EVIDENCE FOR TWO TYPES OF SUBUNITS IN SUCCINYL COENZYME A SYNTHETASE[1]

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SUMMARY: Two species of subunits are derived from Escherichia coli succinyl CoA synthetase by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Their molecular weights are approximately 38,500 and 29,500. The active-site phosphohistidine residue occurs in the smaller subunit. The data strongly suggest that the two subunit types are present in equimolar amounts in the native enzyme (molecular weight ca. 140,000).

Succinyl CoA synthetase from Escherichia coli is known to involve a phosphohistidine residue in its catalysis (1, 2). At least one such residue, and perhaps as many as two, may be formed per enzyme molecule (mol. wt. ca. 140,000) by incubation with excess substrate (3-5). Dissociation of this enzyme into subunits by mercurials was first reported by Ramaley et al. (3) and later by Grinnell et al. (6). A more detailed study of the subunit structure was undertaken by Leitzmann et al. (5), revealing that the enzyme could be dissociated by treatment in dissociating medium (sodium dodecyl sulfate, urea or guanidine hydrochloride solutions) and/or succinylation or alkylation, producing what appeared to be homogeneous subunit preparations with molecular weights near 37,000-38,000. The present report, however, describes experiments which clearly demonstrate that the enzyme is composed of at least two species of subunits, with molecular weights of about 38,500 and 29,500, respectively.

EXPERIMENTAL PROCEDURE

Materials. Succinyl CoA synthetase was purified from E. coli (Crooke's strain), grown on succinate-based medium (3). The purification procedure used was essentially that described by Leitzmann et al. (5), with substitution of

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QAE-Sephadex for DEAE-Sephadex for the ion exchange chromatography. A final purification step involved gel filtration through G-100 Sephadex, resulting in homogeneous preparations of enzyme with specific activity near 25 units/mg.

^{32}P-orthophosphate was obtained from Atomic Energy of Canada, Ltd. It was used in the preparation of ATP-γ-^{32}P, according to a method described by Ramaley et al. (3). Sodium dodecyl sulfate (SDS) was the product of Fisher Scientific Co., and was recrystallized from ethanol. Other reagents were of the highest purity available and were used without further purification.

Preparation of ^{32}P-phosphoenzyme. 2 mg (1.4×10^{-2} μmoles) of succinyl CoA synthetase (specific activity 25.6 units/mg) was incubated for 5 minutes at 25° in a final volume of 1 ml in the presence of 0.05 M potassium phosphate, pH 7.2, 0.05 M KCl, 5 mM $MgCl_2$ and 1.5×10^{-2} μmoles of ATP-γ-^{32}P (specific activity 1.8×10^9 cpm/μmole). The reaction was stopped by the addition of 25 μmoles of sodium EDTA (pH 7.0), and the phosphoenzyme was purified by passage through a 2.5 x 40 cm column of G-100 Sephadex equilibrated with 0.05 M potassium phosphate, 0.05 M KCl, 0.1 mM EDTA, pH 7.2. The peak tube, used in the following experiments, contained 0.51 mg succinyl CoA synthetase per ml, and was found to contain 0.83 phosphoryl groups per 140,000 daltons of enzyme.

Polyacrylamide gel electrophoresis. For analytical runs in the absence of SDS, the method of Ornstein was used (7), with 0.5 mM EDTA present in all gel components. Electrophoresis in the presence of SDS was performed essentially as described by Weber and Osborn (8), using the normal amount of cross-linker in the separating gels. The enzyme sample was prepared by mixing 0.1 ml of the solution described above (0.051 mg ^{32}P-enzyme) with 0.2 ml of 0.01 M sodium phosphate, pH 7.0, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol for 150 minutes at 37°. Protein samples for molecular weight calibrations were prepared similarly, except the 37° incubation was preceded by 3 minutes at 90° to assure the inactivation of trypsin. For electrophoresis, 25 μl of the dissociated protein solution was mixed with 3 μl 0.05% bromophenol blue, 50 μl glycerol, 5 μl 2-mercaptoethanol, 25 μl gel buffer and 25 μl water, and the mixture was

