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Isolation, amino acid analyses and refolding of subunits of pig heart succinyl-CoA synthetase

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For the first time, pig heart succinyl-CoA synthetase has been refolded from its isolated subunits after denaturation. Amino acid analyses of pig heart succinyl-CoA synthetase and its subunits were performed. Subunits were isolated by gel filtration in neutral 6 M-urea. The amino acid composition of the native enzyme bears a strong resemblance to that of the *Escherichia coli* enzyme. Application of the various methods for comparing amino acid compositions [Cornish-Bowden (1983) Methods Enzymol. **91**, 60–75] shows that the degree of relatedness between the α-subunits of the pig heart and *E. coli* enzymes and between the β-subunits of the two synthetases is intermediate between 'strong' and 'weak'. As for the *E. coli* synthetase, it is unlikely that the α-subunit arises from the larger β-subunit by post-translational modification. The pig heart enzyme contains a single tryptophan residue, which is located in the β-subunit. Excitation of the enzyme at 295 nm resulted in a typical tryptophan emission spectrum. Refolding of enzyme denatured in 6 M-guanidine hydrochloride or of α- and β-subunits isolated in this solvent required the presence of either ethylene glycol or glycerol, optimally at 20–25% (v/v). GTP–Mg^{2+} did not stimulate reactivation of the enzyme, in contrast with the result obtained with ATP–Mg^{2+} in the reconstitution of the enzyme from *E. coli*. Yields of 60% and 40% were obtained in the refolding of denatured enzyme and isolated subunits respectively. The fluorescence spectrum of the refolded protein was essentially the same as that of native enzyme. Unrecovered activity could not be accounted for in the form of protein aggregates. The specific activity of refolded enzyme that had been separated from inactive protein on a Bio-Sil TSK 250 column was the same as that of native enzyme. K_m values for GTP of 27 μM and 14 μM were determined for native and refolded enzyme respectively.

INTRODUCTION

Succinyl-CoA synthetase (SCS) (EC 6.2.1.4) of pig heart catalyses the reaction:

$$\text{succinate} + \text{GTP} + \text{CoA} \leftrightarrow \text{succinyl-CoA} + \text{GDP} + P_i \quad (1)$$

The enzyme has an $\alpha\beta$ subunit structure (Brownie & Bridger, 1972), whereas the *Escherichia coli* enzyme has an $\alpha_2\beta_2$ structure (Bridger, 1971). The body of evidence suggests that in either case the active site is located at the points of contact between α- and β-subunits (Bridger *et al.*, 1968; Pearson & Bridger, 1975*a*,*b*; Collier & Nishimura, 1978; Vogel & Bridger, 1983).

Considerable progress has been made in the elucidation of the structure of *E. coli* SCS. Amino acid analyses of the whole enzyme and of the individual subunits have been reported (Bridger, 1974). Prasad *et al.* (1983) have determined that all of the tryptophan residues are located in the β-subunit and that at least one of the residues acts as a reporter group for binding of substrates. Buck *et al.* (1985) have deduced the amino acid sequences of α- and β-subunits from the nucleotide sequences of the respective genes. Wolodko *et al.* (1984) have crystallized the enzyme and described preliminary X-ray-crystallographic studies of the protein. Previously, Pearson & Bridger (1975*a*) had reported that refolding of isolated α- and β-subunits required the presence of ATP, which appeared to exert its effect by phosphorylating the α-subunit (Pearson & Bridger, 1975*b*).

Knowledge of pig heart SCS is less advanced than that of its counterpart from *E. coli*. However, the former is an important protein because of its simpler structure and potential as a model for refolding, assembly and control of multisubunit enzymes. In this paper, we describe isolation of the α- and β-subunits of pig heart SCS and determination of their amino acid compositions. We also report for the first time the successful refolding of enzyme from its isolated subunits.

