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THE INACTIVATION AND DISSOCIATION OF *ESCHERICHIA COLI* SUCCINYL-CoA SYNTHETASE BY SULFHYDRYL REAGENTS

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SUMMARY

1. In immunodiffusion experiments antiserum to carboxymethylated succinyl-CoA synthetase (succinate:CoA ligase (ADP), EC 6.2.1.5) reacted strongly with subunits prepared by treatment of the enzyme with merthiolate (ethylmercurithiosalicylate), but not with native enzyme.

2. Various parameters of inactivation and reactivation of the enzyme in the presence of merthiolate were studied. These included the effects of pH, temperature, protein concentration and merthiolate concentration.

3. Titration of succinyl-CoA synthetase with *p*-chloromercuri[^{14}C]benzoate led to the binding of 4 moles of mercurial per mole of enzyme. The enzyme was dissociated by either merthiolate or *p*-chloromercuribenzoate into what appeared to be two different kinds of subunits.

INTRODUCTION

Recent experiments conducted in this laboratory[1] with succinyl-CoA synthetase (succinate:CoA ligase (ADP), EC 6.2.1.5) from *Escherichia coli* led to the conclusion that formation of the phosphoryl enzyme intermediate involved binding of two phosphoryl groups per mole of enzyme. This number was based on a molecular weight of 140 000 for the enzyme[2]. Immunochemical studies[3] indicated that succinyl-CoA synthetase was dissociated into subunits by treatment with merthiolate (sodium ethyl mercurithiosalicylate) and that this process could be reversed by treatment with dithiothreitol.

In this report we describe several parameters affecting reaction of merthiolate with succinyl-CoA synthetase. Also described are experiments in which *p*-chloromercuribenzoate (PCMB) was employed in place of merthiolate. When [^{14}C]PCMB

Abbreviation: PCMB, *p*-chloromercuribenzoate.

was used to dissociate the phosphorylated enzyme, two different ^{14}C-labeled species were formed. However, only one of these subunits contained a phosphoryl group.

We also report the preparation of antiserum to carboxymethylated succinyl-CoA synthetase. This antiserum was also reactive with merthiolate-produced subunits but not with native enzyme.

MATERIALS AND METHODS

Merthiolate (sodium ethyl mercurithiosalicylate) was obtained from Eli Lilly Co., Indianapolis. Sephadex G-75 and G-100 were obtained from Pharmacia Fine Chemicals, N.J. [*carboxy*-^{14}C]PCMB (10 mC/mmole) was purchased from Calbiochem, Calif.

Antisera were obtained as described previously[3] by immunization of New Zealand white rabbits with either native succinyl-CoA synthetase or carboxymethylated succinyl-CoA synthetase. Carboxymethylation was carried out by the procedure of MORINO AND SNELL[4], using 6 M guanidium chloride as solvent.

Succinyl-CoA synthetase was isolated from *E. coli* (ATCC 4517) and assayed as described previously[1]. Protein was estimated by the method of LOWRY *et al.*[5] with bovine serum albumin as standard. In the present study all of the enzyme employed had an initial specific activity of 1000. Several of the experiments were carried out using succinyl-CoA synthetase of specific activity 460 but this was a preparation which had lost activity on storage.

Immunodiffusion experiments[6] were performed as described previously[3]. The enzyme concentration was 0.1 mg/ml in all experiments.

Sephadex G-75 and Sephadex G-100 were equilibrated with 50 mM potassium phosphate–50 mM KCl–1 mM EDTA (pH 7.5), according to the manufacturer's recommendations. The gels were poured into glass columns of 2.5 cm diameter and packed under a constant hydrostatic pressure head of 20 cm. The final column heights were 60 cm. The excluded volume of each column was determined through the use of "Blue Dextran 2000" as a marker. The Sephadex G-75 column was calibrated for molecular weight estimations with the following markers (all obtained from Mann Research Laboratories, New York): whale myoglobin (mol. wt. 17 700), ovalbumin (mol. wt. 45 000) and bovine serum albumin (mol. wt. 67 000). All elutions from these columns were carried out with the equilibration buffer.

RESULTS

Immunodiffusion experiments with native and dissociated enzyme

Double diffusion experiments comparing reaction of antibody to native enzyme with carboxymethylated succinyl-CoA synthetase and native enzyme are shown in Figs. 1a and 1b. It can be seen that the carboxymethylated enzyme and native enzyme were serologically dissimilar (Fig. 1a). On the other hand carboxymethylated enzyme and merthiolate-dissociated enzyme showed fusion of precipitation lines (Fig. 1b), indicating a close serological relationship between carboxymethylated succinyl-CoA synthetase and succinyl-CoA synthetase subunits.

