EVIDENCE FOR AN ACETYL-ENZYME INTERMEDIATE IN THE ACTION OF ACETYL-CoA SYNTHETASE

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SUMMARY: Acetyl-CoA synthetase (EC 6.2.1.1) from yeast is a ligase which catalyzes the synthesis of ATP from ADP and acetyl-CoA or acetyl-dephosphoCoA. The enzyme also catalyzes the rapid and reversible transfer of an acetyl group between CoA and dephospho CoA in the absence of the other components of the total ligase reaction. Such transfer is chemically equivalent to a CoA-acetyl-CoA exchange, and points therefore to an acetyl-enzyme intermediate in the transfer ("exchange") reaction. Since the "exchange" is an intrinsic activity of the enzyme, it seems probable that the acetyl-enzyme mediates the total ligase reaction as well.

Acetyl-CoA synthetase catalyses the synthesis of ATP according to Eq. 1:

$$\text{Acetyl-CoA} + \text{AMP} + PP_i \rightleftharpoons \text{acetate} + \text{CoA} + \text{ATP} \qquad (1)$$

By common testimony, acetyl adenylate - non-covalently bound to the enzyme - is an intermediate of the reaction (Eqs. 2 and 3) (1-3):

$$E + \text{acetyl-CoA} + \text{AMP} \rightleftharpoons E \cdots\cdots \text{acetyl adenylate} + \text{CoA} \qquad (2)$$

$$E \cdots\cdots \text{acetyl adenylate} + PP_i \rightleftharpoons E + \text{acetate} + \text{ATP} \qquad (3)$$

The present report provides some evidence for the proposition that the reaction of Eq. 2 entails the participation of an acetylated enzyme as a component of the reaction. Such participation is represented in simple terms as the sum of Eqs. 4 and 5:

$$E + \text{acetyl-CoA} \rightleftharpoons E\sim\text{acetyl} + \text{CoA} \qquad (4)$$

$$E\sim\text{acetyl} + \text{AMP} \rightleftharpoons E \cdots\cdots \text{acetyl adenylate} \qquad (5)$$

Since acetyl-CoA synthetase can act on acetyl-dephospho CoA in place of acetyl-CoA in the synthesis of ATP, it becomes feasible to study the transfer of acetyl between CoA and dephospho CoA in lieu of the exchange of radioactive CoA into acetyl-CoA (Eq. 4). Radioactive CoA in our hands proved difficult to purify, whereas dephospho CoA of a high purity is readily accessible and the "exchange" is easily measured.

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MATERIALS AND METHODS

Acetyl-CoA, CoA, dephospho CoA, and ATP were purchased from P-L Biochemicals. [^{14}C]Acetic anhydride was a product of Amersham/Searle. Other labeled compounds were supplied by New England Nuclear. [^{14}C]Acetyl-dephospho CoA was prepared from dephospho CoA and [^{14}C]acetic anhydride by the method of Simon and Shemin (4), and purified on a column of DEAE-cellulose (equilibrated with 0.003 N HCl) by elution with a linear gradient of LiCl (0.03-0.2 M) in 0.003 N HCl. [^{14}C]Acetyl-dephospho CoA was separated from salt by Sephadex G-10 chromatography

Enzyme activity was determined by the formation of acethydroxamate as described by Jones and Lipmann (5). Enzyme unitage is defined according to Berg (1). The protein content of crude enzyme was assayed by the method of Warburg and Christian (6), and that of purified enzyme containing dithiothreitol by the method of Ross and Schatz, with bovine serum albumin as standard (7).

The enzyme was purified from dried yeast (El Molino Active Dry Yeast, El Molino Mills, City of Industry, California) purchased in a local health food store. The dried yeast (5 g), suspended in 10 ml of water, stood for an hour at 4°. An ice-cold solution (30 ml) of 0.1 M K_2HPO_4 containing 1 mM dithiothreitol was added, and the suspension was passed through a pre-cooled (-20°) French press at high pressure (5000 psi). The effluent was centrifuged for 20 min at 28,000xg in a Sorvall centrifuge. To the crude extract was added 2% protamine sulfate (6 ml/50 ml of extract), and, after standing 10 min, the mixture was centrifuged and the pellet discarded. The supernatant solution was brought to 60% saturation with solid ammonium sulfate and centrifuged. After washing the pellet with 50% ammonium sulfate solution (pH 6.8, containing 1 mM dithiothreitol), it was dissolved in enough 1 mM dithiothreitol to give a protein concentration of 5 mg/ml. To 35 ml of the protein solution was added 1 ml of alumina C_γ gel (BioRad Laboratories); 10 min later the suspension was centrifuged and the gel discarded. The supernatant solution was treated with 2 ml of gel, stirred for 10 min, and centrifuged. After washing the precipitated gel with 20 ml of water and then 20 ml of 0.05 M potassium phosphate buffer (pH 7.0), the enzyme was eluted with two 15-ml portions of 0.1 M potassium phosphate (pH 7.5) containing 1 mM dithiothreitol. The enzyme solution was concentrated in a Diaflo apparatus (PM 30 membrane) and applied to a Sephadex G-200 column equilibrated with a solution 0.04 M in potassium phosphate (pH 6.8), 1 mM in dithiothreitol, and 0.5 M in EDTA. Fractions containing enzyme activity were concentrated immediately (as above), made 5% in glycerol, and stored at -180°. Yield of enzyme: 48%. Specific activity: 60 units/mg. This enzyme was used in the experiments of Fig. 1.