MATERIALS AND METHODS

Reagents and enzyme

The lithium salt of CoA and the sodium salt of GTP were purchased from Boehringer Mannheim and Sigma respectively. 'Ultra Pure' urea was obtained from Schwarz-Mann. Guanidine hydrochloride (GuHCl) was a product of the Heico Division of Whittaker Corp. (Delaware Water Gap, PA, U.S.A.). All other chemicals were obtained from commercial sources and were of reagent grade.

Pig heart SCS was isolated as described by Murakami & Nishimura (1974). The enzyme was assayed by the method described by Cha (1969). Protein measurements were made by using a value for $A_{280}^{0.1\%}$ of 0.35 for the native enzyme (Murakami & Nishimura, 1974). The specific activity of the preparations used in this study was 16–17 units/mg. This is lower than specific activities

Abbreviations used: SCS, succinyl-CoA synthetase; GuHCl, guanidine hydrochloride.

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previously reported (Cha, 1969; Murakami & Nishimura, 1974). However, the enzyme was electrophoretically homogeneous.

H.p.l.c. gel filtration of the enzyme

This was performed in a LDC h.p.l.c. apparatus equipped with u.v. monitor set at 280 nm and an integrator. A Bio-Sil TSK 250 (300 mm × 7.5 mm) column was used, with 50 mM-Na_2SO_4/20 mM-sodium phosphate (pH 6.8) buffer. The flow rate was 1 ml/min, with a back-pressure of about 400 lb/in^2 (2.76 MPa). Samples were injected in a volume of 50 μl.

Isolation of subunits by gel filtration in neutral urea

For isolation of the α and β subunits, 12 mg of the purified pig heart enzyme contained in 0.6 ml was mixed with 0.6 ml of 6 M-urea/50 mM-Tris/HCl (pH 7.4)/0.1 mM-EDTA/0.5 mM-dithiothreitol and dialysed against 500 ml of this solution for 24 h at 4 °C. The dialysed sample was made 10% (v/v) in glycerol and 1% (v/v) in 2-mercaptoethanol. The resulting solution was placed on a Sephadex G-150 column (2.5 cm × 90 cm bed dimensions) that had been equilibrated thoroughly with the same urea-containing buffer. Elution with this buffer was conducted at 4 °C at a flow rate of 6 ml/h; 4 ml fractions were collected. The emergence of the subunits from the column was monitored by measurement of protein and by SDS/polyacrylamide-gel electrophoresis. These subunits were used for amino acid analysis.

Isolation of subunits by gel filtration in GuHCl

A 12 mg sample of pig heart enzyme contained in 0.53 ml was dialysed against 6 M-GuHCl/50 mM-Tris/HCl/0.5 mM-dithiothreitol/0.1 mM EDTA, pH 7.5, overnight at 4 °C. The sample was made 10% (v/v) in glycerol and 1% (v/v) in 2-mercaptoethanol and applied to a Sephadex G-150 column (2.5 cm × 93 cm) that had been equilibrated with the same GuHCl-containing solution. Elution was effected at 4 °C with the latter at a flow rate of 3 ml/h; 1 ml fractions were collected. Protein determinations were carried out by u.v. spectroscopy to locate the β-subunit and by the method of Bradford (1976). In order to assess purity of the fractions, samples of the fractions were first dialysed against 25 mM-NH_4HCO_3 and then freeze-dried. The dried samples were subjected to SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970). The purified subunit fractions were concentrated in an Amicon concentration cell [N_2 pressure 40 lb/in^2 (276 kPa)], with a PM-10 membrane, to approx. 6 mg/ml. The subunit solutions were kept at 4 °C.

Amino acid analyses

In preparation for amino acid analyses, purified subunits were dialysed thoroughly against distilled water at 4 °C and freeze-dried. Duplicate samples were dissolved in 1 ml of 6 M-HCl and hydrolysed *in vacuo* for 24, 48 and 72 h at 110 °C. Results were corrected for destruction of residues and resistance of certain residues to hydrolysis by extrapolation to zero time (serine and threonine) and infinite time (valine and isoleucine), respectively. Half-cystine content was determined in samples treated with performic acid (Hirs, 1967) and hydrolysed for 24 h. For the analysis of tryptophan, duplicate samples were hydrolysed in 0.2 ml of 4 M-methanesulphonic acid/0.2% tryptamine for 24 h at 110 °C (Simpson *et al.*, 1976). Amino acid analyses were performed in a Durrum D-500 analyser by Dr. Katharine Wall.