The experiment depicted in Fig. 2 compares the reactions of antisera to native succinyl-CoA synthetase and carboxymethylated succinyl-CoA synthetase. The

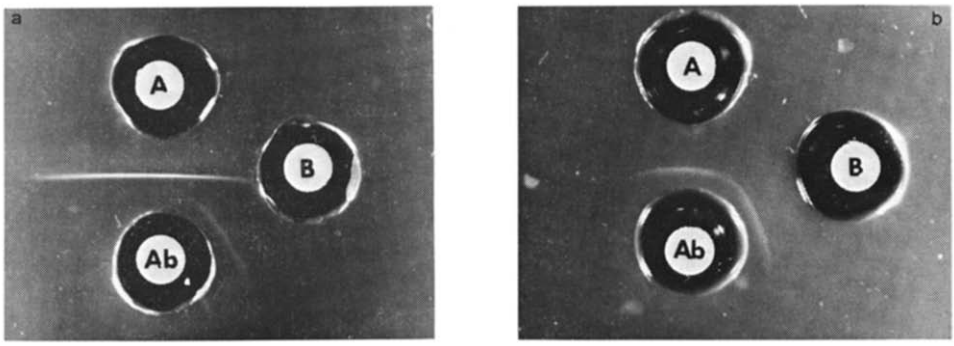
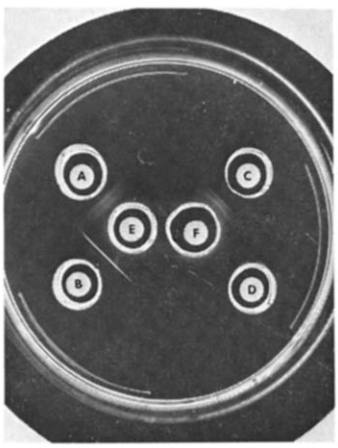


Fig. 1. Immunodiffusion of carboxymethylated succinyl-CoA synthetase. a. Well A contained native enzyme and Well B contained carboxymethylated enzyme. b. Well A contained native enzyme treated with 0.2 mM merthiolate and Well B contained carboxymethylated enzyme. Center wells in a and b contained native enzyme antiserum. Other conditions are described in MATERIALS AND METHODS.

results indicate that both antisera gave similar reactions with subunits produced by merthiolate treatment. On the other hand, only the antiserum to native enzyme gave a reaction with native enzyme.

Effect of time on the inactivation of the enzyme by merthiolate

Previous studies[3] showed that a correlation could be drawn between the loss of succinyl-CoA synthetase activity and dissociation of the enzyme into subunits in the presence of merthiolate. The rate of inactivation is rapid initially and then slows down markedly, as illustrated in Fig. 3. The merthiolate concentration used in this



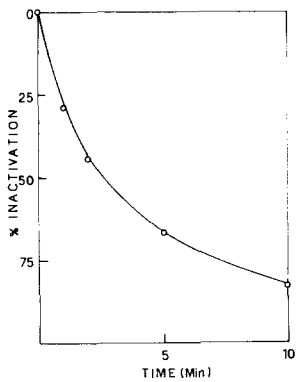
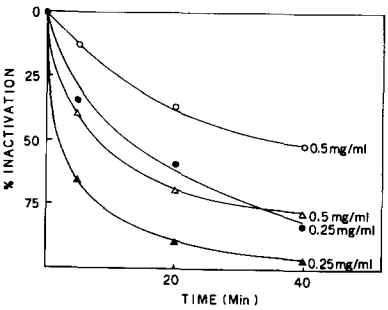


Fig. 2. Comparison of native enzyme antiserum and carboxymethylated enzyme antiserum in immunodiffusion. Wells A and C contained enzyme treated with 0.2 mM merthiolate. Wells B and D contained native enzyme. Well E contained native enzyme antiserum and Well F contained carboxymethylated enzyme antiserum. Other conditions are described in MATERIALS AND METHODS.

Fig. 3. Time dependence of enzyme inactivation by merthiolate. The reaction mixture contained 50 mM potassium phosphate (pH 7.5), 50 mM KCl, 1 mM EDTA, 0.11 mM merthiolate and 20 μg of enzyme (specific activity 1000) in a final volume of 0.17 ml. After incubation at 37° for the specified times aliquots were withdrawn and assayed.



