STUDIES ON THE REACTION MECHANISM OF SOYBEAN CALLUS SUCCINYL CoA SYNTHETASE. PHOSPHORYLATION OF THE ENZYME ON IMMOBILIZED ADENOSINE-TRIPHOSPHATE AND ENZYME ATTACHED TO A SOLID SUPPORT

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1. Introduction

The use of solid supports in biochemistry has received much attention recently and many specific applications have been described [1–2].

This report describes the use of insolubilized ATP and water-insoluble succinyl CoA synthetase as a means of obtaining the phosphoryl enzyme, which is considered to be an obligatory intermediate in the reaction catalyzed by this enzyme, and we also show other useful applications of biospecific adsorbents.

The mechanism of succinyl CoA synthetase (succinate:CoA ligase; ADP, EC 6.2.1.5) has been actively investigated in many laboratories [3–15]. A number of authors [5, 6, 9, 12, 16, 17] have provided evidence for a phosphorylated form of the enzyme. Although there are many uncertainties regarding the over-all mechanism, there is agreement on the participation of the phospho-enzyme in the reaction, which may be formulated as follows:

$$E + ATP \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} E{-}P + ADP \quad (1)$$

$$E{-}P + Succ + CoA \overset{Mg^{2+}}{\rightleftharpoons} E + P_i + SuccCoA \quad (2)$$

$$ATP + Succ + CoA \rightleftharpoons ADP + P_i + SuccCoA \quad (3)$$

By using highly purified soybean callus succinyl CoA synthetase [18] and a new methodology we have studied in detail reactions 1 and 2. In the present investigation, besides obtaining direct evidence for reaction 1, the phosphorylated enzyme was prepared by reaction of immobilized succinyl CoA synthetase with ATP, providing a very stable form of the phospho-enzyme.

Extensive information about reaction 2 has also been obtained, and will shortly be reported [19].

2. Materials and methods

CoA and ATP were obtained from Sigma Chem. Co; cyanogen bromide from Fluka AG; Sepharose 4B from Pharmacia, Uppsala. All other reagents were AR grade from several commercial sources.

Succinyl CoA synthetase was purified and activity was measured as already described [18].

Protein was measured by the method of Lowry et al. [20].

Protein-bound phosphate was determined by adaptation of phenol extract procedure [6] and labile phosphate by the method of Fiske and Subbarow [21]. The molecular weight of the enzyme was assumed to be 160,000 [18]. ATP and enzyme were coupled directly to Sepharose 4B by the cyanogen bromide technique of Cuatrecasas [22]. The activated Sepharose was suspended in cold 0.05 M Tris-HCl buffer, pH 8, in a volume equal to that of the original suspension of Sepharose, and ATP or succinyl CoA synthetase was quickly added in solution (0.2–0.4 ml/ml Sepharose); the mixture was stirred gently at 4° for different times, after which the substituted Sepharose was packed into a column and extensively washed with a large excess of 0.05 M Tris-HCl buffer, pH 8, until negligible amounts of ATP or

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Table 1
Conditions for coupling succinyl CoA synthetase to Sepharose.

Expt.	CNBr/ml Sepharose (mg)	Time of coupling (hr)	Enzyme/ml Sepharose Added (mg)	Enzyme/ml Sepharose Coupled (mg)
1	100	20	1.10	0.2
2	100	40	1.22	0.8
3	200	40	3.70	1.6
4	200	48	1.43	0.7
5	200	64	1.56	1.2

Experimental conditions are described in the text.

protein emerged in the effluent. The quantity of ATP or protein coupled to Sepharose was determined by measuring the amount of ATP or protein added to the activated Sepharose and the total amount of each, present in the washings. These preparations can be stored for long periods at 4° without alteration.

3. Results and discussion

3.1. *Phosphorylation of succinyl CoA synthetase bound to Sepharose by ATP and properties of the Sepharose–enzyme–phosphate complex.*

Different preparations of purified succinyl CoA synthetase were coupled to Sepharose (table 1); it was found that the protein could be coupled to Sepharose with high efficiency, in addition, a general increase in stability of the enzyme attached to Sepharose was observed (Wider, Mendiara and Batlle, unpublished results).