Fluorescence measurements

Fluorescence spectra were obtained in a Perkin–Elmer MPF 44a spectrofluorometer.

RESULTS

Isolation of the α- and β-subunits of pig heart SCS

Subunits were isolated by chromatography on a Sephadex G-150 column in 6 M-urea at pH 7.4, as described in the Materials and methods section. In a typical experiment, the β-subunit emerged in fractions 36–45 and the α-subunit in fractions 41–51. Fractions 36–40 and 46–50 were pooled separately, representing electrophoretically pure β- and α-subunits, in 40% and 20% yields respectively. Attempts also were made to purify the subunits by gel filtration in 6 M-urea/5% acetic acid/0.1 mM-EDTA/0.5 mM-dithiothreitol, the solvent used for separation of the subunits of the *E. coli* enzyme (Pearson & Bridger, 1975*a*). However, resolution of the subunits under these conditions was poor, and this approach was therefore not employed.

Amino acid compositions of the native enzyme and its subunits; comparison with the *E. coli* enzyme

These data are presented in Table 1 with the amino acid content of the native *E. coli* enzyme (Bridger, 1974); there is a striking similarity between the compositions of the two native enzymes, based on an M_r for the pig heart enzyme of 80000 (Murakami & Nishimura, 1974). The apparent relatedness was examined by the method of Cornish-Bowden (1983), by using the data of Bridger (1974) for the *E. coli* enzyme subunits and those for the pig heart enzyme subunits summarized in Table 1. Tryptophan and cysteine residues were not considered in the calculations. The values obtained for the difference index, the composition divergence and the $S\Delta Q$ index were 8.63, 0.0492 and 24.2 respectively for the α-subunits, and 9.18, 0.055 and 30.3 respectively for the β-subunits. Application of the statistical criteria given by Cornish-Bowden (1983) indicates that the values are on the borderline for the 'strong' test of relatedness for the α-subunits, and between the values for the 'strong' and 'weak' test of relatedness for the β-subunits. The distinct dissimilarities in the compositions of the subunits of the pig heart enzyme would appear to indicate that, as for the *E. coli* protein (Bridger, 1974), the α-subunit is not a post-translationally modified form of the larger β-subunit.

Particularly notable was the finding that there is a single tryptophan residue in the pig heart enzyme, and that it is located in the β-subunit. This observation is of considerable interest, since the three tryptophan residues of each $\alpha\beta$ dimer in the *E. coli* enzyme have all been found in the β-subunit, and at least one of these groups functions as a fluorescent reporter group (Prasad *et al.*, 1983).

Fluorescence spectra of native enzyme and subunits

The fluorescence emission spectrum resembled that of the *E. coli* enzyme (Prasad *et al.*, 1983). Thus excitation

Table 1. Amino acid compositions of pig heart succinyl-CoA synthetase and its subunits

Amino acid	Native enzyme (residues/ 80 kDa)	α-Subunit (residues/ 33 kDa)	β-Subunit (residues/ 47 kDa)	Calculated for αβ protein (residues/ 80 kDa)	*E. coli* enzyme* (residues/ 80 kDa)
Lysine	58.7	23.0	34.0	57	51.9
Histidine	13.5	8.9	4.3	13	11.3
Arginine	23.6	9.5	10.3	20	25.3
Aspartic acid	69.7	19.8	51.1	71	61.8
Threonine	44.2	23.9	16.2	40	46.0
Serine	33.6	16.6	19.7	36	23.1
Glutamic acid	83.9	32.7	55.2	88	79.8
Proline	28.2	15.3	14.4	30	31.7
Glycine	78.9	38.7	40.6	79	94.0
Alanine	74.5	27.0	47.1	74	84.7
Half-cystine	14.4	7.4	8.4	16	14.0
Valine	57.3	22.5	36.7	59	68.2
Methionine	13.7	5.3	7.6	13	15.8
Isoleucine	47.8	21.7	29.1	51	55.5
Leucine	62.3	21.6	40.4	62	60.9
Tyrosine	15.6	5.6	8.2	14	13.0
Phenylalanine	27.3	11.5	19.9	31	22.7
Tryptophan	1.2	< 0.1	0.9	1	4.8

* See Bridger (1974).