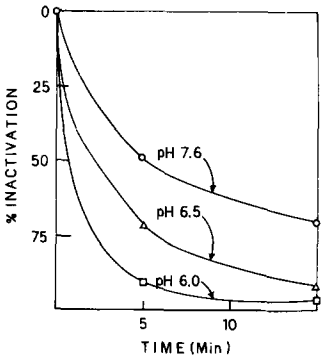


Fig. 4. Effect of protein concentration on inactivation of succinyl-CoA synthetase by merthiolate. The reaction mixtures contained 50 mM potassium phosphate (pH 7.5), 50 mM KCl, 1 mM EDTA, 0.1 mM or 0.2 mM merthiolate (denoted by circles and triangles, respectively) and enzyme (specific activity 460) as indicated in a final volume of 0.47 ml. After incubation at 37° for the specified times aliquots of the reaction mixtures were withdrawn and assayed.

Fig. 5. Effect of pH on inactivation by merthiolate. The reaction mixtures contained 50 mM potassium phosphate (pH as indicated), 50 mM KCl, 1 mM EDTA, 0.1 mM merthiolate and 117 μg of enzyme (specific activity 460) in a final volume of 0.47 ml. After incubation at 37° for the specified times aliquots of the reaction mixtures were withdrawn and assayed.

experiment (0.11 mM) was low enough, so that complete inactivation did not take place even on prolonged incubation. This probably indicates that equilibrium had been attained between native enzyme and merthiolate subunits[3]. The initial rate of dissociation was rapid at all merthiolate concentrations tested.

Effect of protein concentration on inactivation of the enzyme by merthiolate

Both the extent and rate at which enzyme inactivation occurred were affected by the concentrations of enzyme and merthiolate. The results in Fig. 4 show that increased protein concentration protected the enzyme from inactivation, but that this effect was overcome by increasing the merthiolate concentration.

TABLE I

THE EFFECT OF TEMPERATURE DURING INACTIVATION OF SUCCINYL-CoA SYNTHETASE BY MERTHIOLATE ON THE REACTIVATION REACTION AT 37°

The reaction mixtures contained 50 mM potassium phosphate (pH 7.5), 50 mM KCl, 1 mM EDTA, 0.2 mM merthiolate and 117 μg of enzyme (specific activity 460) in a final volume of 0.47 ml. The reaction mixtures were then incubated at the temperatures and times indicated. Following this the reactions were made 9 mM with respect to dithiothreitol and incubated at 37° to effect reversal of merthiolate action. Samples were removed for enzyme assay at the times indicated.

Expt. No.	*Preincubation*		*% Activity remaining*		
	Temp.	*Time (min)*	*After inactivation*	*After reversal*	
				15 min	*60 min*
1	25°	20	11.9	67.8	80.2
2	37°	20	5.9	32.1	43.1
3	45°	20	3.1	4.7	13.3
4	45°	2	14.1	17.0	—

The effect of pH on enzyme inactivation

BOYER[7] has shown that the reaction of PCMB with protein sulfhydryl groups is favored at acidic pH. Inactivation of succinyl-CoA synthetase by merthiolate proceeds at a much faster rate at pH 6.0 than at pH 7.0, as shown in Fig. 5. In the absence of merthiolate there was no inactivation of the enzyme at any of the pH values used. The increased rate of dissociation at lower pH is consistent with the idea that merthiolate and PCMB react with succinyl-CoA synthetase by the same mechanism.

The effect of temperature on enzyme inactivation and reactivation

The rate of reaction of merthiolate with the enzyme was temperature-dependent. An indication of this is seen in Table I. Enzyme preincubated with merthiolate for 2 min at 45° was inactivated to almost the same extent as enzyme incubated with merthiolate for 20 min at 25°. In these experiments there was no inactivation when merthiolate was omitted. It can also be seen in the Table that enzyme inactivated at 45° for either 2 or 20 min could not be reactivated significantly by dithiothreitol. On the other hand enzyme inactivated at 25° and, to a lesser extent, at 37° could be reactivated. It is of interest that the reactivation process was also temperature dependent, as shown in Fig. 6. The rates of reactivation were significantly greater at 40 and 30° than they were at lower temperatures.

Effects of various sulfhydryl titrating reagents on the enzyme

In addition to merthiolate other sulfhydryl reagents inactivated succinyl-CoA synthetase. With merthiolate the least effective, these reagents were (in order of

