Dimeric Pig Heart Succinate-Coenzyme A Transferase Uses Only One Subunit to Support Catalysis[†]

Adrian J. Lloyd[§] and Peter M. Shoolingin-Jordan*

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton, Hampshire, S016 7PX, United Kingdom

Received September 14, 2000; Revised Manuscript Received December 29, 2000

ABSTRACT: Pig heart succinate-coenzyme A transferase (succinyl-coenzyme A: 3-oxoacid coenzyme A transferase; E. C. 2.8.3.5.), a dimeric enzyme purified by affinity chromatography on Procion Blue MX-2G Sepharose, reacts with acetoacetyl-coenzyme A to form a covalent enzyme-coenzyme A thiolester intermediate in which the active site glutamate (E344) of both subunits each forms thiolester links with coenzyme A. Reaction of this dimeric enzyme-coenzyme A species with sodium borohydride leads to inactivation of the enzyme and reduction of the thiolester on both subunits to the corresponding enzyme alcohol, as judged by electrospray mass spectrometry. Reaction of the dimeric enzyme-coenzyme A intermediate with either succinate or acetoacetate, however, results in only one-half of the coenzyme A being transferred to the acceptor carboxylate to form either succinyl-coenzyme A or acetoacetyl-coenzyme A. Reaction of this latter enzyme species with borohydride caused no loss of enzyme activity despite the reduction of the remaining half of the enzyme-coenzyme A thiolester to the enzyme alcohol. That this catalytic asymmetry existed between subunits within the same enzyme dimer was demonstrated by showing that the enzyme species, created by successive reaction with acetoacetyl-coenzyme A and succinate, bound to Blue MX-2G Sepharose through the remaining available active site and could be eluted as a single chromatographic species by succinyl-coenzyme A. It is concluded that while both of the subunits of the succinate-coenzyme A transferase dimer are able to form enzyme-coenzyme A thiolester intermediates, only one subunit is competent to transfer the coenzyme A moiety to a carboxylic acid acceptor to form the new acyl-coenzyme A product. The possible structural basis for this catalytic asymmetry and its mechanistic implications are discussed.

Succinate-coenzyme A transferase catalyses the reversible transfer of coenzyme A from a donor thiolester species to an acceptor carboxylic acid. For the α_2 dimeric mitochondrial porcine heart enzyme (*1*−*3*), the donor and acceptor substrates in vivo are believed to be succinyl-coenzyme A and acetoacetate respectively:

succinyl-coenzyme A + acetoacetate ↔
succinate + acetoacetyl-coenzyme A

Acetoacetate is generated by hepatic catabolism of fatty acids and is an important fuel source in tissues such as the heart and the brain, where, after conversion to acetoacetyl-coenzyme A, it is converted by thiolase to acetyl-coenzyme A and oxidized in the citric acid cycle. Succinate-CoA transferase thus acts as a metabolic "conduit", allowing the extra-hepatic tissues to harness hepatic fatty acid oxidation for their own catabolic requirements (*2*). The importance of this enzyme in man is highlighted by the acute inherited metabolic disorder caused by its genetic deficiency (*4*).

Coenzyme A transferases with widely differing substrate specificities also function in bacterial metabolism where the substrate specificity is defined by the metabolic role of the enzyme (*5*−*8*).

Central to the mechanism catalyzed by coenzyme A transferases is the transfer of the coenzyme A moiety from the donor thiolester, e.g., succinyl-coenzyme A, to a glutamyl moiety of the enzyme (*9*−*12*) shown in Scheme 1. This has been demonstrated not only for the porcine enzyme, where the residue in question is E344, but also for the bacterial transferases characterized from *Escherichia coli* and *Acidaminococcus fermentans* (*13*−*15*). This, and the conservation of this catalytic glutamyl residue in the amino acid sequences of all coenzyme A transferases thus sequenced, suggests that formation of the enzyme coenzyme A thiolester is a universal feature of coenzyme A transferase catalysis (*8*, *16*).

Although it has not been demonstrated directly, the formation of the enzyme-coenzyme A thiolester intermediate is believed to involve nucleophilic attack by the 5-carboxyl of E344 on the thiolester carbonyl of succinyl or acetoacetyl-CoA to form the corresponding succinyl or acetoacetyl-enzyme anhydride with the elimination of coenzyme A (Scheme 1; refs *17*−*19*). The coenzyme A thiol then attacks the succinyl or acetoacetyl-enzyme anhydride at the E344 carbonyl carbon to expel succinate or acetoacetate as the first

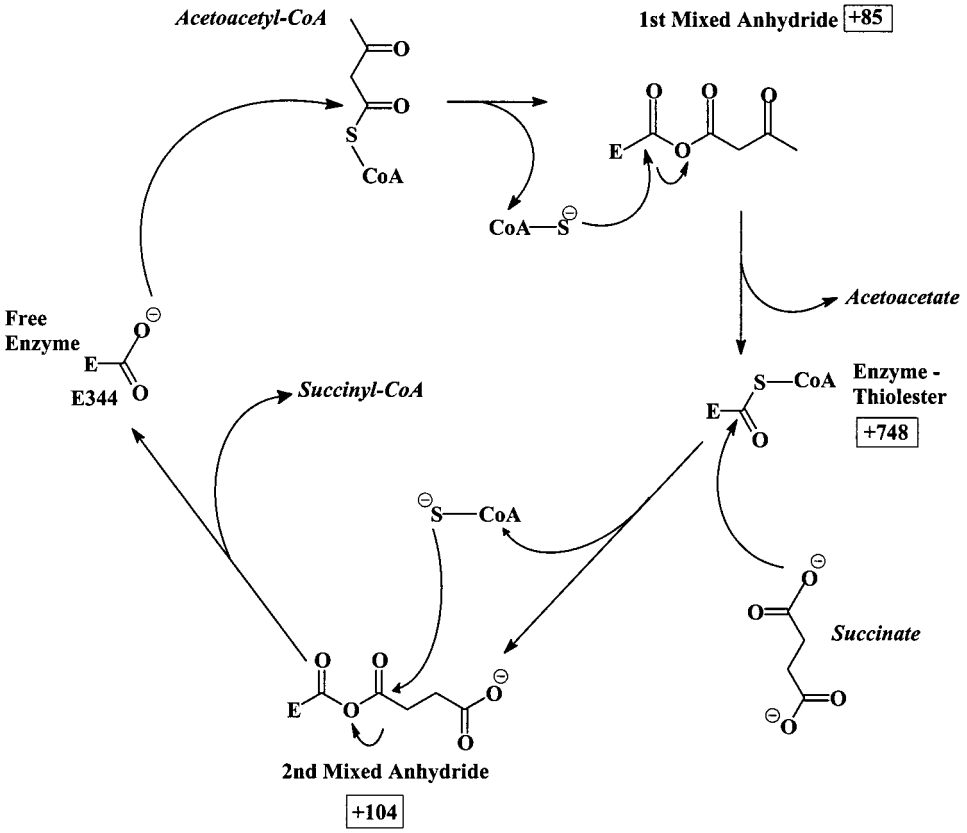
[†] This work was funded by the BBSRC, MRC, and Wellcome Trust. The funding of mass spectrometry facilities by the Wellcome Trust is acknowledged.

* Corresponding author: Division of Biochemistry and Molecular Biology, School of Biological Sciences, Biomedical Sciences Building, University of Southampton, Bassett Crescent East, Southampton, Hampshire, S016 7PX, United Kingdom. E-mail: pmsj@soton.ac.uk. Telephone: +44-2380-594322. Fax: +44-2380-594459.

[§] Present address: Department of Chemistry, Gibbet Hill Road, University of Warwick, Coventry, CV4 7AL, United Kingdom.

10.1021/bi002169v CCC: $20.00
Published on Web 02/02/2001

Scheme 1: Catalytic Mechanism of Transfer of Coenzyme A from Acetoacetyl-Coenzyme A to Succinate by Succinate-Coenzyme A Transferase[a]



[a] The catalytic cycle in the direction of transfer of coenzyme A from acetoacetyl-coenzyme A to succinate is shown. E344 represents the transferase polypeptide catalytic glutamate residue. The boxed values represent the changes in molecular mass (Da) of the polypeptide anticipated during various stages of catalysis relative to that of the mass of the unreacted enzyme polypeptide.

product, resulting in the formation of the enzyme-coenzyme A thiolester intermediate (Scheme 1). Covalent modification studies have revealed that the formation of this intermediate involves a large conformational change in the structure of the protein (*20*).

Transfer of coenzyme A from the enzyme to the acceptor carboxylate substrates acetoacetate or succinate, involves a reversal of the steps involved in formation of the enzyme-coenzyme A thiolester intermediate. Here, a carboxyl oxygen of the acceptor carboxylate attacks the carbonyl of the enzyme thiolester to reform an anhydride between the enzyme E344 carboxyl and the acid. The latter is then attacked by the coenzyme A thiol at the acceptor carbonyl carbon. This regenerates the free enzyme glutamate carboxyl and the succinyl- or acetoacetyl-coenzyme A thiolester product (*17*) as shown in Scheme 1.

The catalytic mechanism of the porcine heart coenzyme A transferase involves intermediates with changes in polypeptide molecular mass that should be amenable to analysis by electrospray mass spectrometry. In this paper, therefore, we used mass spectrometry to study the formation of the enzyme-coenzyme A thiolester from succinate-coenzyme A transferase and acetoacetyl-coenzyme A. We further monitored the transfer of this coenzyme A moiety to succinate and acetoacetate. Our results demonstrate that the porcine heart coenzyme A transferase dimer displays a quantitative catalytic asymmetry between its subunits. The structural and mechanistic implications of these data are discussed.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma with the exception of protein assay dye reagent concentrate and polyvinylidene fluoride membranes (Bio-Rad Laboratories, Ltd.); sodium succinate (BDH); formic acid (Fluka); glycerol (Fisons); sodium borohydride (Aldrich); acetonitrile (Fischer); and pre-packed Sephadex G-25 columns (PD-10 columns; Amersham-Pharmacia Biotech Ltd); dialysis tubing (Medicell International Ltd.) was boiled for 1 h in 10 mM disodium ethylenediamine tetracetate (EDTA)[1] adjusted to pH 7.4 before use. The triazine dyes Procion HE-4R and Procion MX-2G were from ICI Inc., Delaware. These were immobilized on Sepharose 4B according to ref *21*. Succinyl-coenzyme A was prepared immediately before use by acylation of the coenzyme A thiol with succinic anhydride according to ref *22*.

