Chemical modification of *Escherichia coli* succinyl-CoA synthetase with the adenine nucleotide analogue 5'-*p*-fluorosulphonylbenzoyladenosine

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Escherichia coli succinyl-CoA synthetase (EC 6.2.1.5) was irreversibly inactivated on incubation with the adenine nucleotide analogue 5'-*p*-fluorosulphonylbenzoyladenosine (5'-FSBA). Optimal inactivation by 5'-FSBA took place in 40% (v/v) dimethylformamide. ATP and ADP protected the enzyme against inactivation by 5'-FSBA, whereas desulpho-CoA, an analogue of CoA, did not. Inactivation of succinyl-CoA synthetase by 5'-FSBA resulted in total loss of almost four thiol groups per $\alpha\beta$ -dimer, of which two groups appeared to be essential for catalytic activity. 5'-FSBA at the first instance appeared to interact non-specifically with non-essential thiol groups, followed by a more specific reaction with essential thiol groups in the ATP(ADP)-binding region. Plots of the data according to the method of Tsou [(1962) *Sci. Sin.* **11**, 1535–1558] revealed that, of the two slower-reacting thiol groups, only one was essential for catalytic activity. When succinyl-CoA synthetase that had been totally inactivated by 5'-FSBA was unfolded in acidic urea and then refolded in the presence of 100 mM-dithiothreitol, 85% of the activity, in comparison with the appropriate control, was restored. These data are interpreted to indicate that inactivation of succinyl-CoA synthetase by 5'-FSBA involves the formation of a disulphide bond between two cysteine residues. Disulphide bond formation likely proceeds via a thiosulphonate intermediate between 5'-*p*-sulphonylbenzoyladenosine and one of the reactive thiol groups of the enzyme.

Succinyl-CoA synthetase (EC 6.2.1.5) from *Escherichia coli* catalyses the reaction given in eqn. (1):

The enzyme has an $\alpha_2\beta_2$ quaternary structure (Bridger, 1971) with two possible active sites. The M_r of the native enzyme is approx. 140 000 and the α - and β -subunits have M_r approx. 30 000 and 40 000 respectively. A proposal was advanced by Bild *et al.* (1980) for an alternating sites mechanism for the enzyme. Supporting evidence for this hypothesis has been presented by Bridger and his co-workers (Wolodko *et al.*, 1981; Vogel & Bridger, 1982). In the alternating-sites model, it is proposed that only one active site is operative in catalysis at one time and that binding of ATP or succinyl-CoA at the non-productive site is responsible for release of

product (succinyl-CoA or ATP) at the productive site.

It seemed important for us to develop a probe for the ATP(ADP)-binding site as an approach to examining the alternating-sites hypothesis in greater depth. Thus affinity labelling of one site with an ATP(ADP) analogue might permit a study of binding and release of substrates at the other site. We chose to test as such a probe, 5'-*p*-fluorosulphonylbenzoyladenosine (5'-FSBA), a compound that has been used successfully as an affinity analogue of ATP(ADP) (Colman *et al.*, 1977).

The results reported below indicate that 5'-FSBA does modify succinyl-CoA synthetase chemically, at or near the ATP(ADP)-binding site, by effecting disulphide bond formation in the enzyme structure.

Materials and methods

Reagents and enzyme

p-Fluorosulphonylbenzoyl chloride and adenosine were purchased from Aldrich Chemical Co.

Abbreviation used: 5'-FSBA, 5'-*p*-fluorosulphonylbenzoyladenosine.

(Milwaukee, WI, U.S.A.). 5'-FSBA was a product of Sigma Chemical Co. (St. Louis, MO, U.S.A.). [2-³H]Adenosine and *p*-amino[carboxy-¹⁴C]benzoic acid were obtained from Amersham International (Arlington Heights, IL, U.S.A.) and Research Products International (Mount Prospect, IL, U.S.A.) respectively. *p*-Fluorosulphonylbenzoyl-[2-³H]adenosine was synthesized by the procedure of Wyatt & Colman (1977). *p*-Fluorosulphonyl-[¹⁴C]benzoyl-adenosine was synthesized by the method of Esch & Allison (1978). Desulpho-CoA was prepared by the method of Chase *et al.* (1966) as modified by Grinnell & Nishimura (1969). Succinyl-CoA synthetase of *E. coli* was isolated and assayed as described elsewhere (Bowman & Nishimura, 1975).

