ACTA CHEMICA SCANDINAVICA 17 (1963) S 123–S 128

Purification and Charaterization of Succinyl CoA Synthetase from *Rhodopseudomonas spheroides**

BRUCE F. BURNHAM

Charles F. Kettering Research Laboratory, Yellow Springs, Ohio, USA

Succinyl CoA synthetase has been purified 200 fold from the photosynthetic bacterium *Rhodopseudomonas spheroides*. Some of the properties of the purified enzyme are described. The relative ease of preparation of this enzyme and the high activity obtained suggest that succinyl CoA synthetase may have some value in coupled enzyme reactions as a generator of succinyl CoA.

The reversible, substrate level phosphorylation of nucleoside diphosphate is catalyzed by the enzyme succinyl CoA synthetase as shown in eqn. 1.**

$$\text{SUCCINATE} + \text{NTP} + \text{CoA} \longleftrightarrow \text{SUCCINYL} - \text{CoA} + \text{NDP} + \text{PO}_4 \quad (1)$$

The enzyme catalyzing this reaction has been called the phosphorylating enzyme (P enzyme)[1], succinic thiokinase[2], and succinyl CoA synthetase[3].

Previous work on this enzyme has centered largely on the cofactor requirements, and upon the mechanism of the phosphorylation reaction. Sanadi *et al.*[4] demonstrated that the enzyme from mammalian sources required either GTP or ITP for activity and that ATP could not replace either of these nucleotides. Kaufman and Alivisatos[5], however, found that the enzyme from spinach was specific for ATP. Kaufman[6], Hager[7], Gunsalus and Smith[8], and Mazumder *et al.*[2] have examined the mechanism of the reaction but have not yet shown definitive evidence for only one of the several postulated reaction mechanisms.

The present paper describes a simple procedure for the purification of this enzyme from *Rhodopseudomonas spheroides*, which is a fairly rich source of the enzyme. The specific activity of the crude bacterial extract is 0.18 μmoles/min/mg protein compared to 7.8×10^{-3} μmoles/min/mg protein in extracts of spinach.

---

* Contribution No. 106 of the Charles F. Kettering Research Laboratory.

** Abbreviations used: NTP, nucleoside triphosphate; ATP, Adenosine triphosphate; GTP, Guanosine triphosphate; ITP, Inosine triphosphate; UTP, Uridine triphosphate; CTP, Cytosine triphosphate; CoA, Coenzyme A; DEAE, Diethylaminoethylcellulose.

The enzyme from *R. spheroides* is relatively stable both in the crude extracts and in its most highly purified form. Because of the relative abundance of the enzyme, its stability, and its ease of preparation, it offers some promise as a method for producing succinyl CoA in reaction systems requiring this compound as a substrate. In studies on the enzyme δ-aminolevulinic acid synthetase, which requires succinyl CoA and glycine as substrates, Burnham and Lascelles[9] used succinyl CoA synthetase to generate succinyl CoA in the ALA synthetase assay mixture using catalytic amounts of CoA. Rowbury also used succinyl CoA synthetase as a generator of succinyl CoA in his studies on cystathionine synthesis by *E. coli* extracts (R. J. Rowbury personal communication).

EXPERIMENTAL

Chemicals: Nucleoside di and tri phosphates, Coenzyme A, and DEAE cellulose were purchased from Sigma Chemical Company, St. Louis Missouri. The DEAE cellulose was treated as described by Peterson and Sober[10] before use. Calcium phosphate gel was prepared according to the method of Keilin and Hartree[11].

Organism: Rhodopseudomonas spheroides was obtained from June Lascelles (Oxford). The organism was cultured anaerobically in the light on medium S as described by Lacelles[12]. After growth for 24 hours, the cells were harvested by centrifugation. They were resuspended in distilled water and stored at − 20°C until used.