Methods. (a) Succinate-Coenzyme A Transferase Assays. All assays were carried out at 30 °C and, unless specified otherwise, followed the enzyme and succinate-dependent consumption of the Mg:acetoacetyl-coenzyme A complex spectrophotometrically at 303 nm. Assays contained 50 mM

[1] Abbreviations: Da: dalton; DTT: D,L-dithiothreitol; DEAE: diethylaminoethyl; EDTA: disodium ethylenediamine tetracetate; MOPS: 3(*N*-morpholino)propanesulphonic acid; PMSF: phenylmethanesulphonyl fluoride; SDS−PAGE: sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tricine: *N*-tris(hydroxymethyl)-methylglycine; Tris-Cl: 2-amino 2-hydroxymethyl propan-1,3-diol hydrochloride.

2-amino 2-hydroxymethyl propan-1,3-diol hydrochloride (Tris-Cl), pH 8.5, 10 mM $MgCl_2$, 50 μM acetoacetyl-coenzyme A, and enzyme. Control rates were monitored for one minute, prior to the addition of succinate to 50 mM to initiate transferase activity. Otherwise, assays followed the enzyme and acetoacetate-dependent production of acetoacetyl-coenzyme A from succinyl-coenzyme A and contained 50 mM Tris-Cl, pH 8.5, 10 mM $MgCl_2$, 50 mM acetoacetate and enzyme. After determining any background rate, transferase activity was initiated by addition of succinyl-coenzyme A to concentrations specified in the text. Enzyme rates were calculated assuming an absorption coefficient for acetoacetyl-coenzyme A of 20 500 M cm^{-1} (*23*).

(b) Protein Determinations. These were performed using a protein assay kit from Bio-Rad Ltd., according to ref *24*.

(c) Purification of Succinate-Coenzyme A Transferase from Pig Heart. All steps were performed at 4 °C. Porcine heart (950 g) obtained from the local abattoir was homogenized at a ratio of 1 g of tissue/1.5 mL of ice-cold 50 mM potassium phosphate, pH 7.4, containing 1 mM EDTA, 0.2 M KCl, 1 mM D,L-dithiothreitol (DTT) and 0.2 mM PMSF (phenylmethanesulfonyl fluoride) (homogenization buffer) in a Waring blender operated on full power for 10 bursts of 30 s duration. The homogenate was supplemented with a further 1.5 mL of homogenization buffer/g of tissue and was blended again, as before except with the blender at half power. The final homogenate was then clarified by centrifugation at 12300*g* for 1 h. The supernatant was then filtered through muslin.

Freshly ground ammonium sulfate was added to the supernatant with overhead stirring to a final concentration of 40% of saturation. After 45 min, the suspension was centrifuged for 1 h at 12300*g*, and the pellet was discarded. The ammonium sulfate concentration was raised to 65% of saturation and stirred for 45 min. The suspension was centrifuged for 1 h at 12300*g* and the pellet was resuspended to a volume of less than 250 mL in 5 mM potassium phosphate adjusted to pH 7.0 containing 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 10% (v/v) glycerol (low phosphate buffer). The resuspended pellet was dialyzed overnight against 5 L of low phosphate buffer and re-dialyzed for the same period again in a fresh 5 L volume of low phosphate buffer. Precipitated protein was then removed by centrifugation for 1 h at 100000*g*.

The supernatant was applied to a 5 × 50 cm column of diethylaminoethyl (DEAE) Sephacel anion-exchange resin equilibrated in low phosphate buffer. The enzyme was eluted in the same buffer. Active fractions were then concentrated over an Amicon YM-30 membrane by ultrafiltration and then dialyzed overnight against 20 mM *N*-tris(hydroxymethyl)-methyl-glycine (Tricine), pH 8.2, containing 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 10% (v/v) glycerol (Tricine buffer).

The enzyme solution was loaded onto a 5 × 30 cm column of Yellow HE-4R coupled to Sepharose 4B and the column was then washed extensively with Tricine buffer to remove unbound protein. Succinate-coenzyme A transferase was then eluted using a linear 2.2 l gradient from 0.0 to 0.4 M KCl in Tricine buffer. Active fractions were pooled and dialyzed overnight against 20 mM 3(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.2, containing 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 10% (v/v) glycerol (MOPS buffer).

The succinate-coenzyme A transferase preparation was then loaded onto a 5 × 7 cm column of Blue MX-2G coupled to Sepharose 4B. The column was washed with MOPS buffer to remove unbound protein. The transferase was then eluted at 180 mL/h by MOPS buffer containing 0.3 mM freshly prepared succinyl-coenzyme A. The enzyme was then dialyzed overnight against 5 L of MOPS buffer. The buffer was replaced and the enzyme was redialyzed again overnight.

Finally, the protein was applied to a 2.5 × 14 cm column of Blue MX-2G coupled to Sepharose 4B previously equilibrated in MOPS buffer overnight. After washing the column with MOPS buffer, the enzyme was eluted with a linear 500 mL gradient of 0.0 to 0.5 M KCl in MOPS buffer to yield the final product of the purification.

(d) Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) Analysis. Proteins were analyzed on polyacrylamide gels (8.5% total acrylamide unless specified otherwise) according to ref *25*. Gels were stained with 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol and 10% (v/v) acetic acid or silver as described in ref *26*. For experiments where the electrophoresed species were to be subsequently analyzed in a second SDS−PAGE experiment, gels were stained with copper II chloride, and the protein species of interest were then excised and destained as in ref *27*. The polyacrylamide gel fragments were then washed at 40 °C in water for 20 min and then in acetonitrile for 20 min. The dehydrated fragments were allowed to dry overnight at room temperature and were then placed in the wells of a freshly prepared 8.5% SDS−PAGE gel, overlain with loading buffer (*25*) and allowed to rehydrate for 1 h prior to re-electrophoresis.

(e) N-Terminal Sequencing. Proteins separated by SDS−PAGE were electroblotted onto poly(vinylidene difluoride) membranes and subjected to *N*-terminal sequencing by automated Edman degradation as described in refs *28* and *29*.

(f) Succinate-Coenzyme A Transferase Incubations with Substrates and Preparation of Samples for Analysis by Electrospray Mass Spectrometry. Incubations were carried out at 25 °C in a final volume of 100 μL, in 50 mM Tris-Cl, pH 8.5, containing 1 mM EDTA. Succinate-coenzyme A transferase was present in all incubations at a subunit concentration of 17.8 μM (0.93 mg/mL). Incubations were carried out for 2 min in the presence or absence of 19.6 μM acetoacetyl-coenzyme A (1:1.1 molar ratio of enzyme subunit/acetoacetyl-coenzyme A). For some experiments, the acetoacetyl-coenzyme A incubation was followed by incubation for 10 min with either freshly prepared 5 mM sodium borohydride or 0.2 M succinate or 0.2 M acetoacetate as described in Results. Additionally, samples of enzyme treated sequentially with 19.6 μM acetoacetyl-coenzyme A for 2 min, then 0.2 M succinate for 10 min, were also subsequently treated for 10 min with 5 mM sodium borohydride.

After the appropriate incubation time, samples of enzyme were plunged immediately into ice and rapidly desalted into 1 mM HCl at 4 °C using pre-packed PD-10 columns of Sephadex G-25. Fractions were assayed for protein, and the peak fractions were diluted with an equal volume of 98% (v/v) acetonitrile and 2% (v/v) formic acid. Preparation times for samples for electrospray mass spectrometry by this method were typically less than 12 min from the termination of the incubation.

Table 1: Purification of Succinate-Coenzyme A Transferase from Pig Heart[a]

step	vol (mL)	total activity (μmol/min)	total protein (mg)	specific activity (μmol min^{-1} mg^{-1})	recovery (%)	purification (-fold)
crude extract	2610	11293	67818	0.17	100	none
$(NH_4)_2SO_4$ fractionation	305	9755	7360	1.33	86.3	7.9
DEAE Sephacel	317	9000	1409	6.37	79.6	38.3
Sepharose 4B/Yellow HE-4R (0−0.4 M KCl)	277	8468	101.6	83.34	75	499.1
Sepharose 4B/Blue-MX2G (0.3 mM succinyl-coenzyme A)	104	8499	39.5	215.21	75.3	1292
Sepharose 4B/Blue-MX2G (0−0.5 M KCl)	159	7394	33.1	225.80	65.5	1338

[a] Purification of the enzyme was conducted from 950 g of pig heart.

(g) Electrospray Mass Spectrometric Analysis. Samples (11.5−18.2 pmol of protein) were injected in 10 μL aliquots into an acetonitrile/water solvent stream directed into the electrospray source of a V. G. Quattro II triple quadrupole mass spectrometer, operating with a source temperature of 80 °C at cone, capillary, and HV lens voltages of 30 V, 3 kV, and 0.63 kV, respectively. Mass spectra in the range 600−1600 *m*/*z* were derived from data collected over at least 100 scans of 5 s duration each. Mass spectra were then interpreted over the mass range 45 000 to 55 000 with the Maximum Entropy algorithm (Fisons/V. G.) using equine myoglobin as a calibrant.

