# THE ACETATE-ACTIVATING MECHANISM IN *RHODOSPIRILLUM RUBRUM**

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## INTRODUCTION

A stable purified enzyme preparation obtained from *Rhodospirillum rubrum* (*R. rubrum*) has been shown to activate added acetate to form acetylcoenzyme A in the presence of ATP and coenzyme A**[1]. The properties of this enzyme preparation were shown to be similar to those described for yeast, heart muscle, and plant enzymes[2,3,4]. Preliminary exchange experiments[5] between P-$^{32}$P and ATP and between 2-$^{14}$C acetate and acetyl CoA gave results which were not in harmony with the mechanism proposed by LIPMANN, LYNEN and their associates for the yeast enzyme[6]. It was simultaneously reported by BOYER *et al.*[7] using $^{18}$O-labeled acetate and the heart muscle enzyme preparation that an anhydride must be the intermediary form in any mechanism proposed for the acetate activation process. It was subsequently shown by BERG[8] that the anhydride intermediate was acetyl adenylate and the following mechanism was proposed:

$$\text{acetyl} + \text{ATP} \rightleftharpoons \text{acetyl adenylate} + \text{P-P} \quad (1)$$

$$\text{acetyl adenylate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{AMP} \quad (2)$$

The present investigation describes the results of the exchange experiments between P-$^{32}$P and ATP and between 2-$^{14}$C acetate and acetyl CoA using the acetate-activating enzyme of A. *rubrum*. The results are discussed in relation to the proposed mechanism.

## MATERIALS AND METHODS

The enzyme preparations used were either those previously described[1] or slightly modified. The calcium phosphate gel step was replaced with a third ammonium sulfate fractionation when it was found that freshly prepared gel would no longer give the same degree of purification as the older gel. The fraction between 0.40–0.70 saturation[9] was adjusted to pH 6.0 and the fraction between 0.50 and 0.60 containing the enzyme was removed. This step gave the same degree of purification as the original calcium phosphate gel step.

Radioactive pyrophosphate was obtained by the pyrolysis of radioactive phosphate. 0.5–1.0 mc of radioactive phosphoric acid was adjusted to pH 9.0 with 1 *N* NaOH in a silica crucible. 0.2–0.5 ml of 0.2 *M* $Na_2HPO_4$ was added and the mixture taken to dryness in an oven at 90°C. The dried salt was then heated over a Fischer burner for 1 hour. After cooling, the salt was dissolved in a small amount of water and the radioactive pyrophosphate separated from the radioactive inorganic phosphate by the method of KORNBERG AND PRICER[1'] using a Dowex-Cl$^-$ column. The

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** The abbreviations used in this paper are as follows: ATP = adenosine triphosphate, AMP = adenosine 5'-phosphate, CoA = coenzyme A, acetyl CoA = acetyl coenzyme A, P-P = pyrophosphate.

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eluate containing the radioactive pyrophosphate was reduced to one quarter its original volume with a flash evaporator. The pH was adjusted to 4.5, 1.0 ml of 0.1 *M* $ZnSO_4$ added, and the pyrophosphate precipitated as the zinc salt. The precipitated pyrophosphate was removed by centrifugation, washed with 1.0 ml of 0.01 *M* $ZnSO_4$, and then dissolved in 0.1 *N* HCl. $H_2S$ was passed through the solution and the precipitated zinc sulfide removed by centrifugation. The supernatant solution was aerated to remove the excess $H_2S$, the pH adjusted to 7.5, and solution stored as such in the refrigerator. The material assayed by the pyrophosphatase method of KUNITZ[9] 95–97% pyrophosphate. Radioactive pyrophosphate solutions of similar purity could also be obtained using the zinc precipitation procedure alone. The cooled salts were dissolved in a small volume of water and the pH adjusted to 4.5. The pyrophosphate was precipitated as the zinc salt described above. The precipitate was dissolved in 0.1 *N* HCl and 200–300 $\mu$moles of inorganic phosphate added. The pH was readjusted to 4.5 and the precipitation of pyrophosphate repeated. The subsequent steps were the same as described above.