Further purification of the enzyme was achieved through affinity chromatography on agarose-hexane-coenzyme A (type 5), in which the SH group of CoA is bound to the column spacer. After washing the column with 0.06 M potassium phosphate (pH 6.6) containing 1 mM dithiothreitol, enzyme from the Sephadex G-200 step was applied at 4° in the same buffer. Elution was conducted at 4° with 0.1 M potassium phosphate (pH 7.6). The enzyme was stored in 5% glycerol at -180°. Recovery of enzyme was 37% and the specific activity was 145 units/mg. This enzyme was used in the "exchange" experiments of Fig. 2.

A somewhat purer enzyme was prepared in small quantity by affinity chromatography on agarose-hexane-coenzyme A (type 1), in which the CoA is bound to the column by the adenine ring and the SH group is free. The column was equilibrated with 0.1 M potassium phosphate (pH 7.5) containing 10 mM dithiothreitol. Enzyme from the Sephadex G-200 step was applied at 4° in 0.1 M potassium phosphate (pH 6.6) containing 1 mM dithiothreitol and 5% glycerol. Thereafter the column was washed with 0.5 M potassium phosphate (pH 7.5) until the eluate was free of protein. The enzyme was eluted at room temperature with 10 ml of potassium phosphate (pH 7.5) containing 0.01 M potassium acetate, 0.01 M magn-

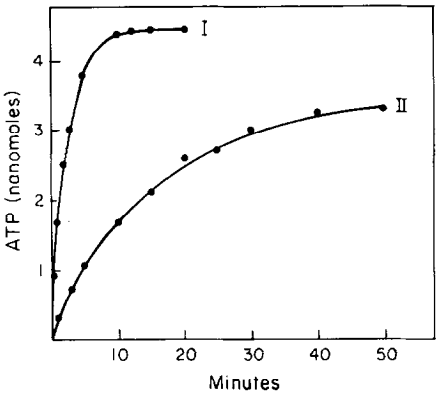

Fig. 1. ATP synthesis with acetyl-CoA and acetyl-dephospho CoA. An incubation volume of 50 μl contained (in nmoles): magnesium chloride, 50; AMP, 50; [^{32}P]PP$_i$ (40,000 cpm/nmole), 50; potassium phosphate (pH 6.6), 5000; and acetyl-CoA synthetase (60 units/mg), 0.25 unit. In addition, for curve I, acetyl-CoA, 4.5; and, for curve II, acetyl-dephospho CoA, 4.5. Temperature, 37°. Aliquots (2 μl) were removed at the indicated times and applied to polyethyleneimine cellulose plates. These were developed (9) for 2-3 hr at room temperature in 1.5 M potassium phosphate, pH 3.4. Zones corresponding to ATP were scraped into vials and counted in a Nuclear-Chicago scintilllation counter with Bray's solution.

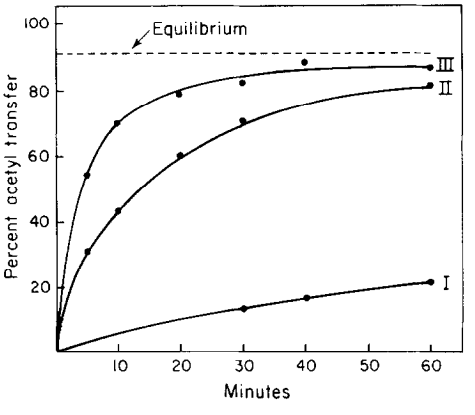

Fig. 2. Acetyl transfer between CoA and dephospho CoA. An incubation volume of 50 μl contained (in nmoles): potassium phosphate, pH 6.6, 5000; acetyl-CoA synthetase, 0.5 units (145 units/mg). In addition, for curve I, [^{14}C]acetyl-dephospho CoA (58 μCi/μmole), 4.0; CoA, 40; for curve II, [^{14}C]acetyl-CoA (51 μCi/μmole), 4.0; dephospho CoA, 40; and, for curve III, same as for curve II with the addition of AMP, 10. Temperature, 37°. Aliquots (2 μl) were removed at the indicated times and applied to polyethyleneimine cellulose plates that were previously moistened at the origin with 1 N formic acid. The plates were developed for 3 hr at room temperature in 0.4 M lithium chloride - 0.25 M ammonium formate, pH 4.2. Zones corresponding to acetyl-dephospho CoA (R_f: 0.67) and acetyl-CoA (R_f: 0.19) were scraped into vials and counted in a Nuclear-Chicago scintillation counter with Bray's solution. For each time point, the radioactivity in the two zones was summed, and the percentage of acetyl transfer to CoA or to dephospho CoA was plotted on the ordinate.

