubation at 30 °C. The small peak eluting just before ppppA is probably pppppA.

here permits one to conclude that ATP is the most efficient substrate for yeast AppppA phosphorylase in reaction with APS.

Discussion

Despite the fact that AppppA has been known in biological systems for over 2 decades (Zamecnik et al., 1966), its role in cellular physiology is still a mystery. In vitro AppppA has been shown to interact with various enzymes [reviewed in Zamecnik (1983)], but the in vivo significance of these interactions is unknown. In our efforts to elucidate the role(s) of AppppA in cellular physiology, we are studying AppppA-degrading enzymes both in procaryotes (Guranowski et al., 1983) and in eucaryotes (Jakubowski & Guranowski, 1983; Guranowski & Blanquet, 1985) with the expectation of finding a clue in properties of any of the AppppA-degrading enzymes that might suggest in vivo function(s) of AppppA. This expectation was substantiated by the discovery of AppppA pyrophosphorylase in yeast (Guranowski & Blanquet, 1985), a multifunctional enzyme that, in addition to phosphorolysis of AppppA, also supports NDP–P_i exchange and phosphorolysis

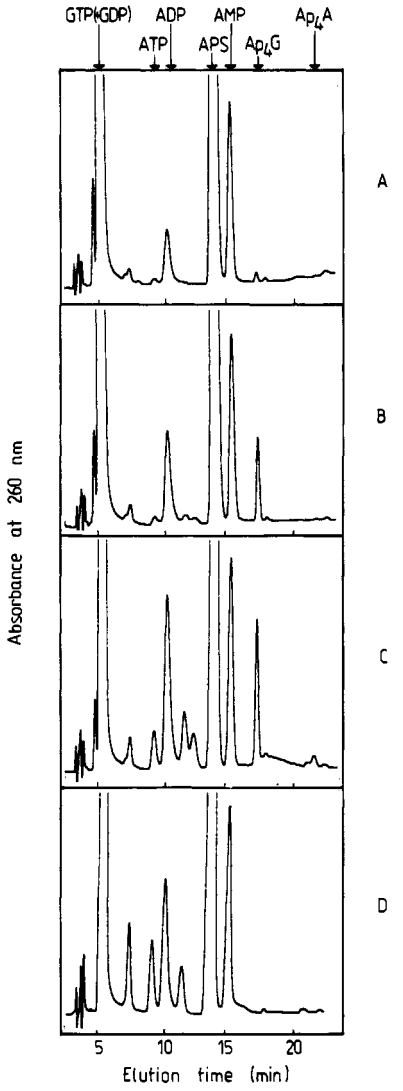

FIGURE 4: Demonstration by high-performance liquid chromatography that yeast AppppA phosphorylase catalyzes synthesis of AppppG from APS and GTP. The assay mixture (200-μL final volume) was the same as that described in the legend to Figure 1 except that GTP was substituted for ATP. At appropriate times, 25-μL aliquots were heated for 3 min in a boiling water bath to stop the reaction, chilled, and centrifuged, and 5-μL portions of the supernatant were analyzed on the reverse-phase column as described under Materials and Methods. Panels A, B, and C show elution profiles of the reaction mixture after 0-, 10-, and 40-min incubation at 30 °C. Panel D depicts a chromatogram of the reaction mixture shown in panel C that has been additionally treated with lupin asymmetrical AppppA hydrolase (0.2 unit/25 μL of the heat-treated mixture from panel C, 15 min, 30 °C)9 In addition to the peaks marked by the arrows, there are four peaks that can be interpreted as follows: a peak that eluted immediately before GTP is probably ppppG (seen on panels A–C), which is known to be present as a contaminant in commercial GTP preparations (see text); a peak that increased during incubation and eluted before APS and may be AppppppG (seen on panels B and C); the identities of the two peaks that are not degraded by lupin AppppA hydrolase are unknown.

of APS (Guranowski & Blanquet, 1986a) and synthesis of AppppA from ADP and ATP in a reverse reaction to phosphorolysis (Guranowski & Blanquet, 1985; Brevet et al., 1987). The data presented in this paper identify a novel reaction catalyzed by yeast AppppA phosphorylase, synthesis of AppppA from APS and ATP, thus extending the versatility of the enzyme.

The synthesis of AppppA from APS is irreversible, and in this respect it differs from the synthesis of AppppA from ADP and ATP, which allows only about 2% (at pH 7.0, or much less of higher pH values) conversion due to the equilibrium constant favoring degradation of AppppA rather than its synthesis (Brevet et al., 1987).