The succinyl CoA synthetase bound to Sepharose was phosphorylated with ATP and Mg^{2+} ions as follows: an amount of Sepharose–enzyme (Seph–E) suspended in 0.05 M Tris-HCl buffer, pH 8, was incubated with ATP (20 μmoles/ml) and Mg^{2+} (250 μmoles/ml) for 5 min at 4°, then the whole suspension was packed into a column (0.7 cm × 5.0 cm); the excess ATP and Mg^{2+} were washed with buffer; a column of packed Sepharose–enzyme–phosphate (Seph–E–P) was thus obtained; alternatively washing the excess ATP and Mg^{2+} was carried out in a batch procedure and the Seph–E–P complex kept in suspension at 4°.

Table 2
Utilization of the Sepharose–enzyme–phosphate complex for the synthesis of succinyl CoA.

Reaction mixtures	Succinyl CoA formed (nmoles)
1. Seph–E	0
2. Seph–E + ATP	785
3. Seph–E–P	375
4. Seph–E–P + ATP	806
5. Seph–E*	196
6. Seph–E* + ATP	788

The reaction mixtures contained 1 ml of Seph–E (15 nmoles enzyme/ml Seph) or 1 ml of Seph–E–P (15 nmoles E/ml Seph; 1.2 nmoles bound P/nmole E) in 0.05 M Tris-HCl buffer pH 8.0; $MgCl_2$ (10 μmoles), glutathione (10 μmoles) CoA (0.37 μmoles), Na succinate (100 μmoles) and ATP (10 μmoles) as indicated in the table, in a final volume of 2 ml. After incubation for 30 min at 37° with vigorous shaking, the reaction mixtures were chilled to 0° and treated as described in the text; the resulting Seph–E* obtained after incubation was reincubated under the same conditions with the reagents indicated. The Seph–E was obtained by coupling succinyl CoA synthetase to Sepharose, then the enzyme attached to the gel was phosphorylated with ATP^{3-} and Mg^{2+} as described in the text; protein bound to Sepharose was determined as described in Methods and the amount of phosphorus bound to enzyme was estimated by already reported procedures [6, 21].

It was important to confirm whether the Seph–E–P complex was indeed formed and if it was a reactive intermediate in the succinyl CoA synthetase reaction; to test this, the Seph–E–P complex, suspended in buffer, was then incubated with and without ATP, with all the remaining components of the reaction mixture, and the succinyl CoA formed was measured in the usual way [18]. Vigorous shaking during all the period of incubation was necessary, after which the reaction mixture was chilled to 0° and immediately centrifuged at 12,000 *g* for 10 min. Succinyl CoA was measured in the supernatant while the sedimented Sepharose–enzyme complex was washed 3 times with 2 vol of 0.05 M Tris-HCl buffer, pH 8.0. This material was designated Seph–E*. Control experiments (table 2) were carried out incubating Seph–E or Seph–E* with and without ATP.

As shown in table 2, succinyl CoA was formed with Seph–E only in the presence of ATP; with

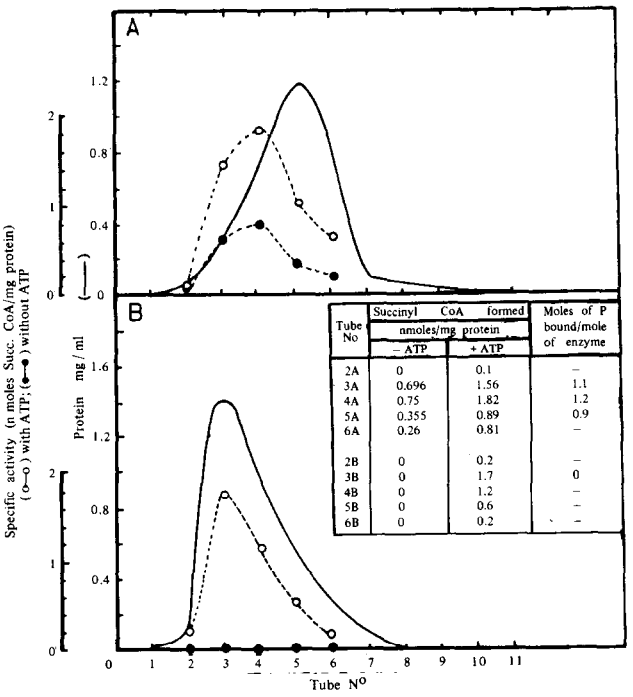
Seph–E–P, succinyl CoA was formed both in the absence and presence of ATP and finally, the fact that Seph–E* in the presence of ATP formed approximately the same amount of succinyl CoA as the Seph–E and Seph–E–P, shows that the enzyme attached to Sepharose is very stable and is suitable for repeated use; in addition, it is also seen that Seph–E* in the absence of ATP still formed some succinyl CoA so, under these conditions, the release of bound phosphate was not complete. These findings clearly indicate that the enzyme–phosphate is a reactive intermediate in the reaction, and that this complex, in the presence of succinate and CoA, formed succinyl CoA with the concomitant liberation of phosphorus.