RESULTS

Purification of Porcine Heart Succinate-Coenzyme A Transferase. Succinate-coenzyme A transferase was purified in a five-step procedure summarized in Table 1. From this it can be seen that the key step in the purification is the Yellow HE-4R/Sepharose 4B gradient elution with potassium chloride. This stage yielded a 12-fold purification, with overall yields of enzyme activity being typically between 50 and 70% with 30 to 40 mg of protein being obtained from 950 g of tissue. The specific activity of the purified enzyme (± standard deviation) was 214.6 ± 28 μmol min^{-1} mg^{-1} ($n = 7$ purifications), with overall purification factors typically exceeding 1000-fold.

SDS−PAGE analysis of samples from each stage of the purification revealed that the final product of the procedure apparently contained two protein species with molecular masses close to 55 000 Da (Figure 1, lanes 6 and 7). The two species clearly co-purified (Figure 1, lanes 4−6) and were present even in purifications where the protease inhibitors 0.2 mM PMSF, 1 mM EDTA, 1 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′*N*′-tetracetic acid, 2 μM pepstatin, or 2 μM leupeptin were present.

Origins of the Apparent Molecular Heterogeneity within Purified Succinate-Coenzyme A Transferase. To determine the identity of the two polypeptide species obtained from the succinate coenzyme A transferase purification, both species were subjected to *N*-terminal sequencing. The results showed that both proteins shared the *N*-terminal sequence Thr−Lys−Phe−Tyr−Thr−Asp−, identical to the sequence predicted from the cDNA sequence of the succinate-coenzyme A transferase mRNA (*14*). Having established that both species were from succinate-coenzyme A transferase, we then addressed the possibility that they might be different conformations of the same polypeptide. The two species separated by SDS−PAGE, were visualized using copper (II) chloride (*27*) and were excised from the gel. After destaining and drying, they were rehydrated in the wells of a fresh

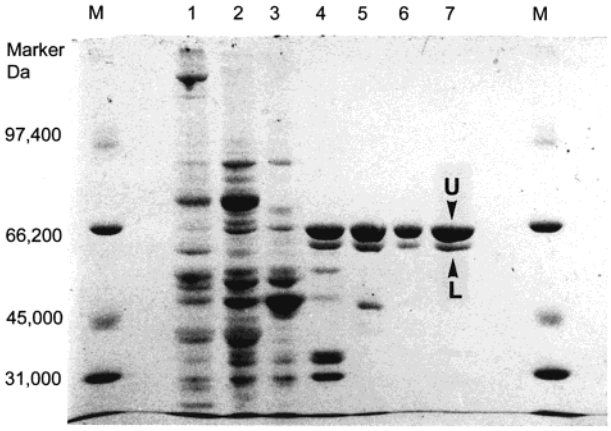


FIGURE 1: SDS−PAGE analysis of the purification of succinate-coenzyme A transferase. Electrophoresis of samples from the purification of the enzyme from pig heart through a Coomassie Blue-stained 8.5% SDS−polyacrylamide gel are shown. Lanes labeled M refer to molecular weight markers rabbit muscle glycogen phosphorylase, bovine serum albumin, hen ovalbumin, and bovine carbonic anhydrase with polypeptide molecular masses of 97 400, 66 200, 45 000, and 31 000 Da, respectively. Lanes 1−6 are samples (μg of protein loaded in square brackets) of crude extract [50], 40−65% saturation ammonium sulfate fraction [50], DEAE sephacel eluate [30], Sepharose-4B/Yellow HE−4R eluate [20], Sepharose 4B/Blue MX-2G succinyl-coenzyme A eluate [10], and Sepharose 4B/Blue MX-2G 0−0.5 M KCl gradient eluate [10]. Lane 7 is loaded with 10 μg of a sample of the Sepharose 4B/Blue MX-2G 0−0.5 M KCl gradient eluate from another succinate-coenzyme A transferase purification. The labels U and L correspond to the apparent higher and lower molecular weight polypeptide species that co-purify throughout the purification of succinate-coenzyme A transferase.

SDS−PAGE gel through which they were re-electrophoresed. This showed that the apparent higher molecular mass (Figure 2, panel a, lane U) and the apparent lower molecular mass transferase species (Figure 2, panel a, lane L) each generated a proportion of apparent lower molecular mass species and apparent higher molecular mass species, respectively, and were thus probably different conformers of the same polypeptide.

We ascertained that both polypeptides were enzymically active by exploiting the ability of the enzyme, as its coenzyme A-thiolester, to undergo autocatalytic cleavage to give fragments with molecular masses of ~37 000 and ~18 000 Da at temperatures ≥ 70 °C (*11*, *12*, *29*). SDS−PAGE in 10% polyacrylamide of samples of succinate-coenzyme A transferase that were treated with 2 mM succinyl-coenzyme A to form the enzyme thiolester and boiled for 2 min in disruption buffer, revealed that two species of approximately 40 000 Da were generated from the 55 000 Da polypeptides (Figure 2, panel b, lane 2). Lower molecular mass material was also generated, but this was

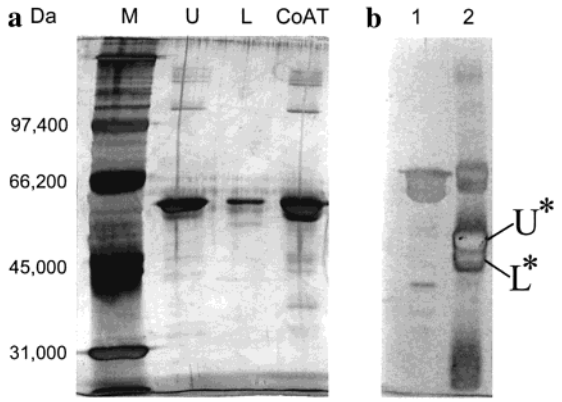


FIGURE 2: SDS−PAGE analysis of different electrophoretic species of porcine succinate-coenzyme A transferase Panel a: silver-stained 8.5% SDS−polyacrylamide gel of the re-electrophoresis of the higher and lower molecular weight succinate-coenzyme A transferase species isolated from a copper-stained 8.5% SDS−polyacrylamide gel that had been loaded with 10 μg/lane of transferase. Lane M: molecular weight markers (see legend to Figure 1 for identity of these standards); lane U: higher molecular weight polypeptide species; lane L: lower molecular weight species; lane COAT: 3 μg of previously un-electrophoresed succinate-coenzyme A transferase. Panel b: silver-stained 10% SDS−polyacrylamide gel demonstrating the autocatalytic cleavage of the succinate-coenzyme A transferase polypeptide by succinate-coenzyme A: lane 1: succinate-coenzyme A transferase preincubated for 1 min in MOPS buffer and then boiled for 2 min prior to electrophoresis; lane 2: succinate-coenzyme A transferase preincubated with 2 mM succinate-coenzyme A in MOPS buffer for 1 min prior to boiling for 2 min; U* and L* indicate the ~40 000 Da species generated from the higher and lower molecular weight transferase polypeptides, respectively. Both lanes were loaded with 10 μg of protein.

beyond the resolving power of the gel to focus into well-defined bands (Figure 2, panel b, lane 2). Nevertheless, these data allowed us to confirm that both apparent higher and apparent lower molecular weight species of succinate-coenzyme A transferase originated from the intact native enzyme and that both could form enzyme-coenzyme A intermediates and were thus enzymically active species.

Electrospray Mass Spectrometric Analysis of Succinate-Coenzyme A Transferase and its Catalytic Mechanism. The succinate-coenzyme A transferase catalytic mechanism is believed to involve formation of a number of enzyme−substrate covalent intermediates. Therefore, we employed electrospray mass spectrometry to analyze the catalytic cycle of this enzyme and to observe any mechanistically significant species.

(a) Electrospray Mass Spectrometric Analysis of Native Succinate-Coenzyme A Transferase. Electrospray mass spectrometric analysis of the enzyme, prior to reaction with substrate revealed a series of molecular ions within the *m*/*z* range of 650 to 1400 (Figure 3, panel aii). When this was interpreted by the Maximum Entropy algorithm (Fisons plc), it became apparent that there was a single polypeptide detectable by this technique in our highly purified succinate-coenzyme A transferase preparation with a molecular mass of 52 166.2 ± 0.8 Da (Figure 3a, panel ai). This result is entirely consistent with our conclusion that the behavior of this enzyme on SDS−PAGE is due to generation of two conformers of a single transferase polypeptide, although we cannot, of course, exclude the remote possibility that only one of the species gives rise to a mass on analysis by electrospray mass spectrometry.

(b) Characterization of the Enzyme-Coenzyme A Thiolester of Succinate-Coenzyme A Transferase Catalysis by Electrospray Mass Spectrometry. The only covalent intermediate thus far detected in the catalytic cycle of succinate-coenzyme A transferase is the thiolester formed between the coenzyme A thiol and the 5-carboxyl of E344 (*9*−*12*). Theoretically, this should increase the mass of succinate-coenzyme A transferase polypeptide by 748 Da. Therefore, we analyzed the polypeptide mass of a sample of transferase that had been previously reacted with acetoacetyl-coenzyme A in a 1.0:1.1 molar ratio of enzyme subunit: acetoacetyl-coenzyme A. Interpretation of these data (Figure 3b, panel bii) by the Maximum Entropy algorithm revealed that the mass of the unreacted enzyme polypeptide had been completely replaced by species with masses of 52 914 ± 2.5 Da and 52 962.4 ± 1.1 Da (Figure 3b, panel bi). The species of lower mass was 747.8 Da greater than the mass of the unreacted enzyme and was thus likely to represent the mass of the enzyme-coenzyme A thiolester intermediate (expected $\Delta = +748$ Da). The species of higher mass was 47.3 Da larger than the mass of the presumptive enzyme-coenzyme A thiolester intermediate. As the acetoacetyl-coenzyme A used in these experiments was the disodium salt, it was considered likely that the extra mass resulted from the formation of the disodium adduct of the coenzyme A moiety in the enzyme-coenzyme A thiolester. Indeed, coenzyme A species are known for their ability to form metal ion adducts on analysis by electrospray mass spectrometry (*30*).

