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PORPHYRIN BIOSYNTHESIS: IMMOBILIZED ENZYMES AND LIGANDS

VI. STUDIES ON SUCCINYL CoA SYNTHETASE FROM CULTURED SOYA BEAN CELLS

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Summary

Soybean callus succinyl CoA synthetase (succinate : CoA ligase, (ADP-forming), EC 6.2.1.5), has been chemically bound to Sepharose 4B and some of its properties have been studied. The optimal conditions for binding have been determined. The immobilized enzyme retained 48% of the activity of the soluble enzyme and the coupling yield amounted to 50%. Sepharose · succinyl CoA synthetase can be stored at 4°C for periods up to 90 days with only 25% loss of activity; it can also be repeatedly used without alteration of its enzymic activity.

The complex showed enhanced thermal stability; pH optimum was between 7.0 and 8.0 for the bound enzyme, and 8.0 for the free enzyme. A general decrease in the Michaelis-Menten constants for the different substrates of the insoluble enzyme, as compared with values obtained for the free enzyme, was found. Plots of the rate product formation against ATP concentration changed from sigmoideal for the soluble succinyl CoA synthetase to hyperbolic for the immobilized enzyme.

Introduction

For some time we have been interested in the study of reaction mechanisms and in the use of immobilized enzymes and ligands of components of the tetrapyrrole pathway [1—5] and we reported the application of an enzyme · gel to the investigation of the mechanism of action of succinyl CoA synthetase [6—8].

Succinyl CoA synthetase (succinate : CoA ligase, (ADP-forming), EC 2.6.1.5), catalyses the formation of succinyl CoA from succinate, a nucleotide triphosphate and CoA; this enzyme is involved in the biosynthesis of δ-amino-

laevulinic acid, an early precursor of porphyrins, and it has been extensively studied in our laboratory [6—12].

Studies of such polymer-bound enzymes, showed that immobilization may alter both the chemical and physical properties of the enzyme, besides simply restricting its physical movement. We report here results of studies on a succinyl CoA synthetase · sepharose complex.

Experimental

All reagents used were those described in ref. 6. Source material of enzyme, succinyl CoA synthetase purification and activity measurement were as already reported [10]. Sepharose 4B activation, enzyme coupling and determination of activity of the coupled enzyme, followed procedures detailed in ref. 6. All other methods not specified here were those described in refs. 6 and 10.

Results and discussion

Chemical coupling. Preliminary experiments were performed to determine the different variables, controlling the coupling yield and remaining activity of the enzyme bound to agarose. It was found that optimal conditions for coupling were obtained by using 100 mg of CNBr/ml of packed Sepharose; the temperature should be kept throughout below 6°C, the pH above 8.0 and a yield of 50% could be obtained using 3—4 mg of added protein/ml activated Sepharose, after 48 h coupling with a low but constant stirring [6]. Under these conditions, the activity retained in the gel · enzyme complex was the highest, reaching average values of 48%. Total enzyme activity decreased towards 50% within the first 20 h of coupling, while the amount of bound protein rose from 0 to 20%. Subsequently, binding increased up to 75% after 64 h, but activity decreased to 32% in the same interval, probably due to inactivation of the free enzyme during the binding process and gradual depletion of suitable coupling sites. This was shown by keeping a sample of soluble succinyl CoA synthetase and unreacted Sepharose under the same mechanical conditions as those of coupling, and taking an aliquot for activity measurement at different time intervals. It was thus observed, that, even under the mild conditions of binding, some 30% inactivation of the soluble enzyme occured.

Stability of water-insoluble soybean callus succinyl CoA synthetase. A number of new properties may be imposed on the insoluble enzyme by the chemical nature of the artificial matrix.

Storage stability. For bound succinyl CoA synthetase suspended in 0.05 M Tris · HCl buffer (pH 8.0) this was investigated by assaying activity over a period of 90 days (Table I). It was observed that they could be stored at 4°C for 3 months, with only 25% decrease in activity.

Repeated use. The repeated use of the same preparation of sepharose succinyl CoA synthetase was also studied, and found that it could be used up to 10 times without alteration of activity.