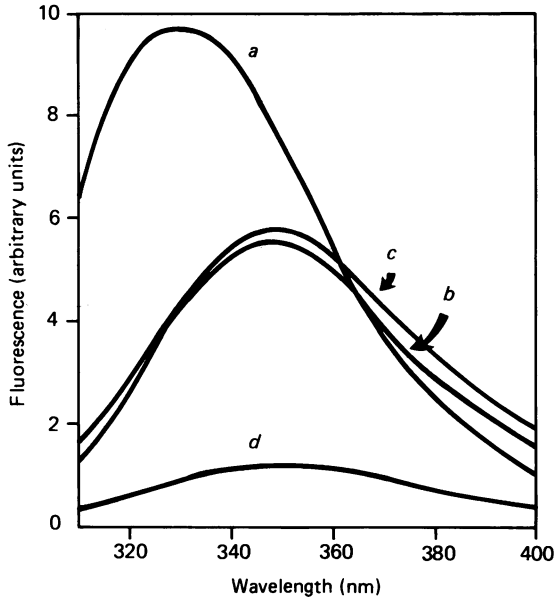

Fig. 1. Fluorescence emission spectra of pig heart succinyl-CoA synthetase and its subunits

Protein concentration was 2.1 μM. Excitation was at 295 nm. Spectra were corrected for the buffers. (*a*) Spectrum of native αβ dimer in 50 mM-potassium phosphate, pH 7.0. (*b*) Spectrum of denatured enzyme in 6 M-urea/5% acetic acid/0.1 mM-EDTA/0.5 mM-dithiothreitol. (*c*) Spectrum of isolated β-subunit in acidic urea. (*d*) Spectrum of isolated α-subunit in acidic urea.

at 295 nm resulted in an emission peak typical of tryptophan fluorescence at about 330 nm, as shown in Fig. 1. This wavelength maximum is consistent with the emitting tryptophan residue being out of contact with the solvent (Burstein *et al.*, 1973). As expected, this peak shifted to about 350 nm under denaturing conditions. Comparison of the spectra of denatured enzyme and β-subunit (also unfolded) shows that the latter accounts for all of the fluorescence of the enzyme under these conditions. The intrinsic tryptophan fluorescence exhibited by the α-subunit was due to a small contamination by the β-subunit in this particular preparation.

Refolding of denatured pig heart SCS

Pearson & Bridger (1975*a*) demonstrated the refolding of *E. coli* SCS from subunits that were isolated by gel filtration in acidic urea solution. ATP was required for optimal refolding. As yet, refolding of the pig heart enzyme has not been demonstrated.

Initial studies in our laboratory were concerned with testing whether pig heart enzyme that had been first denatured in either 6 M-urea or 6 M-GuHCl would refold after dilution of the denaturant. Negligible activity was recovered in the presence or absence of GTP. This was in contrast with the results obtained with the *E. coli* enzyme, which showed that significant activity could be recovered when enzyme, denatured with urea or with GuHCl, was diluted and incubated in the presence of ATP.

More extensive studies were conducted to find appropriate conditions for refolding of the pig heart enzyme. We found that addition of the cryoprotectant ethylene glycol resulted in substantial refolding of denatured enzyme. This is illustrated in Table 2. Thus enzyme that was denatured in either urea or GuHCl could be partially refolded in 25% ethylene glycol or 25% glycerol at pH 8. Recovery was better when the enzyme had been denatured in GuHCl solution. Therefore, subunits were isolated by gel filtration in 6 M-GuHCl, as described in the Materials and methods section.