To confirm our observation of the formation of the enzyme-coenzyme A thiolester of succinate-coenzyme A transferase, we analyzed the products of the incubation of succinate-coenzyme A transferase with acetoacetyl-coenzyme A after reduction by sodium borohydride. This would be expected to reduce the enzyme thiolester to the corresponding enzyme alcohol with elimination of coenzyme A (*10*). Interpretation of resulting electrospray mass spectrum (Figure 3c, panel cii) by Maximum Entropy revealed a single component with a mass of 52 149 ± 0.3 Da (Figure 3c, panel ci). The loss of the 52 914 and 52 962 Da enzyme-coenzyme A species on treatment with borohydride was consistent with their suspected identity as coenzyme A thiolesters. Further, the reduction of the polypeptide mass of succinate-coenzyme A transferase by 17 Da from 52 166.2 ± 0.8 Da to 52 149.0 ± 0.3 Da was entirely consistent with the overall reduction of the carboxylic acid moiety of E344 to the alcohol (expected $\Delta = 14$ Da).

Our data therefore led us to conclude that both subunits within the succinate-coenzyme A transferase dimer react quantitatively with acetoacetyl-coenzyme A to form the enzyme-coenzyme A thiolester, an intermediate complex central to the catalytic activity of this protein.

(c) Electrospray Mass Spectrometric Analysis Reveals Only Half of the Thioesterified Succinate-Coenzyme A Transferase Subunits Can Transfer Their Coenzyme A Moiety to Carboxylate Acceptor Substrates. Having shown that succinate-coenzyme A transferase forms a coenzyme A thiolester with both subunits of the dimeric enzyme on reaction with acetoacetyl-coenzyme A, we attempted to demonstrate the second half reaction catalyzed by this enzyme, namely, the transfer of coenzyme A from E344 of the enzyme to the acceptor carboxylate. Therefore, after incubation of succinate-coenzyme A transferase with ac-

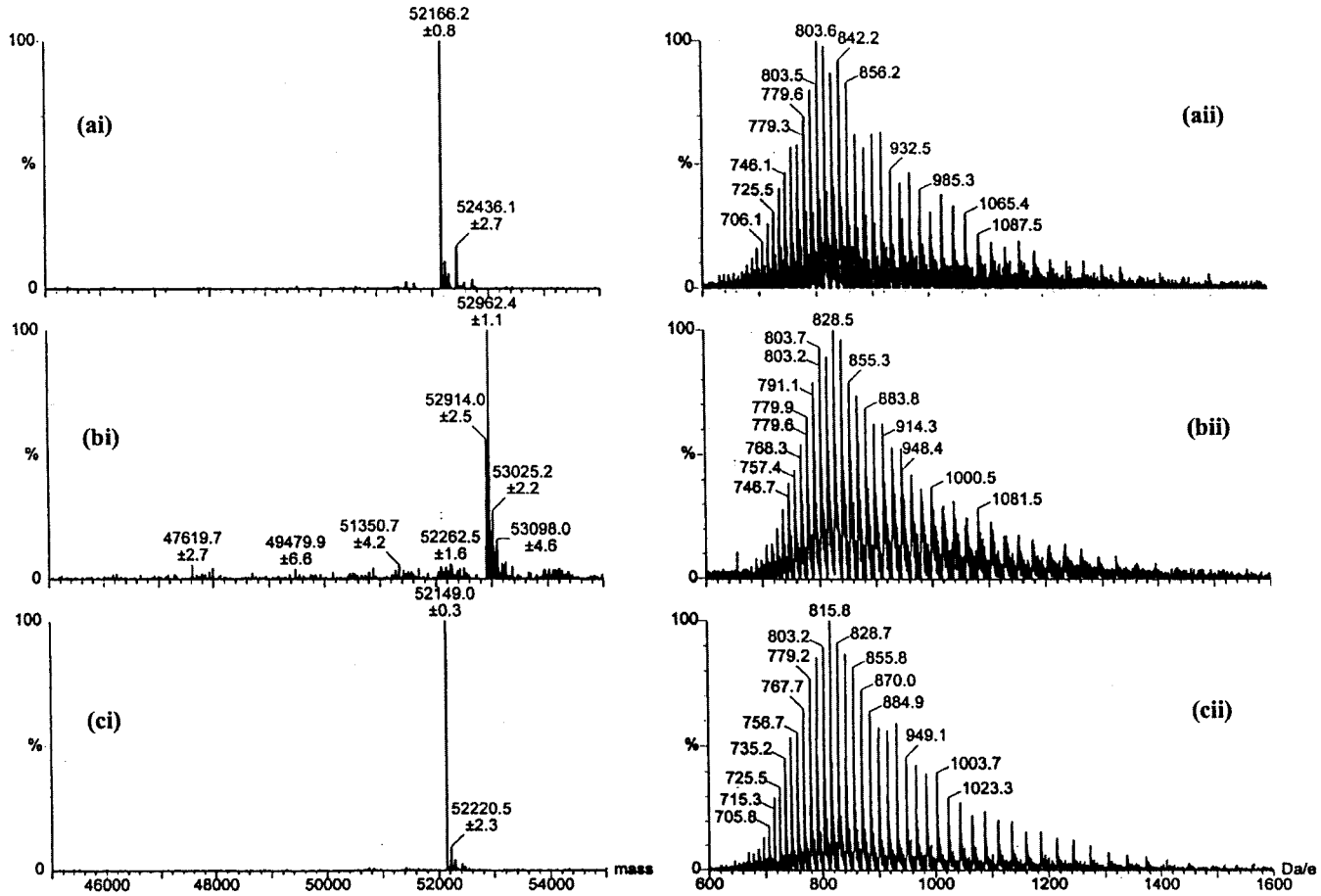


FIGURE 3: Electrospray mass spectrometric analysis of succinate-coenzyme A transferase before and after reaction with acetoacetyl-coenzyme A and after sequential reaction with acetoacetyl-coenzyme A and sodium borohydride Electrospray mass spectra {panels (aii), (bii), and (cii)} and corresponding Maximum Entropy interpretations of these spectra in the mass range 45 000 Da to 55 000 Da {panels (ai), (bi), and (ci)}. Panels (ai-ii), (bi-ii), and (ci-ii) are data obtained, respectively, from injection of 11.5 pmol of succinate-coenzyme A transferase prior to reaction with acetoacetyl-coenzyme A; injection of 11.7 pmol of succinate-coenzyme A transferase after reaction with acetoacetyl-coenzyme A for 2 min in a 1:1.1 molar ratio of transferase polypeptide/acetoacetyl-coenzyme A; and injection of 14.2 pmol of succinate-coenzyme A transferase after reaction with acetoacetyl-coenzyme A as described for (bi-ii) and then treatment with 5 mM freshly prepared sodium borohydride for 10 min.

etoacetyl-coenzyme A, we incubated the enzyme-coenzyme A thiolester with a large excess of sodium succinate (11 240: 1 molar ratio of succinate to enzyme). After 10 min, the protein components were analyzed by electrospray mass spectrometry. The mass spectrum of the enzyme incubated serially with acetoacetyl-coenzyme A and succinate contained two well-defined series as shown in Figure 4a (panel aii). Interpretation of the mass spectrum by Maximum Entropy (Figure 4a, panel ai) revealed that the two species were present in approximately equal proportions. One-half possessed a mass of 52 164.9 ± 5.0 Da similar to that of the native enzyme. The other half was 802 Da larger, being 52 966 ± 2.5 Da, close to that of the disodium adduct of enzyme-coenzyme A intermediate (expected mass = 52 960.2 Da). Similar results were obtained when acetoacetate was used, rather than succinate.

The identity of the 52 966 Da species was probed further with sodium borohydride. Thus, the enzyme was incubated sequentially with acetoacetyl-coenzyme A and succinate and then for 5 min with sodium borohydride. Analysis of the polypeptide products of this incubation by electrospray mass spectrometry revealed that the 52 966 Da species had been eliminated by sodium borohydride, consistent with its existence as a coenzyme A-enzyme thiolester. The resulting species possessed a mass of 52 159.1 ± 1.0 Da (Figure 4b, panel bi), slightly lower than the molecular mass of the native enzyme and close to the average masses of the enzyme and enzyme alcohol.

We had anticipated that we might detect a mass for the unesterified transferase polypeptide and also a mass of equal intensity 14 Da smaller corresponding to the enzyme alcohol generated from reduction of the remaining enzyme-coenzyme A thiolester. However, electrospray mass spectrometry could not resolve the series generated by a 1:1 mixture of enzyme and enzyme alcohol (data not shown). Therefore, the 52 159.1 Da mass obtained on reduction of the transferase species generated by incubation with acetoacetyl-coenzyme A and succinate was most likely contributed to both by the succinate-coenzyme A transferase polypeptide and its counterpart reduced at E344 to the corresponding enzyme alcohol.

Finally, we determined if the loss of only half of the coenzyme A from the central succinate-coenzyme A transferase-coenzyme A thiolester intermediate to succinate was a peculiarity of the enzyme preparation used in these experiments. We obtained results from enzyme from three independent purifications, similar to those reported here, indicating that this phenomenon could not be attributed to an artifact of a spurious batch of enzyme.

