THE ACETATE-ACTIVATING ENZYME OF *RHODOSPIRILLUM RUBRUM**

by

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It was demonstrated in a previous investigation[1] that the acetate-activating enzyme system of *Rhodospirillum rubrum* is not identical with that found in most microorganisms[2,3]. The absence of the enzyme phosphotransacetylase and the requirement of coenzyme A indicated that the enzyme system in this organism was similar to the one first described by NACHMANSOHN AND MACHADO[4] for the acetylation of choline in brain tissue and further elaborated on by LIPMANN *et al.*[5,6] during an extensive investigation of the mechanism of acetylation in pigeon liver. It was subsequently shown by LIPMANN *et al.*[7], with a yeast preparation, that the acetate-activation involved a pyrophosphorolytic split of ATP. With the identification of "active acetate" as the thiolester of coenzyme A by LYNEN *et al.*[8], the overall equation could be formulated as follows:

$$(1) \quad \text{ATP} + \text{CoA} + \text{Acetate} \rightleftharpoons \text{Acetyl CoA} + \text{AMP} + \text{P–P}$$

A similar enzyme system has also been purified from heart muscle[9,10] and more recently it has also been found in a variety of plants and plant tissues[11]. Further information concerning the detailed mechanism of the acetate-activating reaction has recently been obtained by JONES, LIPMAN, HILZ AND LYNEN with the aid of isotopes[12].

The present investigation was undertaken with the purpose of studying the acetate-activating reaction in greater detail with purified enzyme preparations from *Rhodospirillum rubrum*. The present report concerns itself with the purification procedure and the properties of the purified enzyme system.

MATERIALS AND METHODS

Barium ATP**), coenzyme A, and adenosine-3′-phosphate were products of Pabst Brewing Co. Barium ADP, adenosine-5′-phosphate and calcium phosphate gel were purchased from Sigma Chemical Co. Potassium acetate, potassium pyrophosphate, hydroxylamine hydrochloride, and glutathione were commercial products. All acidic solutions were adjusted to pH 7.4 with KOH. Acetyl coenzyme A was prepared by the method of SIMON AND SHEMIN[13]. Crystalline yeast pyrophosphatase was generously supplied by Dr. M. KUNITZ, and a purified preparation of yeast hexokinase was kindly furnished by Dr. W. H. STEIN.

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** The abbreviations used are as follows: ATP-adenosine triphosphate, ADP-adenosine diphosphate, AMP-adenosine 5′ phosphate, CoA-coenzyme A, AcetylCoA-acetyl coenzyme A, P–P-inorganic pyrophosphate.

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Inorganic phosphate was determined by the method of FISKE AND SUBBAROW[14]. Pyrophosphate was estimated by a slight modification of the procedure of KUNITZ[15], and also by the manganese precipitation method of KORNBERG[16]. ATP, ADP and AMP were separated by the column technique of COHN AND CARTER and their respective concentrations determined spectrophotometrically[17]. ATP was also determined by the hexokinase method in which the acid-labile phosphate is measured, ADP by the acid-labile phosphate after 10 minutes' hydrolysis in 1 *N* HCl at 100° C, and AMP by the absorption at 260 mμ. Coenzyme A was assayed by the arsenolysis test with acetyl coenzyme as a standard for CoA[18]. Acetyl CoA was determined spectrophotometrically by measuring the decrease in the optical density at 232 mμ in the presence of arsenate and phosphotransacetylase[19]. The method of WARBURG AND CHRISTIAN[20] was used to measure the protein concentrations. ATPase activity was assayed by the method of KIELLEY AND KIELLEY[21], and myokinase activity by the method of COLOWICK AND KALCKAR[22]. Acetyl coenzyme A deacylase activity was determined by the decrease in optical density at 232 mμ or by the formation of aceto hydroxamic acid.