Table 2. Refolding of pig heart succinyl-CoA synthetase denatured in GuHCl or in urea solutions

Enzyme was mixed at a final protein concentration of 6 mg/ml with 6 M-GuHCl/50 mM-Tris/HCl/0.5 mM-dithiothreitol/0.1 mM-EDTA, pH 7.5, or with 6 M-urea/50 mM-Tris/HCl/0.5 mM-dithiothreitol/0.1 mM-EDTA, pH 7.5. The resulting solutions were kept at 4 °C for 24 h. Denaturant and protein were diluted to 0.1 and 100 μg/ml respectively with 50 mM-Tris/HCl (pH 8)/25% (v/v) ethylene glycol (or glycerol)/50 mM-dithiothreitol and, where indicated, 100 μM-GTP/110 μM-$MgCl_2$. Incubations were carried out for 40 min at 25 °C with ethylene glycol-containing solutions and for 60 min at 25 °C for those containing glycerol. Enzyme assays were performed as described in the Materials and methods section.

Denaturant	Diluent	Addition	Activity regained (%)
None (control)	Ethylene glycol	None	100
GuHCl	Ethylene glycol	None	60
GuHCl	Ethylene glycol	GTP/$MgCl_2$	60
Urea	Ethylene glycol	None	22
Urea	Ethylene glycol	GTP/$MgCl_2$	22
None (control)	Glycerol	None	100
GuHCl	Glycerol	None	58
Urea	Glycerol	None	22

Refolding of isolated subunits

Results of the refolding of isolated α- and β-subunits of pig heart SCS are shown in Fig. 2. A virtually absolute requirement for ethylene glycol is indicated by these results. In other experiments (results not shown), it was demonstrated that 100 μM-GTP plus 110 μM-$MgCl_2$ had no effect on refolding. Practically identical results were observed when glycerol was used in place of ethylene glycol.

The effect of ethylene glycol concentration on refolding of isolated subunits is described in Table 3. The optimal concentration of ethylene glycol was 20–25% (v/v). Comparable results were obtained with glycerol. When the native enzyme was incubated under the same conditions with 20 or 25% ethylene glycol, there was no difference in enzyme activity. However, after 1 h of incubation at 25 °C in 60% ethylene glycol, native enzyme had lost 40% of its activity. Thus it appears that ethylene glycol and, presumably, glycerol have both stabilizing and destabilizing effects on the quaternary structure of pig heart SCS.

Study of possible intermediates in refolding

Samples from refolding solutions of denatured enzyme and mixed isolated subunits were subjected to cross-linking with glutaraldehyde, essentially as described by Hermann *et al.* (1981). No evidence was found for aggregates, such as α_2 or β_2, in solutions of either refolded denatured enzyme or refolded reconstituted solutions of subunits. Moreover, no difference was seen in the formation of large polymers, when native and refolded enzyme preparations were compared. On the other hand, it appears likely that some refolding of monomeric subunits occurs under optimal conditions.

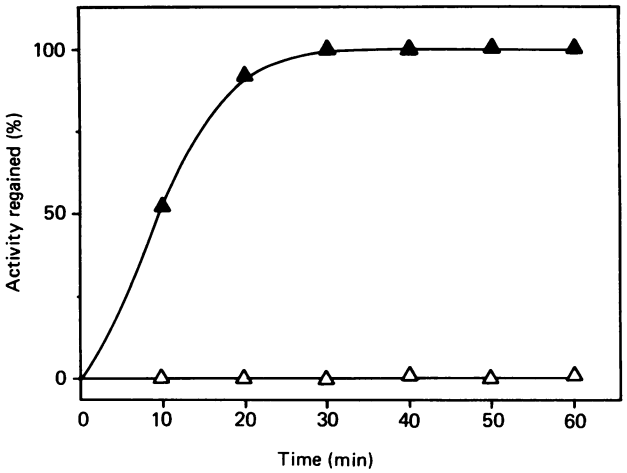

Fig. 2. Refolding of isolated subunits of pig heart succinyl-CoA synthetase

Incubation was carried out at 25 °C with each subunit at 1.43 μM (total protein 100 μg/ml) in 0.1 M-GuHCl/50 mM-Tris/HCl (pH 8)/20 mM-dithiothreitol: ▲, +25% (v/v) ethylene glycol; △, no ethylene glycol. A regain of 100% was equal to 60% recovery of the original activity.