Other characteristics of the synthesis of AppppA from APS catalyzed by yeast AppppA phosphorylase, in particular, substrate specificity of the NTP site, confirm and extend previous data. As shown above, the enzyme uses APS to adenylylate ATP, ppppA, GTP, and ppppG with the formation of AppppA, AppppppA, AppppG, and AppppppG, respectively. Other nucleotides, UTP, CTP, dATP, and ADP, are not substrates in this reaction. Similarly, these four nucleotides were not substrates when ADP was used as the adenylate donor (Brevet et al., 1987). Thus, these results indicate that the NTP site of the enzyme, which functions as the nucleotide acceptor site, requires 5′-triphosphates or 5′-tetraphosphates of purine ribonucleotides. The data presented herein together with the evidence presented elsewhere (Guranowski & Blanquet, 1986a; Brevet et al., 1987) point to the asymmetric nature of the dinucleoside oligophosphate binding site (consisting of NTP and NDP sites) of yeast AppppA phosphorylase. The NDP site, or the nucleotide donor site, has less strict structure requirements than the NTP site (nucleotide acceptor site) and productively interacts with nucleoside 5′-diphosphates (both purine and pyrimidine ribonucleotides and deoxyribonucleotides) and nucleoside 5′-phosphosulfates. Also, the NDP site (nucleotide donor site) can productively interact with NDP but not with NTP, whereas the NTP site (nucleotide acceptor site) interacts productively with 5′-triphosphates and 5′-tetraphosphates of purine ribonucleotides. Asymmetry between the two sites of AppppA phosphorylase is also evident in their interaction with divalent cations. Mn^{2+}, Mg^{2+}, or Ca^{2+} is required for catalysis of AppppA phosphorolysis (Guranowski & Blanquet, 1985) and for AppppA synthesis from ADP plus ATP (Brevet et al., 1987) and from APS plus ATP (this work). No cations are required for NDP–P_i exchange or anionolysis of NDP (Guranowski & Blanquet, 1986a,b). We therefore suggest that the M^{2+} ion(s) participate(s) in productive interaction of AppppA or ATP with the NTP site during phosphorolysis or synthesis of AppppA, respectively.

Two features of the reactions catalyzed by yeast AppppA phosphorylase may suggest possible functions for AppppA in vivo. First, as shown in this work, metabolism of AppppA can be coupled to metabolism of APS via AppppA phosphorylase. Second, the enzyme directly connects metabolism of AppppA and P_i. Thus, in yeast cells an enzyme exists that is able to modify concentrations of AppppA in response to changes in concentrations of APS or P_i. These relationships are provocative enough to warrant further experiments aimed at studying these connections in vivo. Metabolism of phosphate in yeast is elaborate and involves interactions between at least 15 genes (Oshima, 1982). Yeast cells contain two repressible phosphatases. Regulation of expression of the repressible phosphatase is at the level of transcription. A model for the regulatory circuit for the phosphatase system of yeast has been proposed that involves constitutively expressed cytoplasmic regulatory factors. Positive regulatory factors are required to activate the transcription of the phosphatase structural genes. Interaction between these factors is ultimately mediated by an effector. However, there is no evidence as to the nature of the effector, which can be either inorganic phosphate itself or a metabolite thereof. AppppA, being a metabolite of phosphate, could possibly fulfill the role of an effector. A decrease in P_i would lead to an increase in AppppA (mediated by AppppA phosphorylase), which in turn may be available to activate transcription of phosphatase genes by binding either to a positive transcription factor or to another cytoplasmic

regulatory factor. In this model, AppppA would be a positive effector.

APS is a product of the first step of inorganic sulfate reduction, which is catalyzed by ATP-sulfurylase encoded by the *MET3* gene in yeast (Jones & Fink, 1982). Being the first step in a pathway, APS synthesis is tightly regulated both by feedback inhibition of the ATP-sulfurylase (by APS itself, PAPS, SO_3^{2-}, or S^{2-}) and by repression of the enzyme synthesis by *S*-adenosylmethionine. Since APS can also be a substrate for AppppA synthesis, it would not be surprising if AppppA acted as a feedback inhibitor of ATP-sulfurylase. The hypotheses concerning a role of AppppA in phosphate and APS metabolism in yeast are testable experimentally and point toward new directions in studies of functions of AppppA.

Registry No. AppppA, 5542-28-9; APS, 485-84-7; ATP, 56-65-5; ppppA, 1062-98-2; GTP, 86-01-1; AppppA, 41708-91-2; AppppG, 10527-46-5; App(CH$_2$)pA, 101536-13-4; App(CH$_2$)ppA, 88109-92-6; Mn, 7439-96-5; Mg, 7439-95-4; Ca, 7440-70-2; AppppA α,β-phosphorylase, 96697-71-1; adensoine 5′-(α,β-methylenetriphosphate), 7292-42-4; adenosine 5′-(β,γ-methylenetriphosphate), 3469-78-1; adenine, 73-24-5.