Acetohydroxamic acid formation was measured by the method of LIPMANN AND TUTTLE[23]. The reaction mixture in a final volume of 1 ml was mixed with 1 ml of 12% trichloracetic acid, 2 ml of water, 1 ml of 4 *N* HCl, and 1 ml of 5% ferric chloride in 0.1 *N* HCl. The mixture was clarified by centrifugation and the adsorption of the supernatant fluid measured in a KLETT-SUMMERSON photoelectric colorimeter with a #54 filter. A reagent blank was used to set the instrument. 1 μ*M* of acetohydroxamic acid was equivalent to 57 Klett units using as a standard a sample of acetylglutathione kindly furnished by Dr. H. SACHS.

ENZYME PURIFICATION

R. rubrum was grown for 36 hours under continuous illumination in 10–12 liter quantities according to the procedure described previously[1]. The culture was removed from the light cabinet and left for 24 hours at room temperature prior to harvesting. The yield was approximately 3–4 grams of wet cells per liter of culture medium. The cells were washed twice with distilled water and packed tightly in the Sorvall high speed centrifuge at 12,000 × gravity. The packed cells were then stored in the freezing compartment of the refrigerator over night.

Crude extract. The frozen mass of cells was broken up in a cold mortar and ground with 2.5 times its weight of alumina A-303. The tacky paste was taken up with 2.5 times its volume of cold 0.05 *M* phosphate buffer pH 7.4 and centrifuged at 900 × gravity in the International Refrigerated Centrifuge. The supernatant was decanted and the alumina resuspended twice in 10–15 ml of buffer, and recentrifuged. The supernatant fluids were combined and centrifuged at 78,400 × gravity for one hour in the Model E Spinco centrifuge. The supernatant fluid was removed and stored in the deep freeze at —20° C. The activity of the crude extracts was maintained for an extended period of time in the frozen state. Occasionally, the crude extracts showed a lower total enzymic activity than the more purified preparations and in addition increased in activity when kept in the frozen state. This suggested the presence of an inhibitor. Dialysis of these crude extracts did not increase the enzymic activity indicating the inhibitor was of a high molecular weight. The addition of fluoride to the reaction mixture to inhibit ATPase activity resulted only in a loss of activity. No further attempt was made to identify the inhibitor.

1st ammonium sulfate fractionation. To 100 ml of crude extract at 0–2° C were slowly added with continuous stirring 16.6 grams of solid ammonium sulfate to yield a 30% saturated solution. The solution was stirred for another ten minutes and then centrifuged at 8,700 × gravity for 15 minutes. The reddish precipitate contained very little activity and was discarded. Sufficient ammonium sulfate was then added to the supernatant fluid to bring the concentration up to 70% saturation. The heavy precipitate was centrifuged for 15 minutes at 35,000 × gravity. The precipitate was taken up in a

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minimum amount of 0.05 *M* phosphate buffer pH 7.4 and dialyzed against the same buffer over night. The dialysis was continued on the following day using 0.01 *M* phosphate buffer pH 7.4 until the dialysate was free of ammonium sulfate.

Protamine sulfate. The dialyzed 30–70% fraction was diluted with buffer to a final concentration of about 15 mg of protein per ml. A 2% solution of protamine sulfate was slowly added with continuous stirring until a final concentration of 18 mg of protamine sulfate for each 100 mg of protein was attained. The heavy flocculent precipitate was removed by centrifugation and discarded.

2nd ammonium sulfate fractionation. Solid ammonium sulfate was added to the above supernatant to bring the concentration up to 50% saturation. The precipitate was removed by centrifugation and discarded. More solid ammonium sulfate was added and the precipitate formed at 70% saturation removed by centrifugation at 35,000 × gravity and saved. A final precipitate was removed after bringing the ammonium sulfate concentration up to 90% saturation. Both precipitates were dissolved in a minimum amount of 0.05 *M* phosphate buffer pH 7.4 and dialyzed against the same buffer until the solutions were essentially free of ammonium sulfate. About 40% of the total activity was found in the 50–70% fraction as compared to 25% in the 70–90% fraction. However, the latter had about twice the specific activity of the former, and therefore was used for further purification.