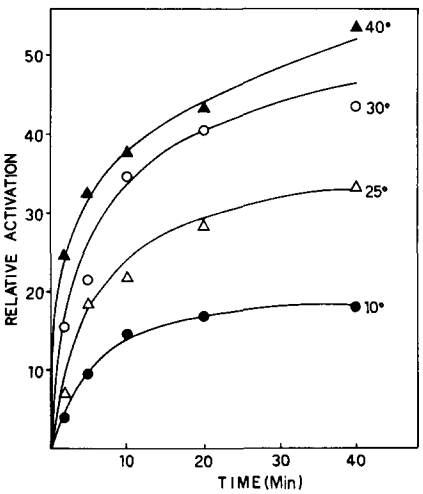


Fig. 6. Effect of temperature on reactivation of enzyme. For inactivation the reaction mixture contained 50 mM potassium phosphate (pH 7.5), 50 mM KCl, 1 mM EDTA, 0.2 mM merthiolate and 117 μg enzyme (specific activity 460) in a final volume of 0.47 ml. The mixture was incubated for 20 min at 37°. For reactivation 0.1-ml aliquots were withdrawn from the inactivation reaction mixture and combined with 0.01 ml of 0.1 M dithiothreitol (final dithiothreitol concentration 9 mM). The resulting solutions were incubated at the temperatures indicated. Aliquots were removed at the specified times for enzyme assay. Relative activation is defined as the difference between activity after reactivation and the activity after inactivation divided by the original activity.

TABLE II

EFFECTS OF DITHIOTHREITOL AND MERTHIOLATE ON BINDING OF [^{14}C]PCMB TO SUCCINYL-CoA SYNTHETASE

The reaction mixtures contained 50 mM potassium phosphate (pH 7.5), 50 mM KCl, 1 mM EDTA, 0.023 mM [^{14}C]PCMB, 9 mM dithiothreitol (where added), 0.2 mM merthiolate (where added) and 117 μg of enzyme (specific activity 460) in a final volume of 0.47 ml. Following incubation at 37° for 5 min the reaction mixtures were subjected to gel filtration on Sephadex G-25 columns and the radioactivity associated with the protein determined.

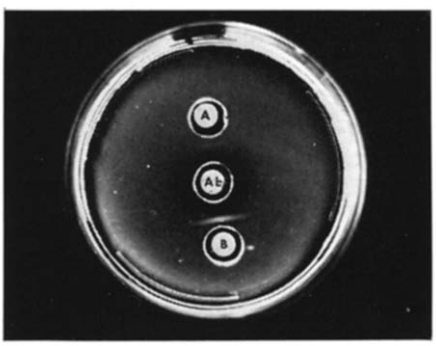
Expt. No.	*Components incubated with enzyme*	*PCMB bound to protein (counts/min)*
1	[^{14}C]PCMB	14 500
2	[^{14}C]PCMB + dithiothreitol*	362
3	[^{14}C]PCMB + merthiolate	1 042

* Enzyme was incubated with [^{14}C]PCMB for 5 min at 37° before dithiothreitol was added.

increasing reactivity): 5,5'-dithiobis-(2-nitrobenzoic acid), PCMB and $HgCl_2$. The effects of each could be reversed by dithiothreitol following inactivation at 37°. The reversibility of PCMB treatment is shown in the double diffusion experiment in Fig. 7. Well A contained enzyme treated with PCMB and Well B contained enzyme treated first with PCMB and subsequently with dithiothreitol.

Reaction of [^{14}C]PCMB with succinyl-CoA synthetase

Succinyl-CoA synthetase was incubated under various conditions with [^{14}C]-PCMB and the reaction mixtures processed on Sephadex G-25 columns to determine



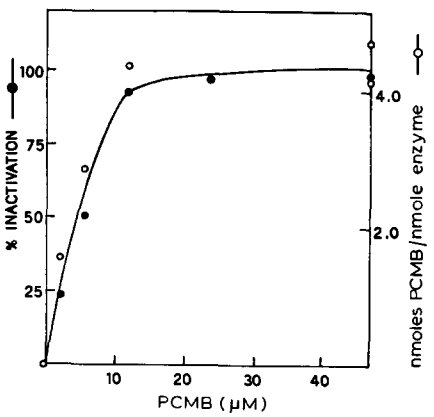


Fig. 7. Reversal of PCMB dissociation of succinyl-CoA synthetase by dithiothreitol. Well A contained enzyme treated with 46 μM PCMB. Well B contained enzyme which had been treated with 46 μM PCMB and then with 9 mM dithiothreitol. The center well contained native enzyme antiserum. Other conditions are described in the MATERIALS AND METHODS.