Thermal stability. Activity of both soluble and insoluble succinyl CoA synthetase was measured over the temperature range 15—60°C, typical results are shown in Fig. 1A. As expected [13] some improved thermal stability of the

TABLE I

STORAGE STABILITY OF IMMOBILIZED SOYBEAN CALLUS SUCCINYL CoA SYNTHETASE

Sepharose-succinyl CoA synthetase was stored at 4°C. Samples were taken at the days indicated and assayed for enzymic activity. *t* represents the specific activity measured at the time shown. Remaining activity is also expressed as a percentage of the original activity.

Time (days)	Specific activity	
	t	remaining (%)
0	0.436	100
7	0.433	99
15	0.425	97
20	0.415	95
30	0.397	91
60	0.392	88
90	0.326	74

insoluble enzyme was observed and the maximal rate for both free and bound succinyl CoA synthetase was found around 37°C. The activation energy values, calculated according to the Arrhenius equation, were 1865 cal/mol for the

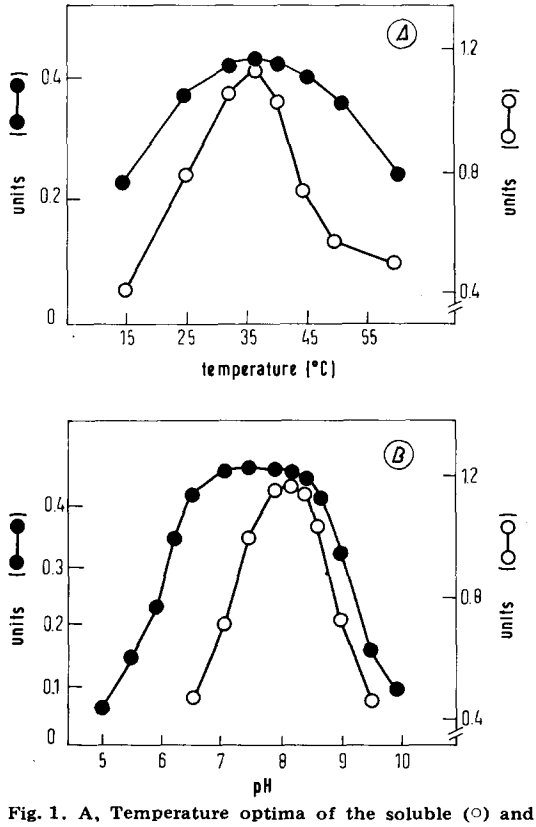

Fig. 1. A, Temperature optima of the soluble (○) and insoluble (●) succinyl CoA synthetase. Activity measurements were performed at the temperatures indicated. Further details of the assay methods are given in refs. 6 and 10. B, pH optima of soluble (○) and insoluble (●) enzyme. Activities were measured under standard incubation conditions (6, 10) at the pH values indicated.

bound enzyme and 5620 cal/mol for the free enzyme.

pH optimum. pH optima of free and bound succinyl CoA synthetase were determined by equilibrating the samples with phosphate and Tris · HCl buffers over the pH range 6.0—9.0 (Fig. 1B). The profiles showed optimum pH between 7.0 and 8.0 for the bound enzyme and 8.0 for the free enzyme.

Kinetic behaviour of immobilized succinyl CoA synthetase. Kinetic studies have shown that diffusion of substrate to the enzyme has become an important factor in the overall kinetics of the reaction of insolubilized enzymes [13].

The effect of the different substrates concentration on the initial rates of product formation by both bound and free succinyl CoA synthetase was