applied to the polyacrylamide gels (0.6 x 9 cm). Electrophoresis was at 8 ma per gel for about four hours. The length of the gel and the distance travelled by the tracking dye were measured with calipers, and the gels (except those to be counted for radioactivity) were stained with Coomassie blue (8). Removal of unbound stain was accomplished with a quick gel destainer (Canalco, Inc.). Densitometer tracings were obtained using a linear transport accessory for a Gilford model 240 spectrophotometer. Protein mobilities were measured from the densitometer tracings and were corrected for expansion of the gels during the staining procedure (8). Peak integration was performed by triangulation.

Radioactivity measurements. Prior to staining, some gels were cut into slices averaging 1.595 mm in width using a lateral gel slicer (Canalco, Inc.). The slices were placed in stainless steel planchets and were treated overnight at room temperature with 0.5 ml of 30% hydrogen peroxide. After drying, radioactivity was measured with a Nuclear-Chicago gas-flow counter.

RESULTS AND DISCUSSION

Figure 1 shows the results of polyacrylamide gel electrophoresis of the phosphorylated enzyme with and without treatment in the presence of SDS. It may be seen that the standard analytical run in the absence of SDS indicates the presence of only one major component, confirming the homogeneity of the enzyme preparation. In contrast, the enzyme gives rise to two components following treatment with SDS and electrophoresis in the presence of the detergent, clearly indicating the existence of two species of subunits in succinyl CoA synthetase. It has been clearly demonstrated (8, 9) that acceptable molecular weight estimations may be made by comparison of the mobilities of the subunits to those arising from proteins of known molecular weights. Such an experiment is represented in Figure 2. Using the standard curve obtained and the average mobilities of the subunits derived from succinyl CoA synthetase in seven experiments (0.486 ± 0.004; 0.363 ± 0.002) the

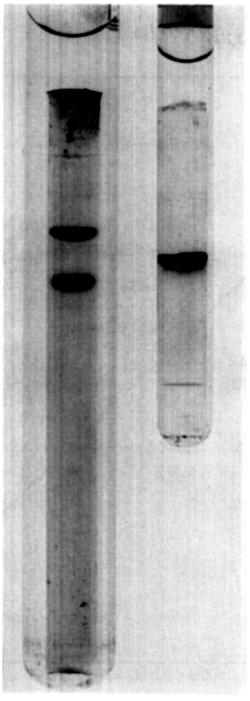

Fig. 1. Polyacrylamide gel electrophoresis of succinyl CoA synthetase. Direction of migration is from top to bottom, toward the anode. Right: Electrophoresis in standard 7% polyacrylamide without SDS treatment. The stacking gel is removed. Left: Electrophoresis in 10% polyacrylamide in the presence of SDS, following incubation with SDS and 2-mercaptoethanol. Details are given in the text.

molecular weights of the subunits may be estimated at 29,600 $\pm$ 500 and 38,700 $\pm$ 300, respectively. Integration of the peaks in seven experiments gives a ratio of area of Peak I to that of Peak II equal to 0.74 $\pm$ 0.02. If it is assumed that the stain reacts equally with both species of subunit, and that the subunits are present in equimolar amounts, a ratio of 0.76 is expected, based on the differences in the apparent molecular weights. Thus, the data are clearly consistent with the existence of equimolar amounts of the two subunit species in the native enzyme, and it is obviously an attractive possibility that there may be two of each type of subunit constituting a tetramer of molecular weight near 137,000.