3.2. *Phosphorylation of succinyl CoA synthetase by Sepharose-ATP*

ATP was coupled to Sepharose with good efficiency (about 70%) and the amount of bound nucleotide was in the range of 30–35 μmoles ATP/ml of Sepharose. The precise mode of binding of ATP to the matrix is not yet definitely established. It has been suggested that it is the 6-amino group of the adenine moiety of NAD which is bound to cellulose when the coenzyme is linked to the solid support [23]; it was assumed that the same group in ATP could be involved in its binding to Sepharose, although there are discrepancies about this [24]. However, since the succinyl CoA synthetase is phosphorylated by the terminal phosphoryl group of ATP, interaction between the enzyme and the immobilized ATP was to be expected, although the efficiency of the process could also depend on the distance between the reactive group and the matrix backbone.

The chromatographic behaviour of succinyl CoA synthetase on a Sepharose-ATP column and on an unmodified Sepharose column are illustrated in fig. 1. The elution of succinyl CoA synthetase on a Sepharose-ATP column (fig. 1A) was slightly retarded compared to its behaviour on unmodified Sepharose (fig. 1B). Each eluate containing protein was assayed both for activity of succinyl CoA synthetase with and without ATP and for bound phosphate.

As was expected, succinyl CoA synthetase was phosphorylated on the column of Sepharose-ATP; the resulting enzyme–phosphate complex was a reactive intermediate in the reaction (inset fig. 1) as succinyl CoA was formed both in the absence and presence of



Tube No	Succinyl CoA formed nmoles/mg protein − ATP	+ ATP	Moles of P bound/mole of enzyme
2A	0	0.1	–
3A	0.696	1.56	1.1
4A	0.75	1.82	1.2
5A	0.355	0.89	0.9
6A	0.26	0.81	–
2B	0	0.2	–
3B	0	1.7	0
4B	0	1.2	–
5B	0	0.6	–
6B	0	0.2	–

Fig. 1. Chromatography of succinyl CoA synthetase (A) on a column (0.7 cm × 10 cm) of Sepharose-ATP (35 μmoles ATP/ml Sepharose) and (B) on a column (0.7 cm × 10 cm) of unmodified Sepharose. The protein (8 mg) and 250 μmoles Mg^{2+} were dissolved in 1 ml of 0.05 M Tris-HCl buffer pH 8.0, and applied to the column at 6–8°. Elution was carried out with the same buffer. Fractions of 2 ml were collected at a flow rate of 0.4 ml/min. In each fraction containing protein, enzymic activity was measured as described [18]; reaction mixtures contained, enzyme or enzyme–phosphate (1 ml), $MgCl_2$ (10 μmoles), succinate (100 μmoles), glutathione (10 μmoles), CoA (0.25 μmoles) and, where added, ATP (10 μmoles) in a final volume of 2 ml. Phosphate bound to protein was also determined [21].

ATP. It was also found that protein was completely recovered from the columns and that the specific activity of the enzyme chromatographed on the Sepharose-ATP column was slightly higher than that of the enzyme chromatographed on unmodified Sepharose.

As judged by the phosphate bound to the enzyme and the amount of total phosphate remaining in the column, it has been observed (Wider, Mediara and Batlle, unpublished results) that the formation of the enzyme–phosphate in the column is quantitative and normally no ATP is wasted. The gel-bound ATP column, which is remarkably stable, can be used more than once to obtain the complex, depending on the amount of ATP bound to Sepharose.

Since ATP can be directly coupled to Sepharose with high yield, the enzyme–phosphate complex of those enzymes which are phosphorylated by ATP in a similar fashion to succinyl CoA synthetase, may well also be efficiently prepared using immobilized ATP.

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