Adenosine 5'-Tetraphosphate and Adenosine 5'-Pentaphosphate Are Synthesized by Yeast Acetyl Coenzyme A Synthetase†

ANDRZEJ GURANOWSKI,² MARÍA ANTONIA GÜNTHER SILLERO, AND ANTONIO SILLERO*
Departamento de Bioquímica, Instituto de Investigaciones Biomédicas del Consejo Superior de Investigaciones Científicas, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain

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Yeast (Saccharomyces cerevisiae) acetyl coenzyme A (CoA) synthetase (EC 6.2.1.1) catalyzes the synthesis of adenosine 5'-tetraphosphate (p₄A) and adenosine 5'-pentaphosphate (p₅A) from ATP and tri- or tetrapolyphosphophosphate (P₃ or P₄), with relative velocities of 7.1, respectively. Of 12 nucleotides tested as potential donors of nucleotidyl moiety, only ATP, adenosine 5'-O-[3-thiotriphosphate], and acetyl-AMP were substrates, with relative velocities of 100, 62, and 90, respectively. The Kₘ values for ATP, P₃, and acetyl-AMP were 0.16, 4.7, and 1.8 mM, respectively. The synthesis of p₄A could proceed in the absence of exogenous acetate but was stimulated twofold by acetate, with an apparent Kₘ value of 0.065 mM. CoA did not participate in the synthesis of p₄A (p₅A) and inhibited the reaction (50% inhibitory concentration of 0.015 mM). At pH 6.3, which is optimum for formation of p₄A (p₅A), the rate of acetyl-CoA synthesis (1.84 μmol mg⁻¹ min⁻¹) was 245 times faster than the rate of synthesis of p₄A measured in the presence of acetate. The known formation of p₄A (p₅A) in yeast sporation and the role of acetate may therefore be related to acetyl-CoA synthetase.

Adenosine 5'-tetraphosphate (p₄A) and adenosine 5'-pentaphosphate (p₅A) were detected as contaminants of adenosine 5'-triphosphate preparations (21, 27), and p₄A was shown to be present in muscle (16, 33), liver (38), and yeast (Saccharomyces cerevisiae) spores (9). The physiological role of nucleoside 5'-tetraphosphates (p₄Nₚ₅) is largely unknown. Adenosine-, guanosine-, and uridine-triphosphates (p₄A, p₄G, and p₄U) are strong competitive inhibitors (nanomolar Ki values) of asymmetrical dinucleoside tetraphosphatase (EC 3.6.1.17) (15, 17, 24, 36), an enzyme cleaving dinucleoside tetraphosphates to the corresponding nucleoside tri- and monophosphates. Since Ap₄A may be important in metabolic regulation (for a review, see reference 23), changes in the level of p₄Nₚ₅ could modulate its concentration and physiological effect. Other enzymes known to be inhibited (micromolar Ki values) by p₄Nₚ₅ are the soluble guanylate cyclase (EC 4.6.1.2) (8) and phosphodiesterase 1 and nucleotide pyrophosphatase (EC 3.6.1.9) from rat liver (2).

The level of p₄A depends on its rate of synthesis and degradation. A specific enzyme (EC 3.6.1.14) which hydrolyzes p₄A to ATP and P₃ exists in muscle (32), and the following enzymes have been reported to synthesize p₄Nₚ₅: adenoacyl-tRNA synthetases and, particularly, the lysyl-tRNA synthetase (EC 6.1.1.6), which catalyzes the synthesis of p₄A from lysine, ATP, and pentadecosyloipolyphosphate (P₃) (37); yeast phosphoglycerate kinase (EC 2.7.2.3), which forms p₄A (33) and p₄G (6) from 1,3-bis-phosphoglycerate and ATP or GTP, respectively; adenylate kinase (EC 2.7.4.3), which was shown to transfer phosphofructose from ADP to ATP (14); a mutated Escherichia coli succinyl-coenzyme A (CoA) synthetase (EC 6.2.1.5) that was unable to catalyze the overall reaction (i.e., the synthesis of succinyl-CoA from ATP, succinate, and CoA) but could synthesize p₄A from ATP through phosphorylation of the enzyme and transfer of the phosphate moiety from the enzyme-phosphate complex to ATP (19); and, finally, firefly luciferase (EC 1.13.12.7), which catalyzes the synthesis of p₄A or p₅A by using luciferin, ATP, and P₃ or hexammonium tetraphosphate (P₄), respectively (25).