Table 3. Refolding of isolated pig heart subunits as a function of ethylene glycol concentration

Subunits were refolded as described in the legend of Fig. 1, at the indicated concentrations of ethylene glycol, for 60 min; 100% represents the maximum re-activation observed with this method (see text).

Ethylene glycol (%, v/v)	Activity regained (%)
0	< 5
10	46
15	92
20	100
25	100
30	83
40	55
60	22

This point is illustrated by the data in Fig. 3. Thus incubation of individual denatured subunits under refolding conditions led to an instantaneous expression of activity at the time of mixing. The amplitude of this activity relative to the total activity regained could be increased by longer preincubation of the subunits. However, as shown in Fig. 3, preincubation of the subunits results in decreased recovery of enzyme activity.

Comparison of various properties of refolded and native enzymes

It was important to establish that the refolded enzyme was indistinguishable from the native enzyme. Thus measurement of the fluorescence of the refolded product gave spectral properties that were virtually the same as those of the native enzyme (see Fig. 1). In addition, native and refolded enzyme preparations were subjected to gel filtration in an h.p.l.c. system, as described in the

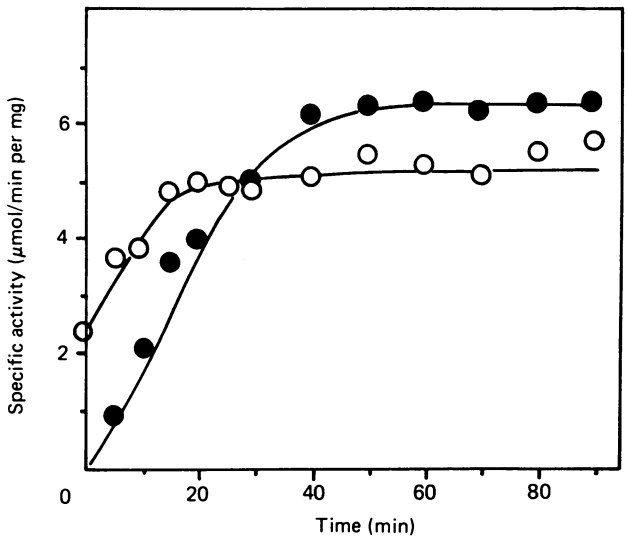

Fig. 3. Effect of preincubation of subunits on kinetics of refolding

Preincubation was carried out at 25 °C for 30 min with each subunit added at 100 μg/ml in 0.1 M-GuHCl/50 mM-Tris/HCl (pH 8)/20% (v/v) ethylene glycol/20 mM-dithiothreitol. Then the solutions were mixed to give a final concentration of 1.43 μM of each subunit. Incubation was resumed at 25 °C. ○, Subunits preincubated; ●, subunits not preincubated.

Materials and methods section. Elution times for native enzyme and for enzyme refolded from denatured enzyme and from isolated subunits were identical, with a relatively broad peak emerging at 10.2 min. Neither of the isolated subunits nor the unsuccessfully renatured protein migrated in this area of the chromatogram. Thus integration of the enzyme peak showed that the specific activity of the refolded enzyme was as least equal to that of the corresponding native enzyme.

Substrate saturation experiments with respect to GTP were carried out. K_m values of 27 μM and 14 μM were obtained with native and refolded enzymes respectively. These values are within the range of values reported by Cha & Parks (1964).