Calcium phosphate gel. The pH of the 70–90% fraction was adjusted to 6.5 with 1 *N* acetic acid and then diluted with buffer to a final protein concentration of 12–15 mg per ml. The gel suspension containing approximately 15 mg of calcium phosphate by dry weight per ml was washed with distilled water until the washings were neutral to brom thymol blue. The gel was then suspended in one half the original volume and a sufficient quantity added to the protein solution to give a ratio of 1:1 of gel to protein. The mixture was stirred for 10 minutes at 0–2° C and then centrifuged for 5 minutes at 400 × gravity. The supernatant was stored in the deep freeze at −20° C under which conditions it was found to be stable for at least 3 months. This preparation was also found to be free of ATPase, acetylcoenzyme A deacylase and pyrophosphatase activity, however, it still contained appreciable quantities of myokinase activity.

In Table I are summarized some typical results obtained with the above fractionation procedure.

TABLE I

ACTIVITY OF VARIOUS FRACTIONS DURING ENZYME PURIFICATION

Fraction	*Volume ml*	*Protein mg/ml*	*Activity μM/ml 30 min*	*Total activity*	*Specific activity*	*Per cent recovery*
Crude	144	22.0	32	4,600	1.5	100
1st Ammonium sulfate (30–70%)	90	15.1	45	4,100	3.0	88
Protamine sulfate	98	8.1	43	4,200	5.2	91
2nd Ammonium sulfate (70–90)	7.4	11.9	130	960	11.2	21
Calcium Phosphate Gel	8.7	6.6	109	950	16.5	20

Hydroxamic acid assay. The absolute requirement for ATP and CoA in the hydroxamic acid assay sytem is indicated in Table II. The high activity observed in the absence of any added acetate was due to the presence of acetate in the ATP preparations

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TABLE II

COMPONENTS OF THE ACETATE-ACTIVATING ENZYME SYSTEM

The complete sytem contained $MgCl_2$ 10 μM, glutathione 10 μM, CoA 0.26 μM, ATP 4.1 μM, K acetate 50 μM, hydroxylamine 400 μM, Tris (hydroxymethyl) aminomethane pH 7.4 50 μM, and enzyme 0.085 mg in a final volume of 1.0 ml. The reaction was carried out at 32° C for 30 minutes.

	Aceto-hydroxamic acid μM
Complete system	0.77
Without glutathione	0.35
Without $MgCl_2$	0.22
Without CoA	0.04
Without ATP	0.03
Without acetate	0.46

used. This enzyme system has been shown to be sensitive to acetate[9]. In Fig. 1 are shown the optimum concentration curves for the various components of the system except acetate. The requirements for high concentrations of hydroxylamine and coenzyme A are in accord with the results previously obtained with the yeast and muscle enzymes. Since the relatively high saturation requirements for hydroxylamine are attributed to the non-enzymic nature of the reaction between acetyl CoA and hydroxylamine, it would appear that at the plateau in the hydroxylamine curve the rate limiting step is the formation of acetyl CoA.

The hydroxamic acid assay has been found to be linear over a ninefold increase in enzyme concentration as shown in Fig. 2. At somewhat higher enzyme concentrations