Reaction of 5'-FSBA with succinyl-CoA synthetase before refolding

In the refolding experiments (Table 3) succinyl-CoA synthetase (7.3 nmol) was incubated for 60 min at 25°C with 25 mM-Tris/HCl (pH 7.6)/28.4% (v/v) dimethylformamide in a volume of 250 µl. Three 25 µl portions of 52 mM-5'-FSBA [in 50% (v/v) dimethylformamide] were added 5 min apart with a 5 min incubation after the last addition. The enzyme was totally inactivated by this treatment. The untreated or control enzyme was handled in the same manner, including incubation with dimethylformamide, but retained 88% of its initial activity. Both enzyme preparations were transferred to columns of PD-10 (Sephadex G-25M; Pharmacia Fine Chemicals, Uppsala, Sweden) that had been equilibrated with 6 M-urea/5% (v/v) acetic acid/0.1 mM-Na₂EDTA. The enzyme was eluted from the columns with the same solvent. The protein fractions were concentrated to approx. 0.5 ml with the use of an Amicon XM-50 membrane (Amicon Corp., Danvers, MA, U.S.A.) under 340 kPa (50 lbf/in²).

Isolation of α- and β-subunits of succinyl-CoA synthetase

The α- and β-subunits were isolated by means of gel filtration on Sephadex G-150 in acidic urea, as described by Pearson & Bridger (1975).

Determination of extent of labelling of the enzyme

Succinyl-CoA synthetase was incubated with radioactive 5'-FSBA exactly as described for the non-radioactive compound. Three methods of analysis were employed. In the first method, 50 µl samples of the reaction mixture were pipetted into 0.5 ml of 5% (w/v) trichloroacetic acid. After the mixture had stood for 1 h at 23°C, 50 µl of a 1% (w/v) solution of bovine serum albumin was added. The precipitate was washed three times with 5% trichloroacetic acid and finally dissolved in 0.1 M-

NaOH. A sample of the latter solution was subjected to scintillation counting of radioactivity.

In the second method, the reaction mixture was dialysed extensively at 4°C against 2 litres of 50 mM-Tris/HCl buffer, pH 7.6 for 24 h with changes of buffer every 6 h. The radioactivity content of the dialysed enzyme was then measured.

In the third method, the reaction mixture was passed through a 15 cm × 0.8 cm column of Sephadex G-50 that had been equilibrated with 50 mM-Tris/HCl buffer, pH 7.6. Fractions of volume 0.5 ml were collected. Radioactivity and protein concentrations were determined by analysis of 100 µl samples.

Measurement of radioactivity

The aqueous samples were mixed with 5 ml of 10% (v/v) Biosolv (Beckman Instruments, Palo Alto, CA, U.S.A.) and 0.7% (w/v) 2,5-diphenyl-oxazole in toluene and their radioactivities counted in a liquid-scintillation spectrometer.

Measurement of protein concentration

Protein was determined by the method of Lowry *et al.* (1951), with succinyl-CoA synthetase as standard (Bowman & Nishimura, 1975).

Determination of free thiol groups

In principle, the method is based on that of Ellman (1959). The reaction mixture consisted of 100 mM-Tris/HCl (pH 8.0)/0.1 mM-EDTA/1% (w/v) sodium dodecyl sulphate containing 1.57 nmol of succinyl-CoA synthetase in a total volume of 1 ml. To this 100 µl of 10 mM-5,5'-dithiobis-(2-nitrobenzoate) in 50 mM-sodium phosphate buffer, pH 7, was added, and the reaction was monitored spectrophotometrically at 412 nm.