The mobility of the ^{32}P-phosphorylated protein under the conditions of SDS treatment is shown in Figure 3, with a densitometer tracing from a standard gel provided for comparison. The phosphorylated subunit clearly corresponds to

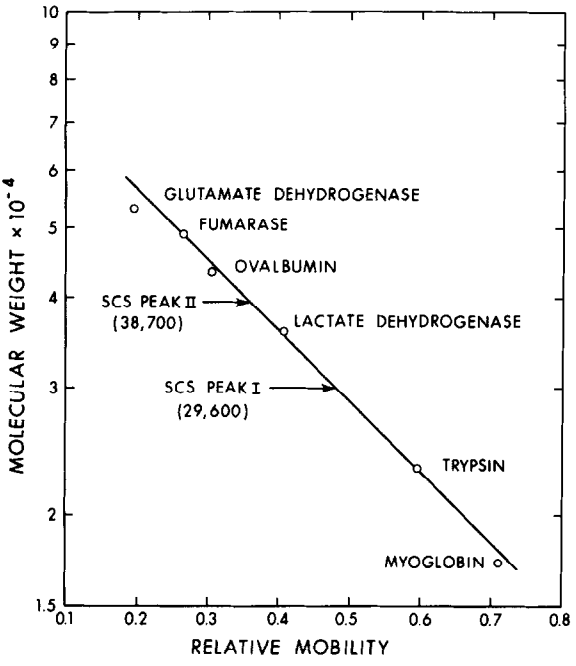

Fig. 2. Determination of molecular weights of subunits derived from succinyl CoA synthetase. The mobilities of the protein standards are averages of those calculated from measurements on three gels; mobilities of succinyl CoA synthetase subunits are averages of those obtained from seven determinations.

the lower molecular weight species. That the ^{32}P label is still in acid-labile phosphohistidine following the SDS treatment and electrophoresis was confirmed by the observation that no significant radioactivity remained in a gel following staining in 7% acetic acid solution and electrophoretic destaining.

These experiments provide an explanation for the observation that no more than two phosphohistidine residues may be formed per 140,000 daltons of enzyme. They do not, however, clarify the observation that the first phosphohistidine residue is formed readily, while it appears to be more difficult to accomplish phosphorylation of the second residue (3, 5). Although there could be undetected heterogeneity in the smaller subunit, a more likely explanation may involve some kind of anti-cooperativity, such that the phosphorylation of one subunit causes subsequent phosphorylation at the other equivalent site to be less favorable.

It should be pointed out that the results of previous experiments of Leitzmann et al. (5) are consistent with the existence of two types of subunits

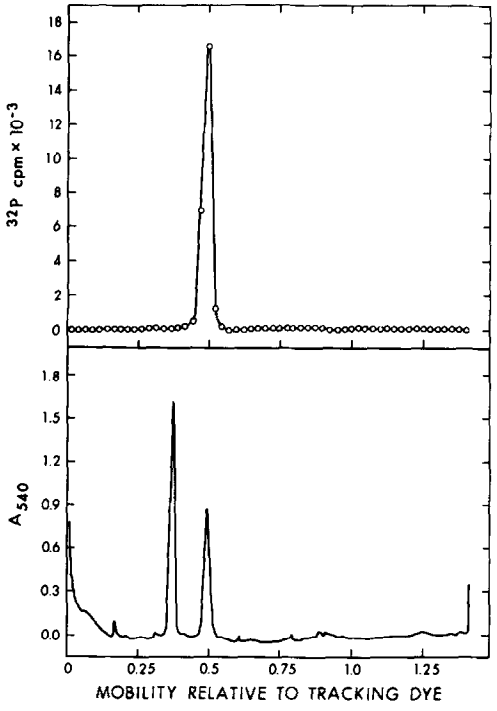

Fig. 3. Top: Mobility of radioactivity derived from ^{32}P-phosphorylated succinyl CoA synthetase in polyacrylamide gel electrophoresis following SDS treatment. Bottom: Densitometer tracing of a stained gel which was run under identical conditions. Experimental details are given in the text.

in this enzyme. Sedimentation analysis of dissociated succinyl CoA synthetase indicated size heterogeneity, and separation of phosphorylated and non-phosphorylated material was achieved by disc electrophoresis following alkylation and succinylation. In those experiments, however, the observed separation could have resulted from either increased negative charge introduced by the phosphoryl residue with otherwise identical subunits, or by differences in subunit size which have now been demonstrated by the experiments reported herein. Our separation must reflect difference in subunit size, since it has been shown that mobility of proteins in SDS-polyacrylamide gel electrophoresis is independent of their relative isoelectric points (8).

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