esium chloride, 0.01 M ATP, and 1 mM dithiothreitol. The enzyme solution was cooled, concentrated, and stored at -180°. Recovery of enzyme was 20%, and the specific activity was 188 units per mg. The "exchange" activity remained intact. The enzyme was judged to be about 85% pure on the basis of gel electrophoresis which revealed the presence of only two bands, one of which was much larger than the other. The small band migrated faster. On progressive inactivation of the enzyme the fast running band grew at the expense of the slower. An approximate molecular weight of 220,000 daltons for the enzyme was ascertained by glycerol density gradient centrifugation. In general, the enzyme at all stages of purification underwent progressive inactivation on repeated freezing and thawing.

The enzyme used in the "exchange" experiments was tested for AMP content by incubating it (8 units) with one nmole of /G-^{3}H/AMP (1 x 10^{6} cpm) for 5 min at 37° followed by filtration over a Sephadex G-50 column. The radioactivity accompanying the enzyme was indistinguishable from background. Thus, the 0.5 unit of enzyme used in the experiments of Fig. 2 contained 0.002 pmole or less of AMP. To remove small amounts of AMP which accompany commercial dephospho CoA, a solution of the latter (7.7 μmoles) in water, mixed with /G-^{3}H/AMP (4 x 10^{5} cpm), was loaded on a Dowex 50W-X8 column (42mm x 6mm), which was then eluted with water (8). Dephospho CoA issued from the column after the void volume. Rechromatography on a twin column afforded dephospho CoA free of measurable radioactivity. Forty nanomoles of dephospho CoA contained 0.1 pmole or less of AMP. Other components of the "exchange" systems of Fig. 2 were assayed for AMP content using the protocol for ATP synthesis detailed in the legend of Fig. 1, with the further addition of glucose and hexokinase. Any AMP was thus converted with /^{32}P/PP$_i$ into /β,γ-^{32}P/ATP which was trapped by conversion to equivalent amounts of /^{32}P/glucose-6-P and /β-^{32}P/ADP. After thin layer chromatography, as described, the zone corresponding to ADP was scraped into vials and counted. Authentic AMP was added to controls. In all cases, including the entire "exchange" systems, the /β-^{32}P/ADP that formed was indistinguishable from background. Thus, the entire "exchange" system after 15 min of incubation could have included no more than 0.1 pmole of AMP.

RESULTS

The course of ATP synthesis from acetyl-CoA and from acetyl-dephospho CoA is depicted in Fig. 1. With acetyl-CoA the initial velocity is about five times greater than with acetyl-dephospho CoA. In the reverse direction of Eq. 1, CoA reacts with ATP and acetate only twice as fast as does dephospho CoA (reported by Jones and Lipmann (5) and confirmed by us). The rate of ATP synthesis is, as expected, a function of the AMP and PP$_i$ concentrations, and can be speeded up or slowed down accordingly.

An "exchange" of dephospho CoA into acetyl-CoA, and of CoA into acetyl-dephospho CoA - in the absence of AMP - is illustrated in Fig. 2. It is clear that the transfer of acetyl from CoA to dephospho CoA proceeds with a higher initial velocity than the reverse transfer. This parallels the more rapid ATP synthesis from acetyl-CoA. It is of some interest to compare the initial velocities of the "exchange" reaction (curve II of Fig. 2) with that of ATP synthesis from acetyl-CoA (that is, the total ligase reaction, curve I of Fig. 1). Even in the favorable conditions of Fig. 1, ATP synthesis is but sevenfold faster

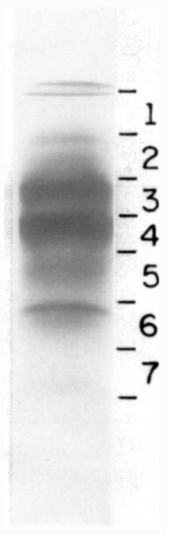

Fig. 3. Gel electrophoresis of the enzyme used in the experiments of Fig. 2. Enzyme (2 units) was applied to duplicate acrylamide (4.5%) gels, and electrophoresis was conducted in 0.035 M Tris-HEPES buffer (pH 7.6) at 2.5 ma/gel (50 volts) for 3.5 hr at 4°. One gel was stained with Coomassie blue. The other was sliced as shown and the slices were assayed as described in the legend of Table 1.