Results

A prerequisite for inactivation by 5'-FSBA was preincubation of succinyl-CoA synthetase in the presence of dimethylformamide, the solvent that was used with the inhibitor (Colman *et al.*, 1977). Thus preincubation of up to 2 h was necessary for optimal inactivation by 5'-FSBA to occur. Shorter intervals of preincubation led to either a partial inactivation or none at all. It should be stressed at the outset that incubation of succinyl-CoA synthetase with 40% (v/v) dimethylformamide (without 5'-FSBA) for 2 h resulted in no loss of catalytic activity. Further, no significant changes in the fluorescence emission spectrum or the u.v.-absorption spectrum of the enzyme occurred in 40% (v/v) dimethylformamide. It would appear, then, that gross changes in the secondary and tertiary structure of succinyl-CoA synthetase did not occur at this concentration of dimethylformamide.

The results of a typical experiment, described in Fig. 1, indicate a linear relationship between 5'-FSBA concentration and loss of succinyl-CoA synthetase activity. It was noted that at the higher concentrations some of the inhibitor tended to be precipitated from solution. This may in part explain the advantage to adding the inhibitor in increments, as shown in Table 1. Thus it can be seen that 6 mM 5'-FSBA, when added in two instalments, totally inactivated the enzyme, whereas the same amount of inhibitor added at one time gave 70% inhibition (see Fig. 1). It is important to mention that the rate of inactivation upon each incremental addition of 5'-FSBA was so rapid as to defy measurement of its kinetics.

Almost complete protection of the enzyme against inactivation by 5'-FSBA was afforded by the addition of either 5 mM-ATP or 5 mM-ADP during the final 30 min of preincubation with dimethylformamide (Table 2). This was consistent with modification by 5'-FSBA of the ATP(ADP)-binding site(s). On the other hand, addition of desulpho-CoA, an analogue of CoA, had no effect on the

inactivation of succinyl-CoA synthetase by 5'-FSBA.

Additional criteria were sought to establish that the action of 5'-FSBA in this system was that of an affinity-labelling agent. For this purpose, both *p*-fluorosulphonyl[¹⁴C]benzoyl adenosine and *p*-fluorosulphonylbenzoyl[2-³H]adenosine were synthesized. However, incubation of succinyl-CoA synthetase with either labelled species of 5'-FSBA led to insignificant binding of radioactivity to the protein, despite the fact that inactivation of the enzyme had occurred. The use of 5'-FSBA labelled in the benzoyl group assured us that after reaction of 5'-FSBA with the enzyme the adenosine moiety was not cleaved by hydrolysis. Assessment of labelling of succinyl-CoA synthetase by radioactive 5'-FSBA was made by three methods, which are described in the Materials and methods section. Being reasonably confident that the inactivation of the enzyme by 5'-FSBA could not involve covalent linkage of any part of the latter to the enzyme, we considered other explanations.

The involvement of thiol groups in the structure

Fig. 1. Inactivation of *E. coli* succinyl-CoA synthetase by 5'-FSBA

Succinyl-CoA synthetase (0.71 nmol) was preincubated for 2 h at 23°C in a 100 µl volume containing 20 mM-Tris/HCl buffer, pH 7.6, and 40% (v/v) dimethylformamide. 5'-FSBA, dissolved in dimethylformamide, was added to give the final concentrations indicated. Enzyme activities were measured 10 min later.

Table 1. Effect of adding 5'-FSBA in increments on the inactivation of succinyl-CoA synthetase

Succinyl-CoA synthetase was preincubated in dimethylformamide, precisely as described in Fig. 1 legend. 5'-FSBA, dissolved in dimethylformamide, was added in two equal portions 10 min apart to give the final concentration indicated. Enzyme activities were measured 10 min after each addition.

Final concn. of 5'-FSBA (mM)	Activity retained (%)	
	After first addition	After second addition
2	82	75
4	71	39
6	54	0

Table 2. Effect of ligands on the inactivation of succinyl-CoA synthetase by 5'-FSBA

Succinyl-CoA synthetase (0.71 nmol) was incubated for 90 min at 23°C in a 100 µl volume containing 20 mM-Tris/HCl buffer, pH 7.6, and 40% (v/v) dimethylformamide. Ligands were added at the concentrations indicated and the incubation was continued for 30 min. Finally, 5'-FSBA was added at a final concentration of 4 mM. Enzyme activity was measured after 10 min at 23°C.