DISCUSSION

Several interesting observations can be made from the amino acid analysis of the subunits of pig heart SCS. First, there is a striking similarity in the amino acid compositions of the native enzymes from pig heart and *E. coli* (Table 1). The comparison has been made on the basis of the M_r of 80000 of the pig heart enzyme, which is more than half that (141000) of the *E. coli* enzyme. Use of the statistical criteria given by Cornish-Bowden (1983) shows that the values of the difference index, the composition divergence and the $S\Delta Q$ for the α-subunits are on the borderline for the 'strong' test of relatedness. According to the same criteria, the degree of relatedness of the β-subunits is intermediate between 'strong' and 'weak'.

The M_r values of the subunits are different: 38500 and 47000 for β-subunit and 28500 and 33000 for α-subunit from the *E. coli* and pig heart enzymes respectively. The basis for the difference in size will have to await elucidation of the amino acid sequences of the pig heart subunits and, perhaps, an accurate assessment of the number of binding sites for substrates in the same enzyme.

The half-cystine content of the two enzymes is also comparable. Both enzymes are known to be extremely sensitive to thiol reagents, and it would appear that thiol-group interactions with other amino acid residues are very similar in the two proteins (Nishimura, 1986). However, there is no evidence that thiol groups are involved in stabilizing $\alpha_2\beta_2$ tetramer against dissociation to $\alpha\beta$ dimer.

From our viewpoint, one of the more interesting findings was that of one tryptophan residue per molecule of pig heart SCS. This was in keeping with the observation that the A_{280} of the enzyme (3.5 for a solution of 10 mg of protein/ml) was relatively low (Murakami & Nishimura, 1974). However, we had found the *E. coli* enzyme to contain three tryptophan residues per β-subunit and none in the α-subunit (Prasad *et al.*, 1983). Quenching of the tryptophan fluorescence by acrylamide was affected significantly by addition of substrates, most notably CoA and ATP. Thus it was proposed that at least one tryptophan residue was located at or near the active site. Modification of a single tryptophan residue resulted in complete loss of enzyme activity, but the enzyme was capable of undergoing phosphorylation by ATP (Ybarra *et al.*, 1986). Fluorescence-quenching studies of the pig heart enzyme have been initiated (J. S. Nishimura, J. Ybarra & P. M. Horowitz, unpublished work). Whereas the effects of substrates and other ligands on fluorescence quenching of pig heart SCS have been small in magnitude, the results indicate that the tryptophan fluorescence is quite sensitive to these perturbations. In addition, since we have observed refolding of the dissociated subunits, it should be possible to investigate the effects of tryptophan modification on the pig heart enzyme.

The need for the presence of ethylene glycol or glycerol in the refolding of the pig heart enzyme should be investigated further. Gekko & Timasheff (1981), in studying the stabilizing effect of glycerol on the native structure of certain proteins, have hypothesized that, when glycerol is mixed with protein, there is an increase in the chemical potential of the glycerol. In this unstable situation, contact between glycerol and the protein is minimized, favouring the native state of the protein. It is likely that the same forces come into play during refolding of pig heart SCS in the presence of ethylene glycol or glycerol. However, it also appears that at higher ethylene glycol concentrations (60%) the enzyme is destabilized.

The refolded enzyme, whether derived from enzyme dissociated in GuHCl solution or from isolated subunits, appears to have chromatographic properties identical with those of the native enzyme. Other properties, such as the regain of native tryptophan fluorescence and similar K_m values for GTP, suggest that the refolded state is very similar to the native state. The yields of approx. 60% from denatured enzyme and 40% from isolated subunits may reflect irreversible modifications in some of the subunit polypeptide chains. Data from cross-linking experiments with glutaraldehyde do not support the notion that the inactive species may consist of polymerized subunits.

The fact that, in contrast with the *E. coli* enzyme, refolding of the pig heart enzyme is not stimulated by the nucleoside triphosphate substrate (GTP) is intriguing. In recent studies (I. A. Khan & J. S. Nishimura, un-

published work), we have been able to show that the secondary structure of denatured *E. coli* SCS is rapidly regained in the form of an inactive $\alpha_2\beta_2$ tetramer. The presence of ATP is required to activate the tetramer. Thus the difference in quaternary structure may be related to the nucleoside triphosphate requirement for reactivation.

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