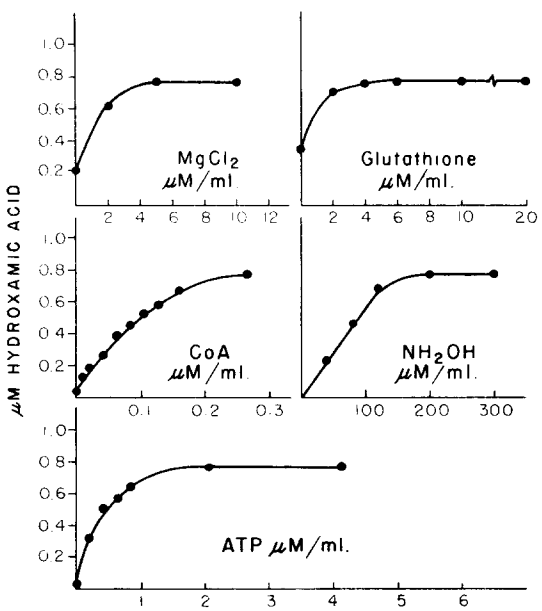

Fig. 1. The optimum concentration curves for the various components of the hydroxamic acid assay system. The standard assay is given in Table II.

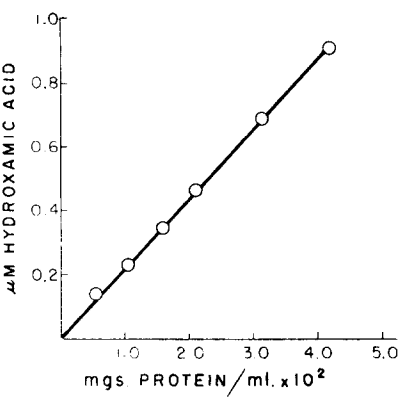

Fig. 2. Activity as a function of enzyme concentration. The reaction mixture contained $MgCl_2$ 10 μM, glutathione 10 μM, CoA 0.11 μM, ATP 3.2 μM, K acetate 50 μM, hydroxylamine 400 μM, Tris (hydroxymethyl) aminomethane pH 7.4 50 μM, in 1.0 ml. The reaction was carried out at 32° C for 30 minutes.

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the activity falls off and the assay becomes unreliable. This may be a reflection of a change in the rate-limiting step of the reaction. With higher enzyme concentrations the rate of acetyl CoA formation increases until essentially all of the CoA is in the form of acetyl CoA at all times. Under these conditions, the rate of the non-enzymic hydroxamic acid formation would be proportional to the hydroxylamine concentration, and since the concentration of the latter was kept constant, one can expect a decrease in activity beyond a certain enzyme concentration range. However, within the limited range of activity (0–1.2 μM hydroxamic acid formed in 30 minutes) the assay has proven to be reliable and quite satisfactory.

The pH-activity curve in Fig. 3 shows the pH optimum for the assay to to be about 7.5 with the activity falling off rapidly on the acid side of the pH optimum. A similar pH optimum was obtained for the yeast and plant enzyme systems[7,11].

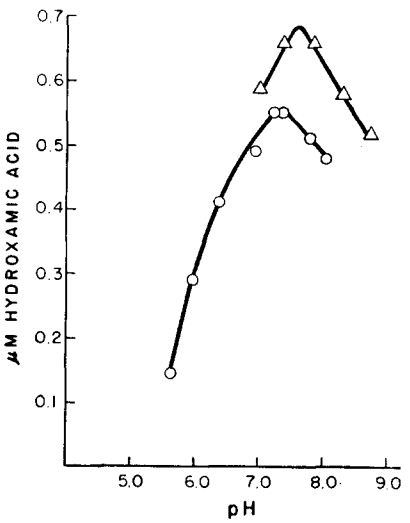

Fig. 3. pH – activity curve. The reaction mixture as in Fig. 2 except 100 μM of buffer used. The pH of the hydroxylamine was adjusted to the pH of the buffer. Enzyme concentration was 0.027 mg in a final volume of 1.0 ml. Incubated for 30 minutes at 32° C. O – phosphate buffer △ – tris (hydroxylmethyl) aminomethane buffer.

Balance study. It is shown in Table III that with catalytic amounts of CoA and with hydroxylamine as a trapping agent, one mole of AMP, P–P, and acetyl coenzyme A is formed for each mole of ATP disappearing. These results have been corrected for the ADP formed during the course of the reaction as the results of the myokinase activity. One half of the ADP formed was added to the ATP disappearing and AMP formed.