Determinations: Enzyme activity was determined by incubating the enzyme for 30 min at 37°C in the following mixture: (μmoles) succinate 100, $MgCl_2$ 10, ATP 4, CoA 0.27, tris buffer (pH 7.4) 50, NH_2OH (freshly adjusted to pH 7.4) 200, enzyme and water to 1.0 ml. The incubation was terminated by adding 1 ml of $FeCl_3$ reagent[13]. The ferric hydroxamine acid was determined by measuring the optical density at 540 mμ. Succinic hydroxamate formation was proportional to enzyme concentration only at very low enzyme levels, *e. g.*, only at concentrations that produced 40 mμmoles of succinic hydroxamate/min or less. Protein was determined by the method of Lowery[14] except following DEAE chromatography. The method of Warburg and Christian[15] was used on fractions from the DEAE column.

Purification of enzyme: Three hundred eighty ml of resuspended cells (100 mg dry wt cells/ml) were thawed and allowed to remain at 2°C for 24 hrs. This suspension was then passed once through a French pressure cell (American Instrument Co., Silver Spring, Md.). Distilled water was added to the disrupted cells to bring the volume up to the equivalent of 50 mg dry wt cells/ml. This viscous preparation was sonicated in 50 ml batches for 1 to 2 min to denature the nucleic acid. The pooled crude cell extract was carefully adjusted to pH 6.2 with molar acetic acid and was then centrifuged at 34 000 × g for 30 min in a Servall Model RC-2 refrigerated centrifuge at 2°C. The total volume of crude supernatant was 670 ml with a protein concentration of 22.4 mg/ml.

Step 1. Protamine sulfate treatment: Freshly prepared 2 % protamine sulfate (maintained at room temperature) was slowly added with thorough mixing to the crude enzyme supernatant. At intervals, (after adding 60 ml samples) the precipitate was removed by centrifugation. Addition of protamine sulfate was continued until the supernatant was clear and free of all pigmented material except cytochrome *c*. Two hundred ml of 2 % protamine sulfate were required for this step. The volume following the final protamine sulfate addition and centrifugation was 730 ml, protein concentration 5 mg/ml. This solution of enzyme was dialyzed with stirring against 4 l of distilled water for 2 hrs, and then overnight against 4 l of 0.015 M PO_4-succinate buffer pH 6.2. The precipitate which formed during dialysis was removed by centrifugation; final volume 750 ml, protein 4.2 mg/ml.

Step 2. Calcium phosphate gel adsorption: Calcium phosphate gel, 27 mg dry wt/ml, was added batchwise to the dialyzed enzyme preparation in the following amounts: 400 mg, 400 mg, 1 350 mg, 675 mg. After each addition the gel was equilibrated 10 min and removed by centrifugation. The first two additions of gel did not result in a significant decrease in the enzyme in the supernatant. The bulk of the enzyme was removed by the third addition of gel. This gel fraction, containing about 90 % of the total enzyme was washed two times with distilled water, and was then equilibrated for 15 min with the following series of eluents: (1) 20 ml 0.015 M PO_4 − succinate buffer pH 6.2, (2) 20 ml 0.03 M PO_4 − succinate buffer

Table 1. Summary of purification.

Step	Volume ml	Protein mg/ml	Specific activity μmole/min/mg protein	Total activity
Uncentrifuged sonicate	720	34	0.180	4 500
Centrifuged sonicate	670	22.4	0.28	4 250
Protamine sulfate treated extract	730	5	1.0	3 750
Combined active $CaPO_4$ gel eluents	73	5.5	3.75	1 490
Off DEAE column	27	0.45	36.2	1 570
Off DEAE column	126	ca 0.4	16.5	

pH 6.2, (3) 17 ml 0.03 M PO_4 – succinate pH 6.2 + 2 ml M KCl + 1 ml substrate mixture (see methods), (4) repeat of number 3, (5) through (10) – successive 15 ml aliquots of 0.2 M PO_4 buffer pH 6.8. The gel was resuspended in the eluting buffers by 15 seconds sonication with a probe sonicator. Elution was continued as long as enzyme continued to be desorbed. Those fractions with a specific activity above 3 μmoles/min/mg protein were pooled and dialyzed against 3 changes of 0.01 M tris buffer pH 7.4 for 24 hrs. The precipitate which formed upon dialysis was removed by centrifugation.