Fig. 8. Binding of [^{14}C]PCMB to succinyl-CoA synthetase. The reaction mixtures contained 50 mM potassium phosphate (pH 7.5), 50 mM KCl, 1 mM EDTA, 117 μg of enzyme (specific activity 460) and [^{14}C]PCMB (10 mC/mmole as indicated in a final volume of 0.47 ml). Following incubation for 5 min at 37° the reaction mixtures were assayed for enzymatic activity and subjected to gel filtration on columns of Sephadex G-25 in order to determine [^{14}C]PCMB bound to the protein.

the amount of radioactivity bound to the protein. The results of these experiments are summarized in Table II. As expected, treatment with dithiothreitol of enzyme to which [^{14}C]PCMB had been bound resulted in a very marked reduction of radioactivity in the protein fraction. In addition, simultaneous incubation of merthiolate and [^{14}C]PCMB with the enzyme resulted in a substantial inhibition of labeling of the enzyme. This does indicate that the two mercurials compete for the same site(s) on the protein. Activity measurements were made of enzyme incubated at various concentrations of [^{14}C]PCMB. It can be seen in Fig. 8 that there was an excellent correlation between the extent of inactivation of the enzyme and the degree of binding of mercurial to the enzyme. It is noteworthy that approx. 4 moles of PCMB were bound per mole of enzyme.

Studies on the separation of enzyme subunits

It was of interest to attempt correlation of the observed binding of 4 moles of PCMB to the enzyme and the report[2] that nonphosphorylated and phosphorylated subunits could be separated from each other after treatment of phosphorylated succinyl-CoA synthetase with PCMB.

TABLE III

DISSOCIATION PRODUCTS FROM TREATMENT OF PHOSPHORYLATED AND UNPHOSPHORYLATED SUCCINYL-CoA SYNTHETASE WITH MERCURIALS

The reaction mixtures contained 50 mM potassium phosphate–50 mM KCl–1 mM EDTA (pH 7.5) in a final volume of 0.5 ml. Expt. 1 contained 0.24 mg ^{33}P-phosphorylated succinyl-CoA synthetase. Expt. 2 contained 0.13 mg succinyl-CoA synthetase. After incubation under the conditions described the reaction mixtures were passed through the calibrated Sephadex G-75 column described in MATERIALS AND METHODS.

Expt. No.	*Form of enzyme*	*Incubation conditions*	*Radioactivity in Sephadex G-75 fraction (counts/min)*	
			Excluded vol.	*Mol. wt. 44 000*
1	^{33}P-phosphorylated enzyme	0.55 mM merthiolate, 16 h at 4°	670	16 600
2	Unphosphorylated enzyme	0.023 mM [^{14}C]PCMB, 5 min at 37°	22 000	27 100

The experiments described in Table III indicate that treatment of succinyl-CoA synthetase with mercurials yielded two products after gel filtration on a calibrated Sephadex G-75 column, each of which contained two titratable sulfhydryl groups. One of these products had an apparent molecular weight of 44 000 and contained the sites for phosphorylation of the enzyme (Expt. 1). The other product was excluded from the gel.

The appearance of a species of molecular weight 44 000 was at odds with a previous report that limited dissociation of *E. coli* succinyl-CoA synthetase by merthiolate gave species of molecular weights 35 000 and 70 000, as measured by two-dimensional immunodiffusion[3]. Partial unfolding of a subunit molecule, which would not be an unreasonable expectation after reaction with a mercurial, could result in increased retardation in gel filtration and might explain the discrepancy between

molecular weights 44 000 and 35 000. As to the identity of the material excluded from Sephadex G-75, it apparently contained little native enzyme, since, as shown in Expt. 1 (Table III), very little ^{33}P was found associated with it. The excluded substance probably has a molecular weight in excess of 70 000, since it is also excluded from Sephadex G-100. It is conceivable that this material represents aggregates of a subunit different from the 44 000 molecular weight species.

DISCUSSION

In immunodiffusion experiments antiserum prepared against carboxymethylated succinyl-CoA synthetase reacted with subunits produced by treatment of the enzyme with merthiolate but not with the native enzyme. On the other hand, antiserum prepared against native enzyme reacted with both native enzyme and subunits. Thus far, these antisera have been useful as devices to ascertain qualitatively dissociation and reassociation of succinyl-CoA synthetase. It should be possible to develop quantitative assays for these changes in quarternary structure of the enzyme through the use of complement fixation analysis.

Various parameters of the reaction of merthiolate with succinyl-CoA synthetase have been studied. The results indicate that the interconversion of native enzyme and subunits can be effectively controlled by manipulation of several factors, including pH, protein concentration, temperature and the concentration of dissociating agent.

In dissociation experiments with [^{14}C]PCMB 4 moles of the mercurial were bound per mole of native enzyme, 2 moles to a subunit species of 44 000 apparent molecular weight and 2 moles associated with what may be an aggregate of another type of subunit.

Attention in our laboratory is being focused on obtaining the apparently different subunit species in sufficient quantity, so that native enzyme reconstitution experiments and physical characterization studies can be carried out with them.

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