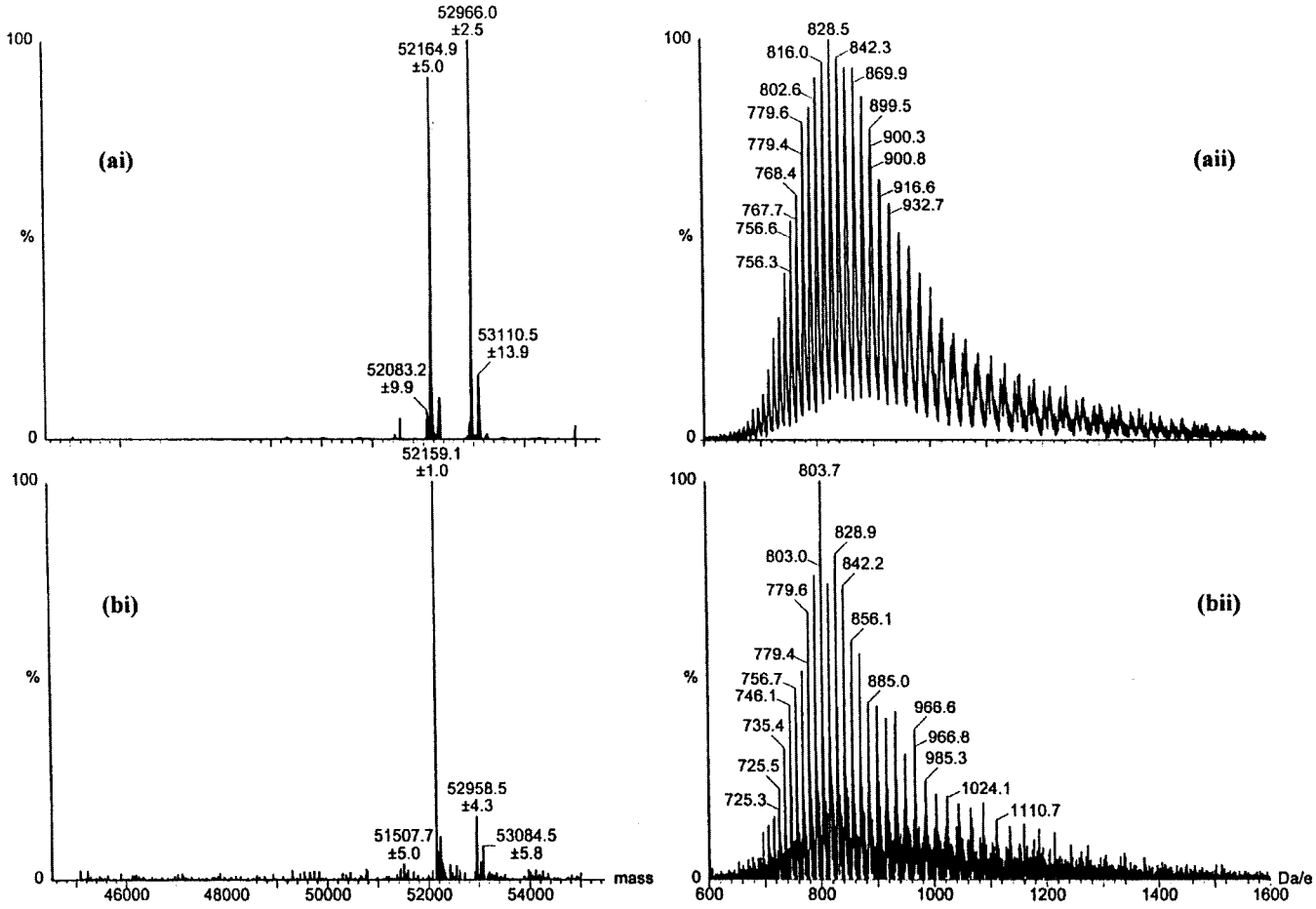


FIGURE 4: Electrospray mass spectrometric analysis of succinate-coenzyme A transferase after sequential reaction with acetoacetyl-coenzyme A and succinate and after sequential reaction with acetoacetyl-coenzyme A, succinate, and borohydride electrospray mass spectra {panels (aii) and (bii)} and corresponding Maximum Entropy interpretations of these spectra in the mass range 45 000 to 55 000 Da {panels (ai) and (bi)}. Panels (ai-ii) show data obtained from injection of 17.1 pmol of succinate-coenzyme A transferase after reaction with acetoacetyl-coenzyme A for 2 min in a 1:1.1 molar ratio of transferase polypeptide/acetoacetyl-coenzyme A and then 0.2 M succinate for 10 min. Panels (bi-ii) show data obtained from injection of 18.3 pmol of succinate-coenzyme A transferase after reaction with acetoacetyl-coenzyme as in panels (ai-ii) followed by 0.2 M succinate for 10 min, followed by 5 mM sodium borohydride for 10 min.

Correlation of Mass Changes in the Succinate-Coenzyme A Transferase Polypeptide on Reaction with Substrates and Sodium Borohydride Reveal Only Half of the Subunits Support Catalysis. Sequential incubation of succinate-coenzyme A transferase with acetoacetyl-coenzyme A and borohydride causes complete inactivation of the enzyme due to the reduction of the active site glutamate-carboxyl of E344 to the corresponding alcohol (*10, 12*). To assess the contribution made to catalysis by the transferase subunit species generated by acetoacetyl-coenzyme A and succinate, we examined the effect of incubation with or without acetoacetyl-coenzyme A, succinate, or sodium borohydride, alone or in combination, on enzyme activity under the conditions used for the electrospray mass spectrometric experiments.

Enzyme incubated alone, or in the presence of either acetoacetyl-coenzyme A or sodium borohydride, suffered no loss of activity whatsoever (Table 2). Transferase incubated sequentially with acetoacetyl-coenzyme A, and then succinate also suffered no loss of activity. As expected, enzyme incubated sequentially with acetoacetyl-coenzyme A and sodium borohydride lost 95% of its activity consistent with reduction of the active site glutamate to its alcohol (Table 2). However, most interestingly, transferase incubated sequentially with acetoacetyl-coenzyme A, succinate, and then sodium borohydride suffered no loss of activity (Table 2), despite reduction by borohydride of the catalytic E344 of half of the subunits of the enzyme to the inactive alcohol. This result demonstrated first, that only half of the enzyme subunits were actually required support catalysis fully and, second, that only half of the enzyme subunits could donate their coenzyme A to the acceptor acid.

Given the dimeric nature of the coenzyme A transferase, two possible models may be considered: (i) All enzyme dimers could form enzyme-coenzyme A intermediates from an acyl-coenzyme A substrate on both subunits but only half of the enzyme dimers could transfer both coenzyme A moieties to an acceptor carboxylic acid or; (ii) only one subunit within each enzyme dimer could transfer coenzyme A to the acceptor carboxylic acid, although all enzyme dimers could become fully thioesterified by coenzyme A at the active site glutamate of both subunits.

Sepharose 4B-Blue MX-2G Affinity Chromatography Demonstrates That the Fully Thioesterified Succinate-Coenzyme A Transferase Dimers Contain Only One Subunit that Donates Coenzyme A to Succinate. Distinguishing between the two models to explain our data required a nondenaturing technique that could separate and analyze native dimeric enzyme and its enzyme-coenzyme A thiolester

Table 2: Effect of Sequential Incubation with Acetoacetyl-Coenzyme A, Succinate, and Sodium Borohydride, Alone or in Combination on Succinate-Coenzyme A Transferase Activity[a]

incubation of enzyme in the presence of	% activity relative to incubation with buffer alone
buffer alone	100.0 ± 2.2 (n = 3 incubations)
+ 19.6 μM acetoacetyl-coenzyme A for two minutes	97.1 ± 3.8 (n = 3 incubations)
+ 19.6 μM acetoacetyl-coenzyme A for 2 min then + succinate for 10 min	96.3 ± 2.8 (n = 3 incubations)
+ 5 mM sodium borohydride for 10 min	113.2 ± 9.3 (n = 3 incubations)
+ 19.6 μM acetoacetyl-coenzyme A for 2 min and then + 5 mM sodium borohydride for 10 min	1.9 ± 0.3 (n = 3 incubations)
+ 19.6 μM acetoacetyl-coenzyme A for 2 min then + succinate for 10 min then + 5 mM sodium borohydride for 10 min	103.8 ± 11.3 (n = 3 incubations)

[a] All incubations were carried out in a final volume of 0.1 mL of 50 mM Tris-Cl, pH 8.5, and 1 mM EDTA. 17.8 μM succinate-coenzyme A transferase was incubated as described in Materials and Methods. After incubation, the enzyme was immediately desalted on pre-packed PD-10 columns of Sephadex G-25 into 50 mM Tris-Cl, pH 8.5, and 1 mM EDTA and assayed for succinate-coenzyme A transferase activity. In this case, 100% activity corresponds to the specific activity of the untreated enzyme which was 180 μmol min^{-1} mg^{-1}. The data in the table are an average of three different enzyme incubations.

intermediate. Nanospray mass spectrometry has been used to analyze subtle noncovalent interactions in subunits from proteins as complex as the ribosome and to observe multi-subunit proteins (*31*); however, in this case, attempts to apply this technique here proved fruitless. Therefore, we exploited the Sepharose 4B-Blue MX-2G affinity column that we had used in the purification of the enzyme. As succinate-coenzyme A transferase could be eluted from this resin with succinyl-coenzyme A, we reasoned that the Blue MX-2G interacted directly with the succinyl-coenzyme A binding site of the enzyme. This was confirmed by demonstration that the dye behaves as a linear competitive inhibitor of this enzyme with respect to succinyl-coenzyme A in the transfer of coenzyme A from succinyl-coenzyme A to acetoacetate (Figure 5, panels a and b).

We surmised that the Sepharose 4B-Blue MX-2G column could bind any dimeric coenzyme A transferase possessing at least one vacant coenzyme A binding site. In contrast, the dimer that had been thioesterified with coenzyme A on both subunits would not be expected to bind to this column. This then would allow us to distinguish between the two models to explain the mass spectrometric data.

As anticipated, succinate-coenzyme A transferase, that had not been reacted with acetoacetyl-coenzyme A, bound tightly to Sepharose-4B-immobilized Blue MX-2G and could be eluted with 0.3 mM succinyl-coenzyme A (Figure 6, panel a). Also as anticipated, the enzyme that had been reacted in a 1.0:1.1 molar ratio with acetoacetyl-coenzyme A for 2 min and quickly desalted by passage through Sephadex G-25 failed to bind to the column and was eluted by buffer alone (Figure 6, panel b). Repetition of the incubation of the enzyme with acetoacetyl-coenzyme A, followed by further incubation with 0.2 M succinate for 10 min prior to gel-filtration, generated the mono-coenzyme A species that bound entirely to the column and could be eluted as a single species with 0.3 mM succinyl-coenzyme A in a manner similar to that of enzyme that had not been reacted with acetoacetyl-coenzyme A (Figure 6, panel c).