During the routine assays in which one ml of reaction mixture was used, it was frequently observed that a slight precipitate was formed during the course of the reaction. In the course of some large scale enzymic runs, this precipitate was much more pronounced. When the manganese precipitation method was utilized to further identify P–P in a sample from the large scale reaction mixture, it was found that the addition of manganese chloride did not enhance the degree of precipitation. This seemed to suggest the possibility of the precipitate being inorganic pyrophosphate. Another sample was, therefore, centrifuged and the precipitate washed with a mixture of cold Tris buffer and $MgCl_2$. The precipitate was dissolved in dilute acetic acid and analyzed for acid labile phosphate and also for pyrophosphate with yeast pyrophosphatase. The value obtained with the latter analysis was 95% of the acid labile phosphorus thus indicating the precipitate to be inorganic pyrophosphate. The chemical nature of the precipitate was not determined, but a mixture of potassium pyrophosphate, $MgCl_2$ and Tris buffer in the concentration used in the reaction mixture did give a similar precipitate suggesting possibly Mg pyrophosphate as the insoluble inorganic pyrophosphate.

Reversibility of the reaction. When acetyl coenzyme A is incubated with AMP and P–P in the presence of the enzyme, there is a decline in the hydroxamic acid formed as shown in Table IV. There is no decline in the absence of pyrophosphate and a small decline in the absence of AMP. The latter result would seem to indicate that AMP

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TABLE III

BALANCE STUDY OF THE ACETATE-ACTIVATING REACTION

The reaction mixture contained $MgCl_2$ 40 μM, glutathione 40 μM, Coenzyme A 0.9 μM, ATP 22.3 μM, hydroxylamine 1600 μM, K acetate 100 μM, Tris (hydroxymethyl) aminomethane buffer pH 7.4 200 μM, and enzyme 0.438 mg in a total volume of 3 ml. The reaction was carried out at 32 °C for one hour. The results are expressed in micromoles.

*ATP**	*ATP corr. f. ADP*	*AMP**	*AMP corr. f. ADP*	*Inorganic* P-P*	*Aceto hydroxamic acid*
—7.1	—6.1	+4.9	+6.0	+6.2	+7.0

* Corrected for the blank without added acetate.

TABLE IV

THE REVERSIBILITY OF THE ACETATE-ACTIVATING REACTION

The complete system contained $MgCl_2$ 10 μM, adenosine 5′ phosphate 9.6 μM, potassium pyrophosphate 100 μM, Tris (hydroxymethyl) aminomethane buffer pH 7.4 50 μM, acetyl coenzyme A 1.74 μM, and enzyme 0.236 mg in a final volume of 1 ml. Reaction mixture incubated at 32° C for one hour. 400 μM neutral hydroxylamine added and after 10 minutes acethohydroxamic acid determined.

	Acetohydroxamic acid μM
acetyl CoA	1.74
acetyl CoA +AMP	1.74
acetyl CoA +P–P	1.47
acetyl CoA +AMP —P–P	0.96

was an impurity in the acetyl CoA preparation. When a sample of CoA was chromatographed according to the COHN AND CARTER procedure[17], it was found that AMP was not a contaminant. However, a small quantity of a nucleotide, which was not further identified, was removed from the column with the eluant for ADP. If the ADP was the contaminant present in the CoA, then in the presence of myokinase one could expect some reversal in the presence of high concentrations of pyrophosphate.

Specificity. The acetate-activating enzyme is active only with acetate and proprionate, the latter showing about 90% of the activity of the former. Butyrate, caproate and octanoate are activated by the crude enzyme preparation but are not with the purified extracts. The crude extracts also contain most of the enzymes for the oxidation of the higher fatty acids*. Since the rate limiting step in the hydroxamic acid assay is the formation of acetyl coenzyme A, the above results indicate the true specificity of the enzyme.