Because of the similarities between the reactions catalyzed by adenoacyl-tRNA synthetases, luciferase, and acetyl-CoA synthetases (7, 22, 25, 31), we checked whether yeast acetyl-CoA synthetase (EC 6.2.1.1) could also catalyze synthesis of p₄A from ATP and P₃. Here, we describe the experimental conditions for the synthesis of both p₄A and p₅A by acetyl-CoA synthetase.

MATERIALS AND METHODS

Three commercial preparations of yeast acetyl-CoA synthetase were used: lot 89F8180 from Sigma (catalog no. A-5269) and two batches of a lyophilized synthetase, lot 127F0650 from Sigma (catalog no. A-1765) and lot 11496526-29 from Boehringer Mannheim GmbH (catalog no. 161675). p₄A-degrading activity was undetectable in those synthetase preparations. As auxillary enzymes, inorganic pyrophosphatase from yeast cells (catalog no. 108987), alkaline phosphatase from calf intestine (catalog no. 108138) (both purchased from Boehringer), and potato apyrase from Sigma (catalog no. A-6132) were used. All of the enzymes were solubilized and/or diluted before use in 25 mM HEPES (N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid)-KOH buffer (pH 7.6) containing 5% glycerol, 0.1 mM dithiothreitol, and 1 mg of bovine serum albumin per ml.

Adenosine 5'-O-[1-thiotriphosphate] S-isomer (ATP₆S) and adenosine 5'-O-[3-thiotriphosphate] (ATP₆S) were from Boehringer Mannheim GmbH. Adenosine 5'-α,β-methylene-triphosphate (pCP₆), adenosine 5'-β,γ-methyleneatetriphosphate (pC₆P₆), adenosine N¹-oxide 5'-triphosphate, adenosine N¹,β-δ-arabinoside 5'-triphosphate, and other nucleotides were from Sigma. Acetyl-AMP was synthesized as described earlier (10). P₃, P₅, and sodium phosphate glass with a chain length of ≥ 2P were from Sigma (catalog no. T5633, T5758, and SS878, respectively). Buffers, salts, and organic

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* Corresponding author. Mailing address: Departamento de Bioquímica, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 4, 28029 Madrid, Spain. Phone: 1-3975413. Fax: 1-3150075. Electronic mail address: ASillero@mvax.fmed.uam.es.

† Dedicated to Professor Severo Ochoa, in memoriam.

‡ Present address: Katedra Biochemii, Akademia Rolnicza, ul. Wołynska 35, Poznan, Poland.

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RESULTS AND DISCUSSION

In Fig. 1, a reaction mixture containing ATP, MgCl₂, acetate, inorganic pyrophosphatase and acetyl-CoA synthetase is shown to accumulate p₄A, dependent on addition of P₃, or at a lower rate, p₃A, dependent on P₃. The reaction could be monitored either by thin-layer chromatography (TLC) (Fig. 1A) or high-performance liquid chromatography (HPLC) (Fig. 1B).

The identity of p₃A and p₃A was established by comigration with standards, UV spectra identical with those of ATP, and sensitivity to alkaline phosphatase (Fig. 1C) and apyrase (data not shown). Smaller amounts of Ap₃A (in the presence of P₃) and traces of Ap₃A (in the presence of P₃) and Ap₃A were also formed and similarly identified (Fig. 1B).

Modifying the standard incubation mixture by adding 100 mM MES (morpholineethanesulfonic acid), HEPES, and CHES (3-N-cyclohexylamino-1-propanesulfonic acid) covering the pH range from 5.1 to 9.5 ([^1]H)ATP and TLC method showed a pH optimum for p₃A synthesis between 6 and 6.5 (MES-KOH buffer), with half-maximum activity at pH 5.2 and 7.4 and no synthesis observed above pH 8. Dependence on divalent cations is shown in Fig. 2.

The following compounds were tested at 3 mM as potential donors of a nucleotidyl moiety onto P₃ by using the (nonradioactive) HPLC assay method: ADP, ATP, dATP, ATPαS and ATPγS, ppC₃Ap, pCH₃pAp, pCH₃pAp, adenosine 5'-triphosphate, adenosine 5'-d-arabinoside 5'-triphosphate, ATP, GTP, UTP, and acetyl-AMP. Only ATP, ATPαS, and acetyl-AMP were active, relative rates of p₄A synthesis being 100, 62, and 80, respectively. Using the[^1]H)ATP assay with TLC, rates with P₃ and P₃ were 7.5 and 1.0 nmol min⁻¹, respectively; P₃ even with 24 h of incubation, was not identified as a substrate. The rates of acetyl-CoA formation (in the presence of 5 mM acetate and 5 mM CoA) at pH 6.3 and 7.5 (pH optimum of the reaction) were, respectively, 1.84 and 7.4 nmol mg⁻¹ min⁻¹. The ATP-PP exchange, reaction, using 2 mM[^32]P)pyrophosphate, (1 pmol is 112 cpm) (in the absence of pyrophosphatase), gave a rate of 0.72 μmol mg⁻¹ min⁻¹ in the presence of 1 mM acetate and about half that value in the absence of added acetate.