ATP, AMP, CoA and potassium pyrophosphate were commercial products and were assayed in the manner previously described[1]. Acetyl CoA was prepared according to the method of SIMON and SHEMIN[11]. The solution was adjusted to pH 1–2 and taken to dryness with a flash evaporator to remove as much of the acetate as possible by distillation or the preparation was chromatographed on Whatman No. 1 paper using ethanol-formate for developing the chromatogram. 2-$^{14}$C sodium acetate was a gift from Dr. DAVID RITTENBERG and contained 0.1 mc per millimole.

Radioactive pyrophosphate was separated from ATP by the charcoal adsorption procedure described by LIPMANN AND CRANE[12]. The same method was initially used to separate acetate and acetyl CoA. However, it was found that very poor recoveries of acetohydroxamic acid were obtained when known amounts of acetyl CoA were adsorbed onto Norit A and the latter then treated with neutral hydroxylamine. Under the conditions used, the hydroxylamine was rapidly broken down to ammonia by the Norit A. Thus, whereas this method separates satisfactorily acetate and acetyl CoA and may be adequate for determining radioactive acetate in acetyl CoA, it was not found to be satisfactory for assaying acetyl CoA with the hydroxylamine method. A distillation diffusion procedure was developed which gave good separation and recovery of acetyl CoA. The reaction mixture was heated for 2 minutes in a boiling water bath to stop the reaction. The mixture was cooled, 400 $\mu$moles of hydroxylamine at pH 7.5 were added, and the solution was permitted to stand for 15 min to permit the conversion of acetyl CoA into the hydroxamic acid. 0.4 ml of 1 *N* $H_2SO_4$ and sufficient water were added to give a final volume of 2 ml. The solution was centrifuged to remove the precipitated protein and 1.0 ml of the supernatant was removed to a 15 ml centrifuge tube. The solution was taken down to dryness by lyophilization. 500 $\mu$moles of glacial acetic acid were added and lyophilization repeated. This procedure was repeated three times. The final sediment was dissolved in 0.3 ml water, the solution was neutralized and brought to a volume of 1.0 ml. Aliquots were then plated on lens paper according to the procedure described by CALVIN *et al.*[13]. All samples were counted with a thin end window counter.

## RESULTS

Initial results for the exchange reaction between radioactive pyrophosphate and ATP with the original calcium phosphate gel enzyme preparation (Enzyme Prep. No. 1) are shown in Expt. 1, Table I. It can be seen that in the presence of the enzyme alone there is an appreciable incorporation of radioactivity in the ATP fraction. The addition of CoA or AMP had very little effect on the extent of incorporation over that of the enzyme alone. However, the addition of acetate reduced the incorporation of radioactivity in the ATP fraction by more than 50 %. In the exchange reaction between 2-$^{14}$C acetate and acetyl CoA (Expt. 2, Table I), very little exchange occurred in the presence of the enzyme alone. The addition of either AMP or P-P produced a two-fold increase in the radioactivity of the acetyl CoA fraction, and when the two compounds were added together the activity was increased four-fold.

Several of these results were completely unexpected on the basis of the mechanism proposed by JONES, LIPMANN, HILZ, AND LYNEN using the yeast enzyme[6]. First, the addition of acetate should not affect the P-$^{32}$P – ATP exchange, whereas Expt. 1 shows a very marked inhibitory effect. Second, CoA should have markedly reduced the exchange reaction whereas it had no effect in the two concentrations used. Third,

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TABLE I

EXCHANGE REACTIONS WITH ENZYME PREPARATION NO. 1 *

| *P-$^{32}$P – ATP exchange* | *Expt. 1 ATP fraction c.p.m.* | *2-$^{14}$C acetate – acetyl CoA exchange* | *Expt. 2 hydroxamic acid c.p.m.* |
|---|---|---|---|
| Without enzyme | 6,600 | Without enzyme | 340 |
| With enzyme | 158,000 | With enzyme | 390 |
| With enzyme + 0.2 $\mu M$ CoA | 146,000 | With enzyme + AMP | 720 |
| With enzyme + 0.4 $\mu M$ CoA | 145,000 | With enzyme + P-P | 760 |
| With enzyme + 10 $\mu M$ acetate | 68,000 | With enzyme + AMP + P-P | 1430 |
| With enzyme + 2.5 $\mu M$ AMP | 140,000 | | |

Expt. 1. Reaction mixture contained per ml: 10 $\mu M$ $MgCl_2$, 2.01 $\mu M$ ATP, 1.94 $\mu M$ P-$^{32}$P, 10 $\mu M$ $H_2S$, 100 $\mu M$ Tris(hydroxymethyl)aminomethane buffer pH 7.5, 5 units of enzyme, and other additions as indicated. The total activity of the P-$^{32}$P added was approximately 800,000 c.p.m. Incubated 30 min at 32°C.