Addition during preincubation	Activity retained (%)
None	56
ADP (5 mM)	85
ATP (5 mM)	97
Desulpho-CoA (1 mM)	56

and function of succinyl-CoA synthetase has been well documented (Nishimura *et al.*, 1973, 1975, 1976, 1980; Collier & Nishimura, 1978). It has also been shown that 5'-FSBA and 5'-*p*-fluorosulphonylbenzoylguanosine can act to modify thiol groups of rabbit muscle pyruvate kinase chemically through disulphide bond formation (Annamalai & Colman, 1981; Tomich *et al.*, 1981).

We therefore measured titratable thiol groups of succinyl-CoA synthetase before and after treatment of the enzyme with 5'-FSBA. The results of this study are shown in Fig. 2. The straight line relating residual enzyme activity to thiol groups consumed was extrapolated to 100% enzyme activity to give 1.7 apparently non-essential thiol groups and to 0% residual activity to give a total of 3.7 thiol groups consumed per $\alpha\beta$ -dimer. The difference between these numbers gives two thiol groups that appear to be important for activity per $\alpha\beta$ -dimer, or for such groups per native $\alpha_2\beta_2$ enzyme. Plotting of the residual activity against number of the latter thiol groups modified, in accordance with the method of Tsou (1962), gave results that are consistent with the essentiality of only one of the two thiol groups. Interestingly, activity was not restored by incubation of the modified enzyme with 100 mM-dithiothreitol at pH 7.5 after the dimethylformamide and excess of inhibitor had been removed. Further, examination of enzyme inhibited by 5'-FSBA by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under

non-reducing conditions revealed that no polymers were formed, ruling out the possibility of inter-subunit cross-linking through disulphide bond formation.

Inability of dithiothreitol to re-activate the 5'-FSBA-treated enzyme might be due to inaccessibility of disulphide bonds to the reducing agent. We therefore reasoned that unfolding of the protein might increase the likelihood that such a reaction might take place. Pearson & Bridger (1975) have demonstrated that α - and β -subunits of succinyl-CoA synthetase can be separated from each other in acidic urea on a Sephadex G-150 column, mixed and then refolded in the presence of thiol to yield active enzyme. We carried out unfolding and refolding of 5'-FSBA-treated enzyme and control enzyme without separation of subunits, as shown in Table 3. The results of this experiment show that a slight re-activation of the 5'-FSBA-treated enzyme occurred (specific activity of 57 $\mu\text{mol}/30\text{ min per mg}$) at 0.1 mM-dithiothreitol, the concentration used by Pearson & Bridger (1975). However, at higher

Fig. 2. Determination of total thiol groups in *E. coli* succinyl-CoA synthetase after modification by 5'-FSBA. Inactivation of succinyl-CoA synthetase was carried out as described in Fig. 1 legend and Table 1 legend, but at twice the scale. Thiol groups were determined quantitatively as described in the Materials and methods section.

Table 3. Effect of dithiothreitol on restoration of succinyl-CoA synthetase activity during refolding

The enzyme preparation was placed in a volume of 20 μl containing 6 M-urea, 5% (v/v) acetic acid and 0.1 mM- Na_2EDTA . This was diluted with 250 μl of 'refolding solution', as described by Pearson & Bridger (1975), except that twice as much Tris buffer was used and the concentration of dithiothreitol was varied. Incubation was carried out at 25°C for 1 h. Samples of each incubation solution were then assayed for succinyl-CoA synthetase activity. The original specific activities were as follows: 5'-FSBA-treated and untreated enzyme 885 $\mu\text{mol}/30\text{ min per mg}$; enzyme from which the subunits were derived, 610 $\mu\text{mol}/30\text{ min per mg}$. The subunits were mixed in an equimolar ratio. Other details are described in the Materials and methods section.