Inhibition. The end products of the acetate-activating reaction were found to be inhibitory to the system. In Fig. 4 is shown the inhibitory activity of AMP and ADP; the latter being about one third as effective as AMP. P–P was also found to be about one third to one half as inhibitory as AMP. The inhibitory action of ADP may be more of a reflection of the myokinase activity than of any action of ADP *per se*. Adenylic-3′-phosphate was almost completely inactive over the same concentration range as AMP.

* Personal communication from Dr. J. STERN.

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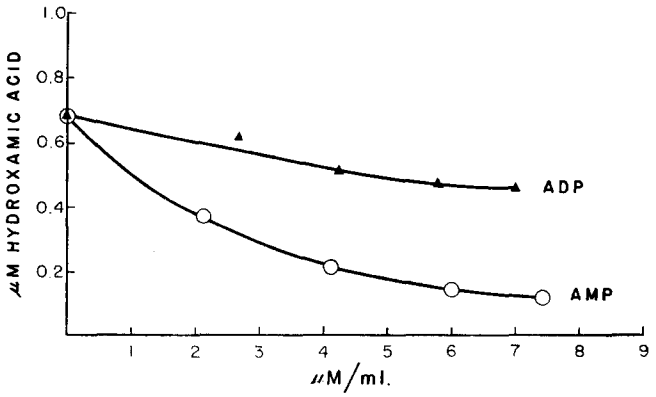

Fig. 4. Inhibition of the acetate-activating enzyme by ADP and AMP. The reaction mixture as in Fig. 2 except CoA 0.27 μM and ATP 2.3 μM. Enzyme concentration was 0.174 mg in a final volume of 1.0 ml. Reaction mixture incubated at 32° C for 30 minutes. O AMP. ▲ ADP.

DISCUSSION

The purified acetate-activating enzyme of *Rhodospirillum rubrum* appears in the absence of high salt concentrations to be a more stable preparation than that of yeast or heart muscle. In addition this preparation is free of pyrophosphatse activity. The presence of the enzyme myokinase still introduces a number of difficulties in many of the assays and work is now in progress to rid the purified preparation of this impurity. Many of the properties of the enzyme described are similar to those found for the yeast, heart muscle and plant enzymes. However, the question of whether the mechanism is the same for this enzyme as for the yeast enzyme must await further investigation.

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SUMMARY

A relatively stable purified enzyme preparation has been obtained from *Rhodospirillum rubrum* which catalyzes the formation of acetyl coenzyme A form ATP, acetate and CoA.

AMP and pyrophosphate were shown to be end products of the reaction and also inhibitors of the reaction.

The properties of the enzyme system have been described and found to be similar to those described for the yeast, heart muscle and plant enzymes.

RÉSUMÉ

Une préparation enzymatique purifiée relativement stable, qui catalyse la formation d'acétyl coenzyme A à partir d'ATP, d'acétate et de CoA a été extraite de *Rhodospirillum rubrum*. L'AMP et le pyrophosphate sont des produits finaux et également des inhibiteurs de la réaction.

Les propriétés de ce système enzymatique sont décrites et sont semblables à celles des enzymes de la levure, du muscle cardiaque et des végétaux.

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ZUSAMMENFASSUNG

Von *Rhodospirillium rubrum* wurde eine relativ beständige Enzyme-Präparation erhalten, die die Bildung von Acetylcoenzym A aus ATP, Acetat und CoA katalysiert.

Es wurde gezeigt, dass AMP und Pyrophosphat Endprodukte und Hemmstoffe der Reaktion sind.

Die Eigenschaften des Enzymsystems wurden beschreiben und es wurde gefunden, dass sie den für Hefe-, Herzmuskel-und Pflanzenenzymen beschriebenen ähnlich sind.

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