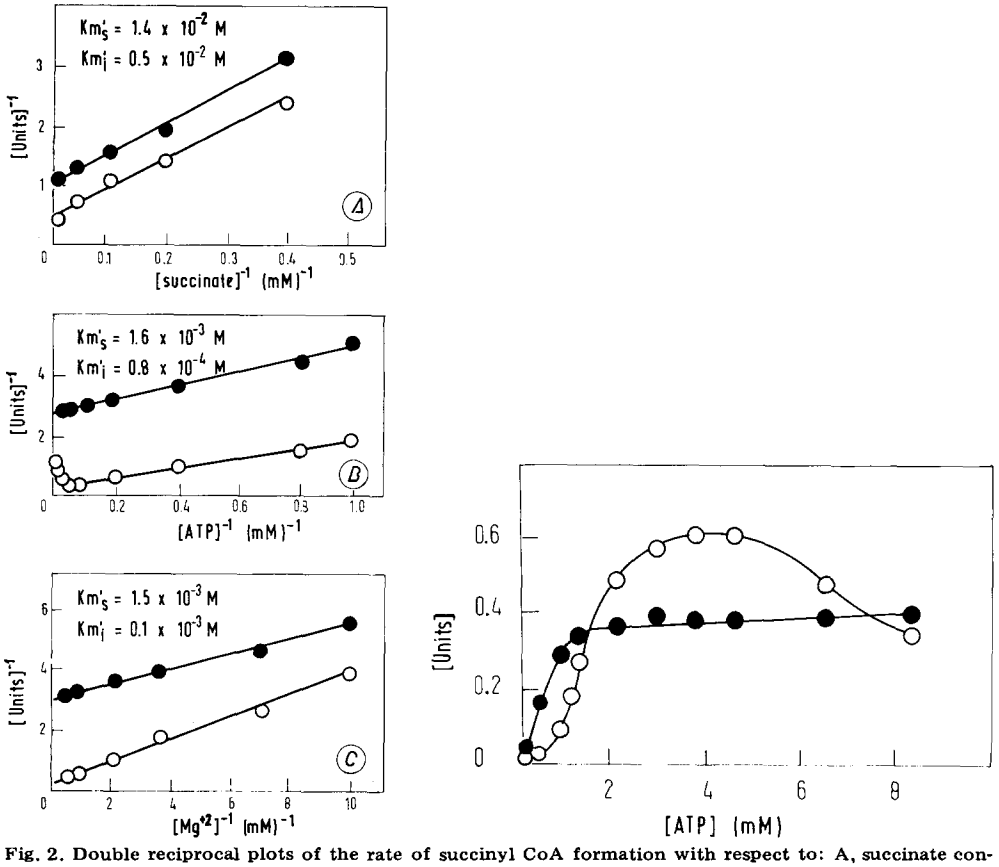

Fig. 2. Double reciprocal plots of the rate of succinyl CoA formation with respect to: A, succinate concentration, at fixed saturation concentrations of ATP, Mg andCoA; B, ATP concentration, at fixed saturation concentrations of succinate, Mg and CoA and C, Mg concentration, at fixed saturation concentrations of succinate, ATP and CoA. The reaction mixtures contained in a final volume of 2 ml, 0.05 M Tris · HCl buffer (pH 8.0), 1 ml of enzyme (8—10 mg of soluble succinyl CoA synthetase (○), specific activity 1.2, or 2—4 mg of insoluble succinyl CoA synthetase (●), specific activity 0.4) glutatione (10 μmol) and CoA (0.25 μmol); saturation levels of the other substrates were as follows: $MgCl_2$ (10 μmol); sodium succinate (100 μmol), ATP (10 μmol). Reaction mixtures were incubated for 30 min at 37°C with vigorous shaking. After incubation they were treated as described in refs. 6 and 10. K'_{m_s} = Michaelis-Menten constant for the soluble enzyme. K'_{m_i} = Michaelis-Menten constant for the insoluble enzyme.

Fig. 3. Effect of ATP concentration on the rate of succinyl CoA formation, at fixed saturation concentrations of succinate, Mg and CoA. Composition of reaction mixtures was as described in Fig. 2.

studied (Fig. 2) and Michaelis-Menten kinetics were observed. A small but general decrease in the K_m values of the insoluble enzyme for the different substrates was also observed. Another difference was that plots of the rate of free succinyl CoA synthetase activity against ATP concentration were sigmoideal and their reciprocals were therefore nonlinear; however, the shape of the ATP saturation curve for the bound enzyme was altered from sigmoideal to hyperbolic, and consequently its reciprocal was linear (Fig. 2B and Fig. 3).

We conclude that fixing the enzyme to a solid support resulted in significant changes in its behaviour.

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