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than the "exchange". Since the rate of ATP synthesis is dependent, among other things, on the AMP concentration, it is possible simply by reducing the latter to bring the two velocities into substantial equality. For this reason, it seems fair to say that the "exchange" and the total ligase reactions have initial velocities of the same order of magnitude.

Fig. 2 (curve III) also makes clear that the addition of AMP (2×10^{-4} M) to the "exchange" system of curve II approximately doubles the initial rate of the "exchange". AMP, of course, evokes the reversible formation of enzyme-bound acetyl adenylate (Eq. 2). Pyrophosphate (2×10^{-4} M), added to the system of curve III, has little effect on the "exchange" rate, but does act to drain away some of the acetyl groups into free acetate as the total ligase reaction (Eq. 1) comes into operation (data not shown). These observations accord with the view that the "exchange" is an intrinsic activity of acetyl-CoA synthetase.

All components of the "exchange" systems are devoid of measurable AMP. It was ascertained, moreover, that no AMP is produced by adventitious enzyme action during the course of the "exchange" reaction (see Materials and Methods). At the very most, 0.1 pmole of AMP (2×10^{-9} M) could have been present during the "exchange" represented by curve II of Fig. 2. This is to be compared with

TABIE 1

Assay of the sliced electrophoretic gel for acetyl-CoA synthetase and acetyl transfer ("exchange") activities

Slice No.	"Exchange"	Acetyl-CoA synthetase
	%	units
1	0.5	0.0
2	0.7	0.0
3	0.5	0.0
4	11.5	0.75
5	1.4	0.0
6	0.9	0.0
7	0.7	0.0

Protein from each gel slice (indicated in Fig. 3) was collected in a 50-μl tubular chamber capped with a dialysis membrane by electrophoresis in 0.035 M Tris-phosphate (pH 7.6) - 1 mM mercaptoethanol for 1.5-2.5 hr at 4° with a current of 3 ma/gel (80-90 volts). A 10-min reversal of polarity completed the process. Eluates were assayed for acetyl-CoA synthetase activity by the hydroxamate method (5) and for "exchange" activity according to the legend of Fig. 2 (curve II).

the 10 nmoles of AMP (2×10^{-4} M) required to double the rate of the "exchange" (curve III, Fig. 2). On the other hand, 1 nmole of AMP (2×10^{-5} M) affected the "exchange" rate of curve II not at all. The threshold of AMP sensitivity lies therefore between 2×10^{-5} and 2×10^{-4} M. The rapid rate of the "exchange" and its relative insensitivity to AMP concentration give confidence that experiments such as those of curve II (Fig. 2) do indeed measure a true "exchange", and not one imputable to the reversible reaction of Eq. 1 or 2.

Fig. 3 shows a gel electropherogram of the enzyme used in the experiments of Fig. 2. A duplicate gel was sliced as shown, and any enzyme in the indicated gel slices was recovered for assay. From the data of Table 1 it is clear that the "exchange" and acetyl-CoA synthetase activities are confined to the protein band of slice No. 4.

DISCUSSION

From gemeral considerations, there is good reason to believe that dephospho CoA occupies the same sub-site as CoA itself in the active center of acetyl-CoA synthetase. The reversible transfer of an acetyl group between CoA and its analogue is thus chemically equivalent to an authentic CoA-acetyl-CoA exchange. Such an exchange, occurring in the absence of the other components

of the total ligase reaction (and especially in the absence of AMP), signals a prominent role for an acetyl-enzyme intermediate in the transfer of an acetyl group from CoA to dephospho CoA in the "exchange", and, by inference, to AMP in the synthesis of enzyme-bound acetyl adenylate.

While acetyl-CoA synthetase is listed officially by the Enzyme Commission as a ligase (10), it operates in practice as if it were a pair of transferases united in one enzyme and acting in tandem (11). Thus, the total ligase reaction (Eq. 1) is seen as a composite of two simple transferase reactions (Eqs. 2 and 3) In the reaction of Eq.2 an acetyl group is transferred reversibly between CoA and AMP; and in the reaction of Eq. 3 the adenylyl group is transferred reversibly between acetate and PP_i. Transferases seem generally to catalyze their reactions by way of a covalent enzyme-substrate intermediate, in which the group undergoing transfer from donor to acceptor is joined to the enzyme by a covalent bond. Arguments upholding this principle of transferase action have been set forth elsewhere (12). The present communication gives some evidence to the effect that the acetyl-transferring phase of the ligase reaction is indeed mediated by an acetyl-enzyme, conforming thus to the general mode of transferase action (12). At this time, little is known of the adenylyl-transferring phase of the ligase reaction (Eq. 3), in which an adenylyl-enzyme intermediate is anticipated.

Acknowledgments

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