For p₄A synthesis the Kₘ value for ATP was 0.16 mM; a range of[^3]H)ATP from 0.15 mM to 2.4 mM was used, with TLC determination of p₄A. The Kₘ for P₃ was 4.7 mM, employing the same assay with 3 mM[^3]H)ATP and 0.3 to 5 mM P₃. The Kₘ for acetyl-AMP, was 1.5 mM; a range of 0.15 to 2.4 mM acetyl-AMP was used along with HPLC determination of p₃A.

p₃A synthesis occurred in the absence of added acetate, but the rate was increased twofold in the presence of 0.5 mM acetate with half-stimulation at 0.065 mM. The possibility that the enzyme preparation contributed acetate was apparently excluded by the lack of effect of addition of preheated enzyme solution. No effect was observed for formate or propionate. Added CoA itself was inhibitory to the standard reaction[^3]H)ATP, TLC method with a 50% inhibitory concentration of 0.015 mM, and such inhibition was prevented by inclusion of 0.5 mM acetate, showing that acetyl-CoA is not inhibitory.

We have recently suggested that enzymes (mainly synthetases and some transferases) which catalyze the transfer of a nucleotidyl moiety via nucleotidyl-containing intermediates and release of PP₃ may produce dinucleoside polyphosphates (7). Aminoacyl-tRNA synthetases (26, 37) and luciferase (7, 25, 31) are examples of this type of enzymes known to catalyze the synthesis of nucleoside 5'-polyphosphates (n > 3) and diadenosine tetraphosphate. Acetyl-CoA synthetase is another enzyme theoretically able to catalyze the synthesis of nucleoside 5'-polyphosphates (n > 3) or Ap₅A (7). The results presented in this paper arose from the observation made very recently with firefly luciferase (25) that the enzyme can catalyze the transfer of the adenylyl moiety from the enzyme-luciferin-AMP complex onto various polyphosphates. The formation of p₄A or p₃A with acetyl-CoA synthetase might analogously involve P₅ or P₄ as the acceptor of the adenylyl moiety of the enzyme-acetyl-AMP complex as follows:

enzyme-acetyl-AMP + P₅ ⇔ P₅A + A + acetate + enzyme (reaction 1)

An alternative mechanism, in view of the apparent absence of involvement of acetate in the reaction, would be

enzyme + ATP ↔ enzyme-AMP + PP₃ (reaction 2)

enzyme-AMP + P₃ + P₃A + enzyme (reaction 3)

In contrast to various aminoacyl-tRNA synthetases (26, 37) and firefly luciferase (7, 25, 31), which require the cognate amino acid and luciferin, respectively, to form an enzyme-X-AMP complex (in which X stands for an organic acid able to form an anhydride bond with AMP), the yeast acetyl-CoA synthetase can catalyze the transfer of adenylyl coming from ATP to a polyphosphate without forming, apparently, an acyl-AMP intermediate. In addition to that, however, the yeast enzyme can use the specific acyl-AMP intermediate acetyl-AMP as a source of adenylate.