Step 3. DEAE Chromatography: A 2 × 16 cm DEAE column was prepared from washed and sized DEAE, equilibrated with 0.01 M tris buffer pH 7.4. The clear pale pink supernatant, (86 ml, protein concentration 3.1 mg/ml) was placed on the column which was then washed with 100 ml of additional 0.01 M tris buffer pH 7.4. The enzyme was eluted from the column with a linear gradient of KCl by increasing the concentration from zero to 0.25 M. The enzyme appeared as a broad band when the KCl concentration reached 0.14 M. All fractions with a specific activity of over 16 μmoles/min/mg were frozen and stored at –20°C for use in the studies on enzyme properties described below. The purification procedure is summarized in Table 1.

RESULTS

Requirements for activity: The enzyme was incubated with the standard assay mixture in a series where single components were omitted. All components of

Table 2. Requirements for enzyme activity. Enzyme was incubated under standard conditions in a series of reactions where a single component was omitted.

Compound omitted	Succinic hydroxamate formed μmoles/min/mg protein
None	36.2
Succinate	0
CoA	0
ATP	0
NH_2OH	0
Mg	0
Minus Mg plus Mn*	27.4
Minus Mg plus Zn**	0

* $MnSO_4$ – Concentration in reaction 5 μmoles/ml.
** $ZnSO_4$ – Concentration in reaction 10 μmoles/ml.

Table 3. Substrate specificity. Succinyl COA synthetase was incubated with 0.1 M acids as shown with the standard assay system.

Substrate	Hydroxamic acid μmoles/min/mg protein
Succinate	33
Malonate	0
Acetate	0
Fumarate	Trace
DL-Malate	0
L-Aspartate	0

the reaction mixture were necessary for activity (Table 2). Manganese, but not zinc could replace magnesium.

Substrate specificity: The enzyme was tested for activity with a number of acids similar in structure to succinate. These compounds were tested (100 μmoles/ml) by substituting them for succinate in the standard assay mixture and incubating them with 4 times the usual amount of enzyme. Succinate was the only compound tested that showed measurable activity (Table 3). The trace of activity observed with fumarate, too low to measure accurately, could very likely have been due to a slight contamination with succinate.

Cofactor requirements: The nucleoside triphosphate requirement of succinyl CoA synthetase was examined using a modification of the usual assay mixture. The results (Table 4) showed that the purine nucleotides were all active, while the two pyrimidine compounds were not active. The activity of the nucleotides was measured at a series of concentrations, so that activity due to contamination

Table 4. Nucleotide specificity. Succinyl CoA synthetase was incubated in standard assay system with the various nucleotides indicated.

Nucleotide	Concentration μmoles/ml	Activity μmoles/min/mg protein	Relative activity
ATP	4	19	100
GTP	4	16	84
UTP	4	0	
ITP	4	14.5	76
CTP	4	0	
ADP	4	0	
ATP	1	11.2	100
GTP	1	10	89
ITP	1	8	72

Table 5. Stability at 37°C at various pH values. Enzyme with a specific activity of 20 μmoles/min/mg protein was added to 0.2 M imidazole buffer at the pH values shown. Volume of each sample = 0.4 ml, protein concentration 0.114 mg/ml. Incubation was at 37°C and at the times shown 0.02 ml samples were withdrawn for activity determination.