Expt. 2. Reaction mixture contained per ml: 10 $\mu M$ $MgCl_2$, 10 $\mu M$ $H_2S$, 100 $\mu M$ Tris(hydroxymethyl)aminomethane buffer pH 7.5, 5 $\mu M$ AMP, 10 $\mu M$ potassium pyrophosphate pH 7.5, 1.44 $\mu M$ acetyl CoA, 1.48 $\mu M$ 2-$^{14}$C sodium acetate with a total activity of approximately 16,000 c.p.m. and 10 units of enzyme. Incubated 60 min at 32°C.

* See text.

P-P either alone or together with AMP markedly enhanced the exchange reaction between acetate and acetyl CoA which could not be anticipated from the scheme proposed. It therefore appeared necessary to re-evaluate the problem and to explore a different mechanism which would explain the facts observed with the *R. rubrum* enzyme in a more satisfactory manner.

The first question to be examined was whether or not acetate plays a more essential or possibly an entirely different role in the exchange reaction between P-$^{32}$P and ATP. The calcium phosphate gel enzyme was prepared however in the presence of acetate. In view of the importance the presence or absence of acetate would assume for interpreting the mechanism involved, it appeared desirable to remove the latter so as to ascertain its role more adequately in the exchange reaction.

The enzyme preparation was dialyzed against phosphate buffer for 4 hours with two changes of buffer. Solid ammonium sulfate was added for complete saturation and the precipitated enzyme centrifuged off. The enzyme was dissolved in a minimum amount of water and then dialyzed against phosphate buffer for 5 hours with three changes of buffer until free of ammonium sulfate (Enzyme Prep. No. 2). This procedure reduced the specific activity of the enzyme to one half the original value. The exchange between P-$^{32}$P and ATP was repeated and the results are shown in Expt. 1, Table II. As in the previous experiment an appreciable exchange occurred in the presence of the enzyme alone. The addition of CoA inhibited the exchange by 86% and increasing the CoA concentration produced no further effect. These results were in agreement with those obtained with the yeast enzyme[6]. However, the addition of acetate again inhibited the exchange reaction, and increasing the acetate concentration resulted in a greater degree of inhibition. The inhibitory effect of acetate in both experiments was 58, 74 and 83% for 10, 20 and 40 $\mu$moles of added acetate respectively. The inhibitory action of CoA after most of the acetate was removed from the enzyme preparation suggested that the two were antagonistic in the exchange reaction. This again was in contrast to the expected results on the basis of the originally proposed

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mechanism. If anything, acetate should enhance the CoA effect by pulling the reaction toward acetyl CoA formation. Therefore, the results support again the view of an active role for acetate in the exchange reaction between P-$^{32}$P and ATP.

The high blank in the absence of any added acetate and the increasing inhibition with increasing acetate concentrations suggested the possibility that acetate may stimulate the exchange in low concentrations and inhibit in high concentrations. This was checked by repeating the exchange reactions in the presence of lower acetate concentrations and these results are shown in Expt. 2, Table II. It can be seen that under these conditions 1 $\mu$mole of acetate doubles the incorporation of radioactivity in the ATP fraction over that of the enzyme alone. Increasing the concentration to 2 $\mu$moles results in a 30% inhibition. 4 $\mu$moles of acetate reduces the rate of exchange almost to the blank level. This indicated that the exchange between P-$^{31}$P and ATP was dependent upon the presence of acetate. The acetate-acetyl CoA exchange reaction was also carried out with this enzyme preparation and the results shown in Expt. 3, Table II, are essentially the same as those obtained previously.