These data are entirely consistent with the conclusion that succinate-coenzyme A transferase contains a homogeneous population of dimers after sequential reaction with acetoacetyl-coenzyme A and succinate, supporting the hypothesis that only one subunit within each enzyme dimer can transfer its coenzyme A to an acceptor acid.

DISCUSSION

The purification method reported here provided a rapid and efficient means for the isolation of succinate-coenzyme A transferase from porcine heart. The specific activity of the enzyme after purification was within the range of values reported for this enzyme in the literature (*32*, *33*). The dye ligands employed here, Procion Yellow HE-4R and Procion Blue MX-2G afforded key steps in this protocol, the latter dye allowing a bio-specific step in the purification. Yellow HE-4R was also found to bind acetoacetyl-coenzyme A thiolase (Lloyd, A. J., and Shoolingin-Jordan, P. M., unpublished data) and thus also provided a step in the purification of this enzyme as well. Procion Yellow HE-4R and Procion Blue MX-2G have not been used previously for the purification of coenzyme A transferases. Other dyes such as Procion Red HE-3B and Cibacron Blue F3GA have found use in the purification of mammalian succinate-coenzyme A transferases (*29*, *32*, *34*). However, in neither of these cases has it been possible to elute the transferase enzymes bio-specifically. It may therefore prove profitable to apply Blue MX-2G to the bio-specific purification of other transferase enzymes, using the appropriate thiolester as an eluant.

Interestingly, purified succinate-coenzyme A transferase appeared to be heterogeneous on SDS−PAGE. We were able, however, to demonstrate that the two species on SDS−PAGE originated from the same polypeptide, were enzymically active and were interchangeable conformers of each other. In support of this conclusion is also the observation that there was only a single polypeptide observable on electrospray mass spectrometric analysis of the purified transferase. Similar observations of two species of succinate-coenzyme A transferase detectable by SDS−PAGE have been made by Williams (*11*) but not by other workers with this enzyme (*32*). We speculate that it is likely that a section of the polypeptide of this enzyme has a particularly stable region of secondary or tertiary structure whose denaturation is incomplete under the conditions used for SDS−PAGE.

The predicted mass of the mature porcine heart succinate-coenzyme A transferase has been calculated from the cDNA sequence to be 52 197 Da. The calculated mass of the immature polypeptide carrying the N-terminal mitochondrial targetting sequence is 56 371 Da (*14*). In all electrospray mass spectrometric analyses carried out, no mass corre-

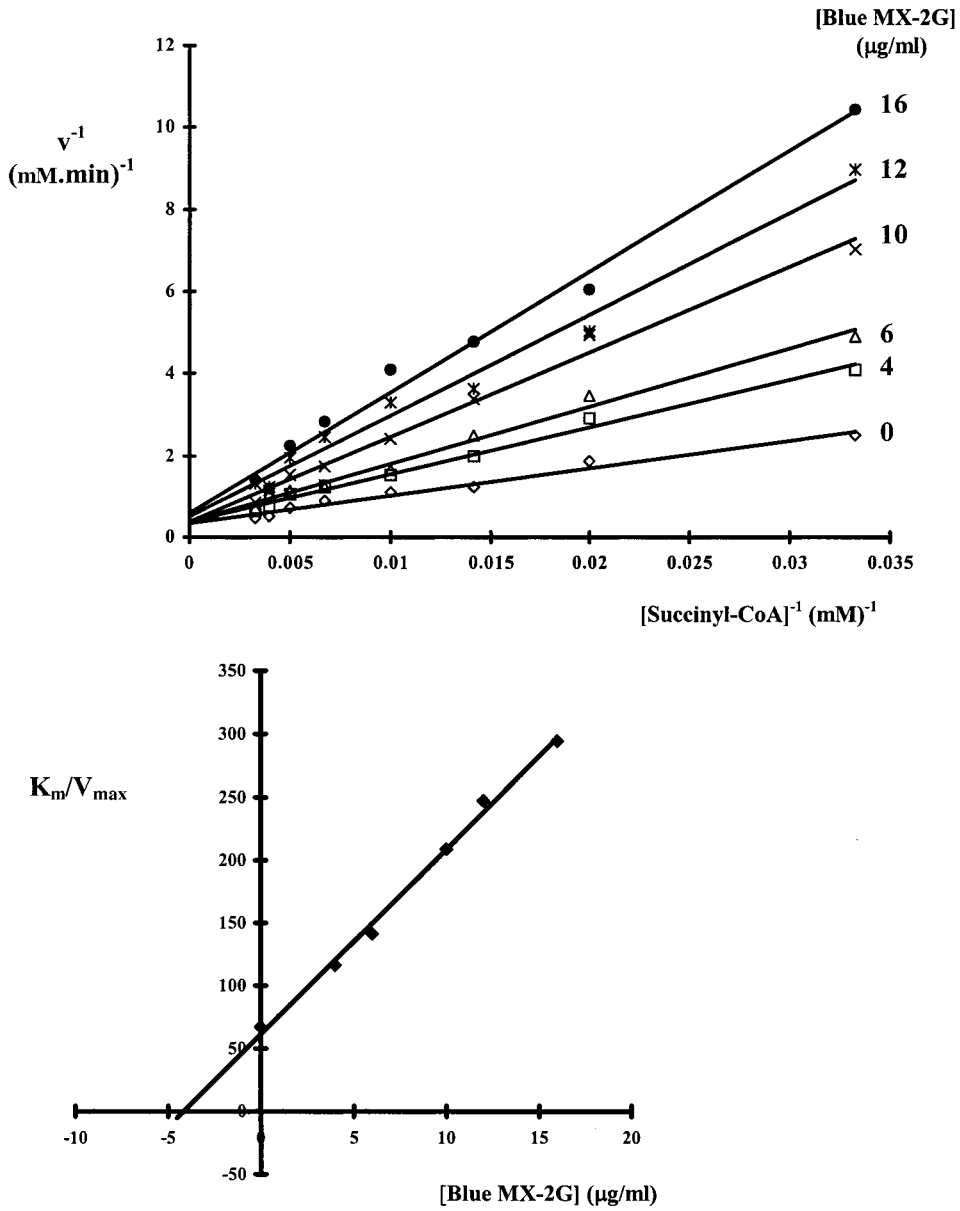


FIGURE 5: Inhibition of succinate-coenzyme A transferase by Blue MX-2G Enzyme activity was assayed in the direction of transfer of coenzyme A from succinyl-coenzyme A to acetoacetate. Panel a: [succinyl-coenzyme A] was varied between 30 and 300 μM at [Blue MX-2G] (symbols in square brackets) of 0 [◇], 4 [□], 6 [△], 10 [**x**], 12 [★], and 16 μg/mL [●]. Data were plotted as double reciprocal plots of v^{-1} vs [succinyl-coenzyme A]$^{-1}$ at each [Blue MX-2G]. Lines of best fit were obtained by linear regression. Panel b: Slopes from panel a (K_m/V_{max}) were replotted against [Blue MX-2G]. The line of best fit was obtained by linear regression.

sponding to the immature species was ever detected. In our hands, however, unreacted succinate-coenzyme A transferase was estimated by electrospray mass spectrometry to have a single mass of 52 166.2 Da. This is 30.8 Da smaller than the mass of the mature protein predicted from the cDNA sequence. The origin of this discrepancy may be caused by a sequence mutation or a DNA sequencing error.

It was of course conceivable that this discrepancy in predicted and experimental mass of the transferase polypeptide was due to contamination of our succinate-coenzyme A transferase preparation with an unrelated polypeptide of mass 52 166.2 Da. However, electrospray mass spectrometry demonstrated that acetoacetyl-coenzyme A causes complete conversion of the 52 166.2 Da polypeptide to a species with an accompanying mass increase entirely consistent with thioesterification of coenzyme A to the active site glutamyl residue of both subunits of succinate-coenzyme A transferase, indicating that this mass corresponded to the unreacted free succinate-coenzyme A transferase polypeptide.

Confirmation that acetoacetyl-coenzyme A formed an enzyme-thiolester with succinate-coenzyme A transferase was obtained by borohydride reduction. This treatment eliminated activity concurrent with the reduction of the active site glutamate, in the form of the glutamyl-coenzyme A thiolester, to the corresponding alcohol. These results are consistent with previous data showing acyl-coenzyme A-dependent borohydride inactivation/reduction of a number of other coenzyme A transferases (*10*, *13*, *16*).