pH of incubation	Specific activity μmoles/min/mg protein 0 hrs incubation	4 hrs incubation	7 hrs incubation
6.2	20	14.4	10.6
6.6	20	14.8	14.3
7.0	20	18	18
7.4	20	16.8	11.7
7.8	20	12.7	9.5

by one of the other nucleotides would be apparent. In the case of all of the active compounds, the ratios of activity at saturating concentrations of nucleotide were close to the ratios at concentrations less than K_m (Table 6). The activity of the 3 purine nucleotides was also examined at two stages during purification. The relative rates following the calcium phosphate gel step were: ATP 100, GTP 71, and ITP 76. These values compare favorably with those obtained using enzyme that had been chromatographed on DEAE cellulose (Table 4). Succinyl CoA synthetase from *R. spheroides* is apparently less specific in its nucleotide requirement than succinyl CoA synthetase from spinach[5] or pig liver[2]. The nucleotide specificity of succinyl CoA synthetase from *E. coli* has not been reported[3].

The comparison of relative activities of the different nucleotides at two stages in purification, indicates that the activity of the nucleotides is not due to the presence of a nucleotide transphosphorylase.

pH Stability: The stability of the enzyme was examined following DEAE chromatography. For this purpose, the enzyme solution was diluted to a con-

Table 6. Michaelis constants. Activity was measured in the standard assay system while the concentration of a single component was varied. K_m values were calculated using the double reciprocal plot of Lineweaver and Burk[16].

Substrate	K_m moles/l
Succinate	1.9×10^{-3}
CoA	0.4×10^{-4}
ATP	1.4×10^{-3}
Mg++	6.4×10^{-3}

centration of 0.114 mg/ml in 0.2 M imidazole buffer at several pH values between 6.2 and 7.8. The tubes containing the diluted enzyme were incubated at 37°C. At intervals, samples were removed and tested for activity. As shown in Table 5 the enzyme is comparatively stable for several hours under these conditions.

Determination of Michaelis constants: In measurements of activity for the purpose of K_m determination, standard assay concentrations were used with the exception of the compound being tested. The values of K_m were obtained using the double reciprocal plot of Lineweaver and Burk[16]. Straight lines were obtained in all cases except with Mg^{++}. The skewed curve observed at high Mg^{++} (2×10^{-2} M) concentrations suggested substrate inhibition. The value given in Table 6 for Mg^{++} was obtained by extrapolation of the "best straight line" obtained at low Mg^{++} concentrations. Half maximum activity was observed with 2×10^{-3} M Mg^{++}.

REFERENCES

1. Kaufman, S., Gilvarg, C., Cori, O. and Ochoa, S. *J. Biol. Chem.* **203** (1953) 869.
2. Mazumder, R., Sanadi, D. R. and Rodwell, V. W. *J. Biol. Chem.* **235** (1960) 2546.
3. Hager, L. P. In Boyer, P. D., Lardy, H. and Myrbäck, K. *The Enzymes*, 2nd Ed., Academic Press, New York, 1962.
4. Sanadi, D. R., Gibson, D. M., Ayengar, P. and Jacob, M. *J. Biol. Chem.* **218** (1956) 505.
5. Kaufman, S. and Alivisatos, S. G. A. *J. Biol. Chem.* **216** (1955) 141.
6. Kaufman, S. *J. Biol. Chem.* **216** (1955) 153.
7. Hager, L. P. *J. Am. Chem. Soc.* **79** (1957) 4864.
8. Gunsalus, I. C. and Smith, R. A. *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957.* Academic Press, New York, p. 77.
9. Burnham, B. F. and Lascelles, J. *Biochem. J.* **87** (1963). *In press.*
10. Peterson, E. A. and Sober, H. A. In Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology.* Academic Press, New York, 1962, Vol. V, p. 3.
11. Keilin, D. and Hartree, E. F. *Proc. Roy. Soc., London* **B 124** (1938) 397.
12. Lascelles, J. *Biochem. J.* **62** (1956) 78.
13. Lipmann, F. and Tuttle, L. C. *J. Biol. Chem.* **159** (1945) 21.
14. Lowry, O. H., Rosenbrough, N. J., Farr, R. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
15. Warburg, O. and Christian, W. *Biochem. Z.* **310** (1941) 384.
16. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.

Received April 2, 1963.