References

Brevet, A., Coste, H., Fromant, M., Plateau, P., & Blanquet, S. (1987) *Biochemistry 26*, 4763–4768.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Guranowski, A., & Blanquet, S. (1985) *J. Biol. Chem. 260*, 3542–3547.

Guranowski, A., & Blanquet, S. (1986a) *J. Biol. Chem. 261*, 5943–5946.

Guranowski, A., & Blanquet, S. (1986b) *Biochimie 68*, 757–760.

Guranowski, A., Jakubowski, H., & Holler, E. (1983) *J. Biol. Chem. 258*, 14784–14789.

Guranowski, A., Biryukov, A., Tarussova, N. B., Khomutov, R. M., & Jakubowski, H. (1987) *Biochemistry 26*, 3425–3429.

Jakubowski, H., & Guranowski, A. (1983) *J. Biol. Chem. 258*, 9982–9989.

Jones, E. W., & Fink, G. R. (1982) in *The Molecular Biology of Yeast Saccharomyces. Metabolism and Gene Expression* (Strathern, J. N., Jones, E. W., & Broach, J. R., Eds.) pp 181–299, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Oshima, Y. (1982) in *The Molecular Biology of Yeast Saccharomyces. Metabolism and Gene Expression* (Strathern, J. N., Jones, E. W., & Borach, J. R., Eds.) pp 157–180, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Vallejo, C. G., Sillero, M. A. G., & Sillero, A. (1974) *Biochim. Biophys. Acta 358*, 117–125.

Zamecnik, P. (1983) *Anal. Biochem. 134*, 1–10.

Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., & Randerath, K. (1966) *Biochem. Biophys. Res. Commun. 24*, 91–97.

Spatial Proximity of the Glycine-Rich Loop and the SH_2 Thiol in Myosin Subfragment 1†

Kazuo Sutoh*,‡ and Toshiaki Hiratsuka§

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan, and Department of Chemistry, Asahikawa Medical College, Asahikawa, Hokkaido 078, Japan

Received July 15, 1987; Revised Manuscript Received November 3, 1987

ABSTRACT: Subfragment 1 (S1) prepared from rabbit skeletal muscle myosin was digested with trypsin to cleave the 95K heavy chain into three pieces, i.e., the 23K, 50K, and 20K fragments. The trypsin-treated S1 was then cross-linked with *p*-nitrophenyl iodoacetate. The cross-linker bridged one of the reactive thiols (SH_2) in the 20K fragment and a lysine residue in the 23K fragment [Hiratsuka, T. (1987) *Biochemistry 26*, 3168–3173]. Location of the lysine residue was mapped along the 23K fragment by "end-label fingerprinting", which employed site-directed antibodies against the N-terminus of the 23K fragment and against the C-terminus of the 24K fragment (the 23K fragment plus nine extra residues at its C-terminus). The mapping revealed that Lys-184 or Lys-189 was the residue cross-linked with SH_2. Since the cross-linker used here spans only several angstroms, the result indicates that Lys-184 or Lys-189 is very close to SH_2 in the three-dimensional structure of myosin head. Examination of the primary structure of the 23K fragment has revealed that these lysine residues are in and very close to the so-called "glycine-rich loop", whose sequence is highly homologous to those of nucleotide-binding sites of various nucleotide-binding proteins.

Heavy chain of myosin subfragment 1 (S1)[1] contains two reactive cysteine residues called SH_1 and SH_2 (Sekine & Kieley, 1964). These residues are located in the tryptic 20K fragment of the S1 heavy chain [trypsin cleaves the heavy chain into the 23K, 50K, and 20K fragments which are aligned in this order (Mornet et al., 1979)] and are only 10 residues apart in the primary structure (Elzinga & Collins, 1977; Gallagher & Elzinga, 1980). The structure around the SH_1–SH_2 region seems flexible; cross-linking experiments showed that these thiols can be as little as 2 Å apart (Burke

† This work was supported by a grant-in-aid for Special Distinguished Research from the Ministry of Education, Science and Culture of Japan and by a basic research grant from the Muscular Dystrophy Association, Inc.

‡ University of Tokyo.

§ Asahikawa Medical College.

[1] Abbreviations: S1, myosin subfragment 1; LEP, lysyl endopeptidase; NPIA, *p*-nitrophenyl iodoacetate; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; IgG, immunoglobulin G.

0006-2960/88/0427-2964$01.50/0