Enzyme preparation and concn. of dithiothreitol	Specific activity ($\mu\text{mol}/30\text{ min per mg}$)
5'-FSBA-treated enzyme (10.6 μg)	
0.1 mM	57
1.0 mM	312
10 mM	354
100 mM	414
Untreated enzyme (10.4 μg)	
0.1 mM	187
1.0 mM	419
10 mM	434
100 mM	485
α -Subunit (3.3 μg) plus β -subunit (4.3 μg)	
0.1 mM	455
1.0 mM	671
10 mM	691
100 mM	770

concentrations of the reducing agent significantly greater re-activation was achieved. Compared with the untreated enzyme, which had been exposed to dimethylformamide but not to 5'-FSBA, 85% of succinyl-CoA synthetase activity was restored at 100 mM-dithiothreitol. A similar effect of dithiothreitol was seen in the reconstitution of the enzyme from isolated α - and β -subunits. In this case, the reconstituted enzyme had a higher specific activity than the native enzyme. We have noted that, in their more recent work, Wolodko *et al.* (1981) used 1 mM-dithiothreitol in the reconstitution of the enzyme.

Discussion

Preincubation of succinyl-CoA synthetase with 40% (v/v) dimethylformamide was required for inactivation of the enzyme by 5'-FSBA to take place. The basis for this phenomenon is not understood. However, it was clear from fluorescence and u.v.-spectroscopy studies that gross changes in the enzyme's structure did not occur. The enzyme was protected against inactivation by ATP and ADP. On the other hand, the CoA analogue desulpho-CoA, which has been shown to protect succinyl-CoA synthetase against thiol-group modification by CoA thiosulphonate (Collier & Nishimura, 1978; Nishimura *et al.*, 1982), did not protect the enzyme against 5'-FSBA. The data are consistent with modification of the enzyme at the ATP(ADP)-binding site. Unfortunately, the rapidity with which the modification took place precluded measurement of the rate of inactivation.

Use of 5'-FSBA labelled in either the adenosine or the fluorosulphonylbenzoyl moiety made it possible to determine unequivocally that the inactivation did not involve incorporation of either residue or derivative into the inactive enzyme species. Thus it was logical to consider the possibility that disulphide bond formation had occurred, as seen with rabbit muscle pyruvate kinase (Annamalai & Colman, 1981; Tomich *et al.*, 1981). Indeed, while a total of 3.7 thiol groups per $\alpha\beta$ -dimer disappeared during complete inactivation of succinyl-CoA synthetase by 5'-FSBA, the loss of only two of these appeared to be critical to the inactivation process (Fig. 2). The other 1.7 groups appeared to be more reactive, but less critical for catalytic activity. With respect to the latter, we have observed disappearance of thiol groups without accompanying loss of succinyl-CoA synthetase activity in the treatment of the enzyme with permanganate ion (Nishimura *et al.*, 1976).

The failure of simple incubation of 5'-FSBA-inactivated succinyl-CoA synthetase with 100 mM-dithiothreitol to restore activity was surprising. The fact that unfolding and refolding of the enzyme, under the conditions described by Pearson &

Scheme 1. *Proposed mechanism of inactivation of succinyl-CoA synthetase by 5'-FSBA*

Bridger (1975), was required to achieve this is consistent with the formation of disulphide bonds that were inaccessible to the reducing agent.

A model that summarizes the findings of this work is presented in Scheme 1. Proximity of a thiol group (S_1) to 5'-FSBA bound to the ATP(ADP)-binding site favours a nucleophilic attack by the thiol group (represented as a thiolate anion) on the sulphonyl group, releasing fluoride ion. This reaction results in the formation of a thiosulphonate intermediate (II). A neighbouring thiol group (S_2) displaces the sulphonyl moiety, yielding the disulphide, which represents an inactive form of enzyme. This is in agreement with the model proposed by Colman and co-workers (Annamalai & Colman, 1981; Tomich *et al.*, 1981) for modification of rabbit muscle pyruvate kinase.

The data in the present paper provide the first quantitative indication of the involvement of two closely spaced thiol groups, one of which is required for catalytic activity, at the ATP(ADP)-binding site of succinyl-CoA synthetase. The subunit location of the disulphide bond formed remains to be established. From what is known, one of the thiol groups is likely to be located on the β -subunit (Collier & Nishimura, 1978). Since the phosphorylation site of succinyl-CoA synthetase is on the α -subunit, there exists the intriguing possibility, also suggested by related work (Ball & Nishimura, 1980), that ATP- and ADP-binding subsites are located on both α - and β -subunits. Whether the thiol group on the β -subunit is the essential thiol group that is modified by the action of 5'-FSBA remains to be demonstrated.

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