Although it is not the object of this work to elucidate the mechanism of the synthesis of p₄A, whether it goes through reaction 1 or reaction 2, (implying the formation of enzyme-acetyl-AMP or enzyme-AMP intermediates, respectively), some comments could be raised on this point. Contradictory reports concerning the acetyl dependence(d) or independence of the ATP-PP exchange date from the early fifties (compare references 12 and 20 with reference 1). Variability in the results has been ascribed, among other causes, to important mechanistic differences between the members of this type of enzymes (acyl-CoA synthetases included), to the occurrence of a hypothetical factor required for the formation of enzyme-AMP complex, which could be lost in the process of the enzyme purification, and to the presence of the contaminant acetate (18). Direct interaction of ATP with the enzyme is also supported by the stabilizing effect exerted by that nucleotide on the acetyl-CoA synthetase through an unknown mechanism (5, 29). Since the molecular weights obtained in the presence and absence of ATP are identical (29), the possibility that adenylylation of the synthetase by ATP contributes to its stability should also be considered. Also, because the enzyme has an α2 structure (5) and, as is shown in this study, the exogenous acetate doubles the rate of p₄A synthesis observed in the absence of acetate, it seems plausible that each subunit of the synthetase participates in that reaction in a different manner—according to reaction 1 or 3. The existence of two interacting substrate sites for ATP was proposed earlier for the acetyl-CoA synthetase from Methanobacterium (11).
FIG. 1. Synthetic products from ATP, P₃, and P₄. (A) TLC. The complete assay mixture (0.05 ml, final volume) contained 50 mM MES-KOH (pH 6.3), 5 mM MgCl₂, 0.1 mM dithiothreitol, 5 mM potassium acetate, 3 mM ATP, 0.05 U of inorganic pyrophosphatase, 5 mM polyphosphate, and 16 μg of acetyl-CoA synthetase. Incubation was carried out at 30°C. At the times mentioned below, 0.002-ml aliquots were spotted on silica gel sheets containing a fluorescent indicator (Merck, Darmstadt, Germany). The chromatogram was developed for 90 min in dioxane-ammonia-water (6:1:6 by volume). This chromatographic system was specifically developed to better separate p₄A (R_f = 0.17) and p₅A (R_f = 0.11) from ATP (R_f = 0.31). The picture was taken under shortwave UV light. The lanes represent the following: a and b, the mixture without enzyme taken after 5 and 24 h, respectively; c to g, the complete mixture containing triply phosphate taken after 0, 1, 5, 10, and 24 h, respectively; h and p, authentic standard of p₄A; i and j, the mixture without polyphosphate taken after 5 and 24 h, respectively; k to o, complete mixture containing tetratopolyphosphate taken after 0, 1, 5, 10, and 24 h, respectively. (B) HPLC. Reaction mixtures corresponding to lanes b, j, g, and o from panel A were diluted 10-fold and analyzed by HPLC as previously described (25). (C) Effect of alkaline phosphatase. A reaction mixture with P₃ (as described above) was incubated overnight at 30°C, heated at 100°C for 3 min, and centrifuged, and 0.05-ml portions of the supernatant were treated with alkaline phosphatase (2 U) for the indicated times and analyzed by HPLC as described in reference 25. Ado, adenosine.
The synthesis of Ap4A is not yet clearly established. We do believe that p3A synthesis is an intrinsic property of yeast acetyl-CoA synthetase; it was observed in four different batches of enzyme. Only one preparation (lot 42H8025, catalog no. A-5269 from Sigma) showed an insignificant capability for the synthesis of p3A. Among others, one can speculate on the following reasons for the insignificant capability: (i) mutational changes in some essential amino acids of the enzyme (a similar example for succinyl-CoA synthetase is described in reference 19), (ii) phosphorylation or dephosphorylation of the native enzyme (phosphorylation of threonyl- and seryl-tRNA synthetases) increased the Ap3A synthesis up to six- and twofold, respectively (3), (iii) the possible occurrence of different isozymic forms, depending upon the yeast growth conditions (13, 28, 29). In an attempt to clarify this point, the commercial preparations of acetyl-CoA synthetase used in this work were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme from Boehringer Mannheim had a main band of 72 kDa. All samples from Sigma had two important bands of 72 kDa (main band) and 82 kDa. The preparation from Sigma (lot 42H8025) with negligible capacity for the synthesis of p3A presented a protein electrophoretic pattern indistinguishable from the Sigma batches which were active in the synthesis of that nucleotide.

Which enzymes contribute most to the p3A and p4A production observed during yeast sporulation (9) is not known.

SYNTHESIS OF p4A BY ACETYL-CoA SYNTHETASE

Yeast 3-phosphoglycerate kinase catalyzes p3A and p4G synthesis at rates of about 10^{-4} times the rate of the transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP (6, 33). The activity of this enzyme is approximately 47 U/mg of protein in crude extract (30). Aminoacyl-tRNA synthetases (9, 26, 37) also can make p3A and p4A. As we show here, acetyl-CoA synthetase may also contribute. Evidence for its possible role in the sporulation-related production of p3A (p4A) is strengthened by the facts that (i) acetate is an inducer of sporulation (4), (ii) the level of the enzyme in yeast cells is relatively high (0.2 U/mg of protein in crude extract (5), and (iii) yeast cells contain high levels of polyphosphates, mainly p3 and p4 (34, 35). The possibility that acetate could stimulate p4A (p3A) formation from ATP and P3 (P4) could then be considered as one of the reasons for the efficiency of acetate as an inducer of sporulation. Acetate has been used for that purpose, on an empirical basis, since 1916 (4). As previously stated (7), when the normal metabolic equilibria are unbalanced (as could happen in yeast cells growing in a sporulating medium), the levels of such cosubstrates as specific tRNA or CoA may decrease, favoring the synthesis of p3A and/or p4A through reactions between various enzyme-X-AMP complexes and polyphosphates.

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REFERENCES