Detection of the formation of the enzyme-coenzyme A thiolester by electrospray mass spectrometry was easily achieved. However, no masses were observed that could be attributed to the anhydrides between the transferase and the acetoacetate or succinate predicted during the transfer of either coenzyme A from acetoacetyl-coenzyme A to the

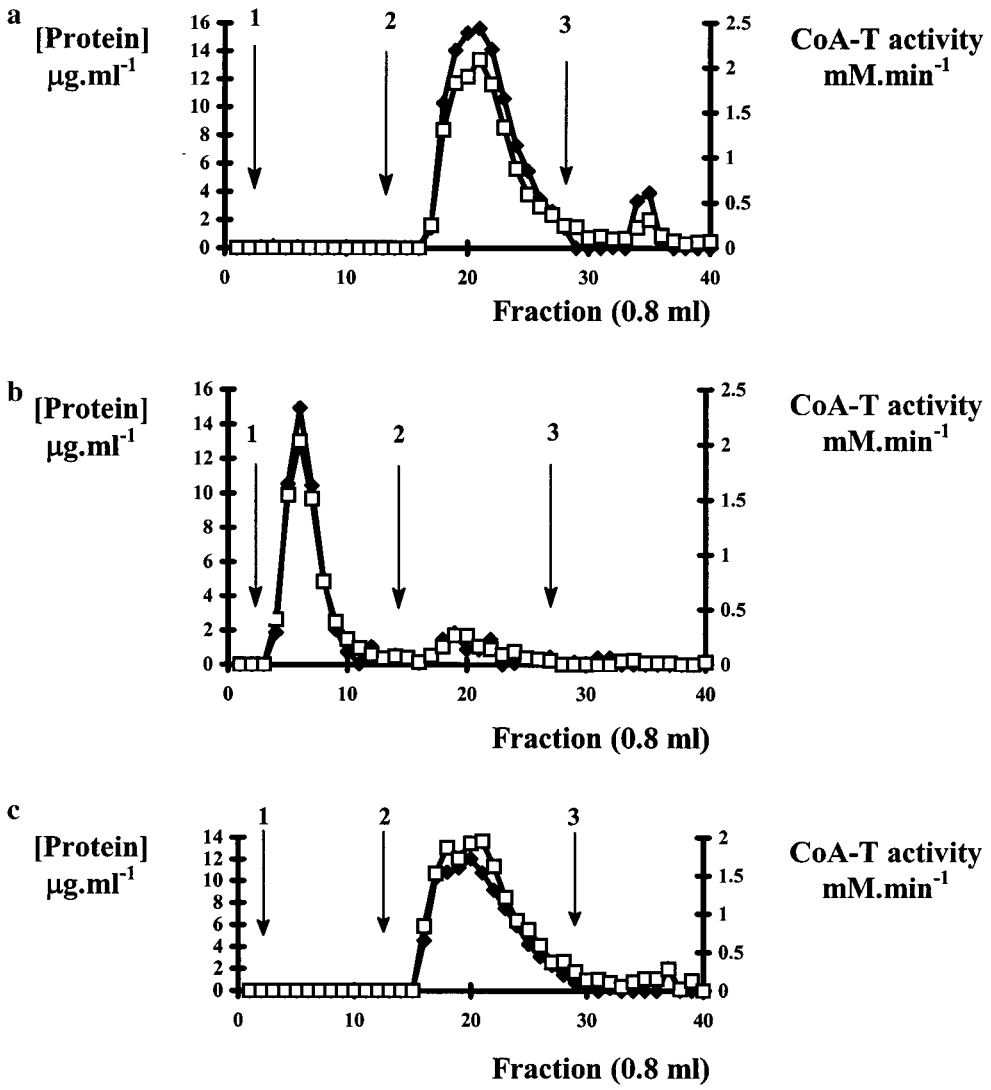


FIGURE 6: Chromatography of succinate-coenzyme A transferase treated with acetoacetyl-coenzyme A or acetoacetyl-coenzyme A and succinate on Sepharose 4B/Blue MX-2G. Panel a: succinate-coenzyme A transferase (2.25 nmol) was incubated in 100 μL of 50 mM Tris, pH 8.5, and 1 mM EDTA for 2 min at 25 °C and immediately desalted by gel filtration through Sephadex G-25 columns (PD-10, Pharmacia) into MOPS buffer at 4 °C. The enzyme was then immediately applied to a 5-mL column of Blue MX-2G immobilized on Sepharose 4B equilibrated in MOPS buffer. The column was then washed in sequence with 10 mL of MOPS buffer, 12 mL of MOPS buffer + 0.3 mM freshly prepared succinyl-coenzyme A, and 10 mL of MOPS buffer + 1 M KCl (applied at arrow 1, 2, and 3, respectively). 0.82 mL fractions were collected and assayed for protein (□) and succinate-coenzyme A transferase activity (◆). Panel b: Succinate-coenzyme A transferase (2.25 nmol) was incubated in 100 μL of 50 mM Tris, pH 8.5, and 1 mM EDTA with acetoacetyl-coenzyme A in a 1:1 molar ratio of transferase subunit to acetoacetyl-coenzyme A for 2 min at 25 °C. The incubation was immediately desalted and chromatographed on immobilized Blue MX-2G as described for panel a. 0.82 mL fractions were collected and assayed for protein (□) and succinate-coenzyme A transferase activity (◆). Panel c: succinate-coenzyme A transferase (2.25 nmol) was incubated as described in panel b with acetoacetyl-coenzyme A. The incubation was then supplemented by 0.2 M succinate for a further 10 min. At this point, the transferase was immediately desalted and chromatographed on immobilized Blue MX-2G as described for panel a. 0.82 mL fractions were collected and assayed for protein (□) and succinate-coenzyme A transferase activity (◆).

enzyme or during coenzyme A transfer from the enzyme-coenzyme A thiolester to succinate. A probable explanation for this is that the anhydride lifetime is too transient to allow its detection. It may, however, be possible to observe anhydride formation by combining rapid-mixing technology with electrospray mass spectrometry. A similar approach has been adopted to examine polymerization of the tetrapyrrole chain on the dipyrromethane cofactor of porphobilinogen deaminase (*35*).

Despite the vast excess of acceptor carboxylate used in these experiments in attempts to transfer coenzyme A from its thiolester at the transferase active site, only half of the subunits of succinate-coenzyme A transferase appeared to be active in this regard. Interestingly, coenzyme A binding proteins, such as fatty acyl-coenzyme A binding protein, maintain noncovalent complexes with their CoA ligands that are stable to analysis by electrospray mass spectrometry (*30*). However, this clearly was not evident in the case here because, first, the enzyme incubated with coenzyme A in place of acetoacetyl-coenzyme A underwent no apparent change in polypeptide mass under these conditions (data not shown). Second, the identity of the remaining enzyme-coenzyme A thiolester was confirmed using sodium borohydride. Third, the types of complexes observed by Robinson et al. (*30*) would not have survived the electrospray probe temperatures used here.

Hersh and Jencks (*9*) have also analyzed the release of coenzyme A from pig heart succinate-coenzyme A trans-

ferase by succinate. After incubation with acetoacetyl-coenzyme A and succinate, the enzyme was allowed to stand, to allow the hydrolysis of any coenzyme A remaining thioesterified to the transferase. Any coenzyme A released was then assayed. As these workers failed to detect any further released coenzyme A, they concluded that the enzyme-thiolester was completely consumed by conversion to succinyl-coenzyme A. Although this result would seem to be in disagreement with our conclusions, it should be remembered that their detection of enzyme thiolester was dependent only on the detection of released coenzyme A. Our results, in contrast, comprise actual molecular masses of the enzyme polypeptide thiolester intermediate. Indeed, the inability of Hersh and Jencks (*9*) to detect any further release of coenzyme A, after treatment of the enzyme-coenzyme A thiolester with succinate, is not inconsistent with our data, which suggests that half of coenzyme A remains thioesterified to the enzyme after treatment with succinate. Their recoveries of succinyl-coenzyme A, trapped as succinyl-hydroxamate (*9*) from incubations of the enzyme-coenzyme A and radiolabeled succinate, were as low as 43%. This figure is quite close to our value of 50% that indicates only half of the subunits of the enzyme-coenzyme A thiolester intermediate transfer CoA to the acceptor dicarboxylate.

The inability of half of the enzyme-coenzyme A thiolester to donate coenzyme A to succinate is also surprising given that equilibrium constants estimated for this step by White et al. (*20*) suggest that only 0.00002% of the enzyme should remain as enzyme-coenzyme A thiolester under these conditions. Again, the apparent inconsistency between our data and that of ref *20* is probably resolvable because they made the assumption that both subunits once thioesterified to coenzyme A were equally reactive to succinate. We, on the other hand, suggest that this is not the case and that the equilibrium constants quoted in ref *20* refer only to the subunit that releases its coenzyme A to succinate. Indeed, our data suggest the equilibrium position of the reaction on the subunit that does not release its coenzyme A to the acceptor acid in the second half reaction lies overwhelmingly in the direction of enzyme-coenzyme A thiolester synthesis. This is consistent with the apparent stability of this species in the presence of a vast excess of the acceptor acid.

Incubation of succinate-coenzyme A transferase with acetoacetyl-coenzyme A, followed by further sequential addition of succinate and then finally borohydride, generated a 1:1 mixture of enzyme subunit and enzyme subunit where E344 had been reduced to its alcohol. Remarkably, this treatment did not cause any loss in enzyme activity, again, adding to the evidence suggesting that only half of the subunits in any succinate-coenzyme A transferase preparation contribute to the full enzyme activity. Furthermore, we demonstrated that within each enzyme dimer, only a single subunit was required for full activity. It is a tantalizing possibility that the catalytic asymmetry seen within the succinate-coenzyme A transferase dimer is reflected structurally in the conformational heterogeneity displayed by this enzyme on SDS−PAGE (Figure 1).

Catalytic asymmetry has also been observed in the *Escherichia coli* coenzyme A transferase. Here, half-site reactivity has been observed with respect to formation of the enzyme-coenzyme A intermediate (*36*). Half site reactivity implies that for every two active sites, only one is reactive at any point in time but both are required for full expression of activity. However, in contrast to this and the *E. coli* enzyme, our results with the porcine heart transferase have shown that, within the dimeric enzyme, one subunit can be irreversibly modified by borohydride reduction without loss of any catalytic efficiency. This implies that only a single subunit is ever active in the transferase dimer and that the catalytically inactive subunit has no direct role in supporting the catalysis of its active neighbor. Currently, our data do not allow us to speculate what the function of the inactive subunit may be in this enzyme, and this issue may only be addressed once a crystal structure becomes available.

There are a number of possible mechanistic and structural explanations for the catalytic asymmetry observed in our studies with porcine succinate-coenzyme A transferase. For example, the two subunits within the transferase dimer may simply exhibit very different affinities for the acceptor acid. Alternatively, subtle differences in the way each transferase subunit interacts with the coenzyme A moiety may contribute to the observed catalytic asymmetry. Whitty et al. (*33*) have determined the relative contributions of binding energy made by sections of the coenzyme A molecule to catalysis by succinate-coenzyme A transferase. They concluded that active site binding of the α, β, and γ carbon atoms of the pantoic acid domain of coenzyme A significantly destabilize the enzyme-coenzyme A thiolester, enhancing its reactivity toward acetoacetate or succinate by a factor of 10^7. These are clearly key interactions between the enzyme and coenzyme A. Thus, our data are consistent with a situation where, within the inactive subunit, interactions of the pantoic acid moiety of coenzyme A, crucial for transition state stabilization during transfer of coenzyme A to the acceptor carboxylate, are sufficiently disrupted to render this subunit incapable of supporting the second half reaction.