Since the exchange reaction between P-$^{32}$P and ATP showed a dependence on acetate in low concentrations, the high blank in the absence of added acetate suggested acetate contamination of the reaction mixture. Various ATP preparations were tested without any appreciable effect on the blank. Solutions of P-P, $MgCl_2$, and Tris buffer were freshly prepared and when tested showed no effect. The substitution of phosphate for Tris buffer did not alter the blank. These results pointed to the enzyme as a possible source of acetate contamination in spite of the extensive treatment to remove acetate. A new enzyme preparation was therefore prepared according to the modified procedure previously outlined, in which case acetate was not introduced at any point in the purification procedure (Enzyme Prep. No. 3). The exchange reactions were repeated and the results are shown in Table III. As can be seen there is little

TABLE II

EXCHANGE REACTIONS WITH ENZYME PREPARATION NO. 2*

| *P-$^{32}$P – ATP exchange* | *Expt. 1 ATP fraction c.p.m.* | *Expt. 2 ATP fraction c.p.m.* | *2-$^{14}$C acetate – acetyl CoA exchange* | *Expt. 3 hydroxamic acid c.p.m.* |
|---|---|---|---|---|
| Without enzyme | 900 | 450 | Without enzyme | 1100 |
| With enzyme | 53,200 | 12,400 | With enzyme | 1500 |
| With enzyme + 0.2 $\mu M$ CoA | 8,600 | | With enzyme + AMP | 1460 |
| With enzyme + 0.4 $\mu M$ CoA | 7,800 | 5,700 | With enzyme + P-P | 2160 |
| Without enzyme + acetate | 20 $\mu M$ 14,000 | 1 $\mu M$ 26,000 | With enzyme + AMP + P-P | 2320 |
| With enzyme + acetate | 40 $\mu M$ 9,000 | 2 $\mu M$ 21,000 | | |
| With enzyme + acetate | | 4 $\mu M$ 14,000 | | |
| With enzyme + 2.5 $\mu M$ AMP | 49,000 | | | |

Expt. 1. Reaction mixture contained per ml: 10 $\mu M$ $MgCl_2$, 2.10 $\mu M$ ATP, 3.00 $\mu M$ P-$^{32}$P, 10 $\mu M$ $H_2S$, 100 $\mu M$ Tris(hydroxymethyl)aminomethane buffer pH 7.5, 6.5 units of enzyme, and other additions as indicated. The total activity of the P-$^{32}$P added was approximately 100,000 c.p.m. Incubated 30 min at 32°C.

Expt. 2. Reaction mixture contained per ml: 10 $\mu M$ $MgCl_2$, 3.06 $\mu M$ ATP, 4.15 $\mu M$ P-$^{32}$P, 50 $\mu M$ Tris buffer pH 7.5, 2 $\mu M$ $NaHB_4$, 3.4 units of enzyme, and other additions as indicated. The total activity of the P-$^{32}$P added was approximately 100,000 c.p.m. Incubated 30 min at 32°C.

Expt. 3. The reaction mixture was identical to that of Expt. 2, Table I, with the exception that only 5 $\mu M$ of potassium pyrophosphate and 3.4 units of enzyme were used. Incubated 60 min at 32°C.

* See text.

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alteration in the pattern with the new enzyme preparation. The antagonistic action of acetate on CoA inhibition is more clearly brought out by the ability of 1 μmole of acetate to reverse almost completely the inhibitory effect of CoA.

TABLE III

EXCHANGE REACTIONS WITH ENZYME PREPARATION NO. 3*

| *$P^{-32}P$ – ATP exchange* | *Expt. 1 ATP fraction c.p.m.* | *$2^{-14}C$ acetate – acetyl CoA exchange* | *Expt. 2 hydroxamic acid c.p.m.* |
|---|---|---|---|
| Without enzyme | 144 | Without enzyme | 55 |
| With enzyme | 12,200 | With enzyme | 3,630 |
| With enzyme + 0.2 μ*M* CoA | 1,380 | With enzyme + AMP | 1,900 |
| With enzyme + 1 μ*M* acetate | 26,300 | With enzyme + P-P | 4,200 |
| With enzyme + 4 μ*M* acetate | 17,750 | With enzyme + AMP + P-P | 4,660 |
| With enzyme + 10 μ*M* acetate | 12,300 | | |
| With enzyme + 1 μ*M* acetate + 0.2 μ*M* CoA | 19,900 | | |

Expt. 1. Reaction mixture contained per ml: 10 μ*M* $MgCl_2$, 20 μ*M* Tris buffer pH 7.5, 3.41 μ*M* ATP, 3.66 μ*M* $P^{-32}P$, 7.7 units of enzyme and additions as indicated. The total activity of the $P^{-32}P$ added was approximately 115,000 c.p.m. Incubated 20 min at 32°C.
Expt. 2. Reaction mixture contained per ml: 10 μ*M* $MgCl_2$, 50 μ*M* Tris buffer pH 7.5, 1.93 μ*M* acetyl CoA, 3.4 μ*M* AMP, 4.3 μ*M* pyrophosphate, 1.48 μ*M* $2^{-14}C$ sodium acetate with a total activity of approximately 16,000 c.p.m. and 23 units of enzyme. Incubated 90 min at 32°C.

* See text.

The inability to reduce the blank with the enzyme prepared by the modified procedure prompted further purification of the enzyme with the hope of either eliminating the contaminating acetate or possibly other contaminating enzymes which could also carry out the exchange reaction between $P^{-32}P$ and ATP. The procedures which gave the best results did so in the presence of acetate only; the latter apparently having a protective action. Alcohol fractionation in the absence of acetate or in the presence of phthalate buffer at pH 6.0 resulted in very little purification with large losses in activity. In the presence of acetate, the alcohol fractionation gave a 3 fold purification with an 80% recovery of the total activity. This enzyme preparation was extensively dialyzed and the activating enzyme adsorbed onto C γ alumina gel. The gel was thoroughly washed with water and the enzyme eluted with phosphate buffer. This step produced another 2 fold increase in purity with a final specific activity of 60. When the exchange was carried out with this enzyme preparation, a high blank was still obtained in the absence of added acetate. Exhaustive dialysis for 4 days while reducing considerably the specific activity of the enzyme, failed to eliminate the blank. The enzyme was also treated with Dowex-1-$HCO_2^-$ and Dowex-1-$OH^-$ for 1 hour but these treatments also failed to eliminate the blank. These experiments indicated the great difficulty in removing entirely the acetate from the enzyme preparation. Considerable efforts probably would have been required to attain this aim, but in view of the data described above and the results obtained in other laboratories this effort was not considered as essential to justify further extensive work.

## DISCUSSION

The data presented are in disagreement with the mechanism of acetyl CoA formation as originally proposed for the yeast enzyme[6]. The increased rate of exchange between $P\text{-}P^{32}$ and ATP in the presence of low acetate concentrations, the inhibitory

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effect of CoA, the antagonism of CoA and acetate, the enhanced rate of exchange between 2-$^{14}$C acetate and acetyl CoA in the presence of P-P and AMP are all in accord with the mechanism proposed by BERG[8] as shown in reactions (1) and (2). This places the acetate-activating enzyme in the large category of activating enzymes, as *e.g.* those which activate pantoic acid, sulfate, bicarbonate, etc.[14,15,26], where P-P and AMP are end-products of the reaction and the exchange between P-$^{32}$P and ATP requires the presence of the substrate to be activated.

The inability to remove acetate completely from the enzyme precluded a more detailed study of some other aspects of the mechanism. The evidence is suggestive that the source of the blank is the acetate strongly bound to the enzyme and difficult to remove by the methods tried. The other possibility that cannot be excluded is the contamination of the enzyme preparation by other activating enzymes mentioned above. An examination of the known activating enzymes which might possibly be responsible for the exchange blank would exclude all but the $SO_4^=$ and $HCO_3^-$ activating enzymes. The former may be excluded on the ground that the Dowex treatment did not reduce the blank, since under such treatment $SO_4^=$ would have been removed. This would leave the $HCO_3^-$ activating enzyme as a possible source of the blank.

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SUMMARY

Acetate in low concentrations was found to stimulate the exchange reaction between radioactive pyrophosphate and ATP. In higher concentrations acetate inhibited the exchange reaction. Acetate was also found to overcome the inhibitory action of CoA.

P-P was found to stimulate the exchange reaction between radioactive acetate and acetyl CoA.

The data do not support the view, proposed for the acetate activation process, that AMP combines with the enzyme and is exchanged with CoA. They are in accord with the mechanism in which acetyl adenylate* is proposed as the anhydride intermediate.

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* The term acetyl adenylate appears preferable to adenyl acetate since it is in line with other forms of activated acetate, such as acetyl phosphate and acetyl CoA.