The mechanistic expression of these changes in binding energy may reside with the relative abilities of the two subunits to undergo the large conformational changes on formation of the enzyme-coenzyme A thiolester that are central to its mechanism (*20*, *36*). The fact that only one subunit could transfer its coenzyme A back to succinate or acetoacetate implies that, unlike the active subunit within the native succinate-coenzyme A transferase dimer, the conformational changes associated with the inactive subunit, on thioesterification to coenzyme A, are quite different from those experienced by the active subunit. Thus, the inactive subunit may possess an environment at its active site that is unable to destabilize the enzyme-coenzyme A thiolester in the presence of the acceptor carboxylate because of an alternative mode of pantoic acid binding in this subunit that is not competent in the active transfer of the coenzyme A moiety.

An alternative interpretation of the results reported here may depend on the position of the active sites relative to each other in the porcine succinate-coenzyme A transferase dimer on formation of the enzyme-coenzyme A thiolester. A crystal structure of the unliganded glutaconyl-coenzyme A transferase from *A. fermentans* is now available (*37*). From this it is clear that in this closely related enzyme, the active site glutamate residues are too far from each other (37 Å) to interact directly. However, this structure is not representative of the enzyme-CoA thiolester intermediate, because of the

large and easily detectable conformational changes that occur on reaction of both bacterial and porcine enzymes with their corresponding acyl-coenzyme A substrates (*20*, *36*). Although such a conformational change is unlikely to be large enough to bring the two active site glutamate residues into close enough proximity for one to interfere with the function of the other, this possibility cannot be discounted.

Another possibility also must be considered. Given the size of coenzyme A, the nucleotide binding sites of both active sites may be considerably closer to each other in the coenzyme A transferase dimer than the E344 residues. Inspection of the crystal structure of butyryl-coenzyme A dehydrogenase, as a complex with acetoacetyl-coenzyme A, shows that the substrate is bound in an extended conformation, such that the distance between the sulfur atom and the 3′-phosphate on the ribose ring is 24.6 Å (*38*). Should the two coenzyme A molecules, bound within the succinate-coenzyme A transferase dimer, be in a similar conformation, the two E344 residues may be close enough to allow the 3′-phosphoadenosine-5′-diphosphate of the coenzyme A thioesterified to the active subunit to distort the productive interaction of the analogous nucleotide portion of the coenzyme A molecule thioesterified to the inactive subunit. This would reduce its reactivity toward succinate or acetoacetate. If this was the case, modification of E344 in this subunit by borohydride reduction of the enzyme thiolester would, as was observed, have no effect on catalysis.

Dimeric enzymes such as glutamate 1-semialdehyde aminotransferase that involve covalent intermediates within their reaction mechanism are known to display half-site reactivity (*39*). The crystal structure of this enzyme reveals a polypeptide loop in one subunit that restricts access to the active site of the other. In the second subunit, the loop is more mobile allowing access of the substrate to the catalytic pyridoxal 5′-phosphate cofactor. Extending these observations to porcine heart succinate-coenzyme A transferase might suggest that such a comparable loop might block access of the acceptor acid to the enzyme-coenzyme A thiolester on one subunit if the other subunit had already reacted to regenerate the carboxyl group of E344. However, this would not be the case if one of the two E344 residues had been reduced to the uncharged alcohol.

In summary, this is the first report, of which we are aware, concerning a dimeric enzyme catalyzing a ping-pong mechanism where half of the subunits are catalytically superfluous with respect to the second half reaction. The observations exemplify the power of electrospray mass spectrometry as an indispensable technique for investigating protein chemistry. As this technique becomes more widely used for enzymological investigations, more examples of this type of phenomenon may be forthcoming. A more definitive explanation for the structural and mechanistic causes underlying the half site reactivity of succinate-CoA transferase is likely to arise from the determination of the crystal structure of not only the free enzyme but also of the enzyme-coenzyme A thiolester. These studies are now well advanced in our laboratory.

ACKNOWLEDGMENT

We thank Drs. N. Wright and P. Skipp of the Biological Mass Spectrometry Unit at the University of Southampton for assistance with and discussion of the mass spectrometric experiments conducted in this paper. We also are grateful for useful and incisive discussions with Professor M. Akhtar, Dr. G. R. Lewis, and Dr. E. Mitchell.

REFERENCES

1. White, H., and Jencks, W. P. (1976) *J. Biol. Chem. 251*, 1708−1711.
2. Mitchell, G. A., Kassovska-Bratinova, S., Boukaftane, Y., Robert, M. F., Wang, S. P., Ashmarina, L., Lambert, M., Lapierre, P., and Potier, E. (1995) *Clin. Invest. Med. 18*, 193−216.
3. Rochet, J.-C., Oikawa, K., Hicks, L. D., Kay, C. M., Bridger, W. A., and Wolodko, W. T. (1997) *Biochemistry 36*, 8807−8820.
4. Song, X.-Q., Fukao, T., Watanabe, H., Shintaku, H., Hirayama, K., Kassovska-Bratinova, S., Kondo, N., and Mitchell, G. A. (1998) *Hum. Mutat. 12*, 83−88.
5. Bukel, W., Dorn, U., and Semmler, R. (1981) *Eur. J. Biochem. 118*, 315−321.
6. Eikmans, U., and Buckel, W. (1990) *Hoppe-Seylers Biol. Chem. 371*, 1077−1082.
7. Baetz, A. L., and Allison, M. J. (1990) *J. Bacteriol. 172*, 3537−3540.
8. Parales, R. E., and Harwood, C. S. (1992) *J. Bacteriol. 174*, 4657−4666.
9. Hersh, L. B., and Jencks, W. P. (1967) *J. Biol. Chem. 242*, 3481−3486.
10. Solomon, F., and Jencks, W. P. (1969) *J. Biol. Chem. 244*, 1079−1081.
11. Williams, W. A. (1990) Structure/Function Relationships within Pig Heart CoA Transferase. Thesis from University of Minnesota, Minneapolis, USA.
12. Rochet, J.-C., and Bridger, W. A. (1994. *Protein Sci. 3*, 975−981.
13. Sramek, S. J., and Frerman, F. E. (1975) *Arch. Biochem. Biophys. 171*, 27−35.
14. Lin, T., and Bridger, W. A. (1992) *J. Biol. Chem. 267*, 975−978.
15. Mack, M., and Buckel, W. (1995) *FEBS Lett. 357*, 145−148.
16. Mack, M., Bendrat, K., Zelder, O., Eckel, E., Linder, D., and Buckel, W. (1994) *Eur. J. Biochem. 226*, 41−51.
17. White, H., and Jencks, W. P. (1976) *J. Biol. Chem. 251*, 1688−1699.
18. Pickart, C. M., and Jencks, W. P. (1979) *J. Biol. Chem. 254*, 9120−9129.
19. Moore, S. A., and Jencks, W. P. (1982) *J. Biol. Chem. 257*, 10882−10892.
20. White, H., Solomon, F., and Jencks, W. P. (1976) *J. Biol. Chem. 251*, 1700−1707.
21. Dean, P. D. G., and Watson, D. H. (1979) *J. Chromatogr. 165*, 301−319.
22. Simon, E. J., and Shemin, D. (1953) *J. Am. Chem. Soc. 75*, 2520.
23. Williamson, D. H., Bates, M. W., Page, M. A., and Krebs, H. A. (1971) *Biochem. J. 121*, 41−47.
24. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
25. Laemmli, U.K. (1970) *Nature 227*, 680−685.
26. Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307−310.
27. Lee, C., Levin, A., and Branston, D. (1987) *Anal. Biochem. 166*, 308−312.
28. LeGendre, N., and Matsudaria, P. (1988) *Biotechniques 6*, 154−159.
29. Child, C. J., Spencer, J. B., Bhogal, P., and Shoolingin-Jordan, P. M. (1996) *Biochemistry 35*, 12267−12274.
30. Robinson, C. V., Chung, E. W., Kragelund, B. B., Knudsen, J., Alpin, R. T., Poulsen, F. M., and Dobson, C. M. (1996) *J. Am. Chem. Soc. 118*, 8646−8653.
31. Benjamin, D. R., Robinson, C. V., Hendrick, J. P., Hartl, F. B., and Dobson, C. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 7391−7395.
32. Moore, S. A., and Jencks, W. P. (1982) *J. Biol. Chem. 257*, 10893−10907.

33. Whitty, A., Fierke, C. A., and Jencks, W. P. (1995) *Biochemistry 34*, 11678−11689.
34. Sharp, J. A., and Edwards, M. R. (1978) *Biochem. J. 173*, 759−765.
35. Aplin, R. T., Baldwin, J. E., Pichon, C., Roessner, C. A., Scott, A. I., Schofield, C. J., Stolowich, N. J., and Warren, M. J. (1991) *Bioorg. Med. Chem. Lett. 1*, 503−506.
36. Sramek, S. J., Frerman, F. E., McCormick, D. J., and Duncombe, G. R. (1977) *Arch. Biochem. Biophys. 181*, 525−533.
37. Jacob, U., Mack, M., Clausen, T., Huber, R., Buckel, W., and Messerschmidt, A. (1997) *Structure 5*, 415−426.
38. Djordjevic, S., Pace, C. P., Stankovich, M. T., and Kim, J. J. (1995) *Biochemistry 34*, 2163-2171.
39. Hennig, M., Grimm, B., Contestabile, R., John, R. A., and Jansonius, J. N. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 4866−4871.

BI002169V