Protein Science (1994), *3*:975-981. Cambridge University Press. Printed in the USA.

Identification of glutamate 344 as the catalytic residue in the active site of pig heart CoA transferase

JEAN-CHRISTOPHE ROCHET AND WILLIAM A. BRIDGER
Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

(RECEIVED January 16, 1994; ACCEPTED April 4, 1994)

Abstract

The enzyme CoA transferase (succinyl-CoA:3-ketoacid coenzyme A transferase [3-oxoacid CoA transferase], EC 2.8.3.5) is essential for the metabolism of ketone bodies in the mammalian mitochondrion. It is known that its catalytic mechanism involves the transient thioesterification of an active-site glutamate residue by CoA. As a means of identifying this glutamate within the sequence, we have made use of a fortuitous autolytic fragmentation that occurs at the active site when the enzyme–CoA covalent intermediate is heated. The presence of protease inhibitors has no effect on the extent of cleavage detectable by SDS-PAGE, supporting the view that this fragmentation is indeed autolytic. This fragmentation can be carried out on intact CoA transferase, as well as on a proteolytically nicked but active form of the enzyme. Because the resulting C-terminal fragment is blocked at its N-terminus by a pyroglutamate moiety, it is not amenable to direct sequencing by the Edman degradation method. As an alternative, we have studied a peptide (peptide D) generated specifically by autolysis of the nicked enzyme and predicted to have an N-terminus corresponding to the site of proteolysis and a C-terminus determined by the site of autolysis. This peptide was purified by reversed-phase HPLC and subsequently characterized by electrospray mass spectrometry. We have obtained a mass value for peptide D, from which it can be deduced that glutamate 344, known to be conserved in all sequenced CoA transferases, is the catalytically active amino acid. This information should prove useful to future mutagenesis work aimed at better understanding the active-site structure and catalytic mechanism of CoA transferase.

Keywords: active-site glutamate; autolytic fragmentation; CoA transferase; HPLC; mass spectrometry; nicked enzyme

Ketone bodies such as acetoacetate and β-hydroxybutyrate constitute the major metabolic fuel for the mammalian heart and kidney. In the mitochondria of these tissues, acetoacetate is converted to acetoacetyl-CoA, which is then further broken down to 2 acetyl-CoA molecules capable of entering the tricarboxylic acid cycle (Fig. 1). The homodimeric enzyme CoA transferase (succinyl-CoA:3-ketoacid coenzyme A transferase, EC 2.8.3.5) is responsible for the formation of acetoacetyl-CoA by transfer of a CoA moiety from succinyl-CoA to acetoacetate. This reaction is characterized by ping-pong kinetics and by the formation of a stable enzyme-CoA covalent intermediate (Hersh & Jencks, 1967a, 1967b). It has been shown that the enzyme's catalytically active moiety, to which CoA is thioesterified, is a glutamate side chain (Solomon & Jencks, 1969).

Reprint requests to: William A. Bridger, Department of Biochemistry, 4-74 Medical Sciences Building, University of Alberta, Edmonton, Alberta T6G 2H7, Canada; email: bill_bridger@darwin.biochem.ualberta.ca.

Abbreviations: CoA, coenzyme A; RP-HPLC, reversed-phase HPLC; MALDI, matrix-assisted laser desorption/ionization; TFA, trifluoroacetic acid; PMSF, phenylmethylsulfonyl fluoride.

A full-length cDNA encoding the cytoplasmic precursor to this enzyme has been cloned from a pig heart library and sequenced (Lin & Bridger, 1992). The deduced amino acid sequence reveals a 481-residue mature subunit of molecular weight 52,197 Da. Furthermore, a hydrophilicity plot for CoA transferase is strongly indicative of a structure characterized by 2 interacting domains that are linked by a hydrophilic hinge region (Lin & Bridger, 1992).

Two distinct cleaved forms of CoA transferase relevant to this study may be prepared. One of these, referred to as the nicked enzyme, is produced by proteolysis in the hydrophilic region that connects the 2 proposed domains. SDS-PAGE analysis indicates that this linker region is nicked when the enzyme is purified in the absence of the serine proteinase inhibitor phenylmethylsulfonyl fluoride. This proteolytic cleavage generates N- and C-terminal fragments of 28 kDa and 24 kDa, respectively (Lin & Bridger, 1992). The proteolytically nicked enzyme retains full catalytic activity.

The second cleaved form of CoA transferase involves an autolytic fragmentation at the active-site thiol ester. Several plasma proteins that have an internal thiol ester involving glutamate and

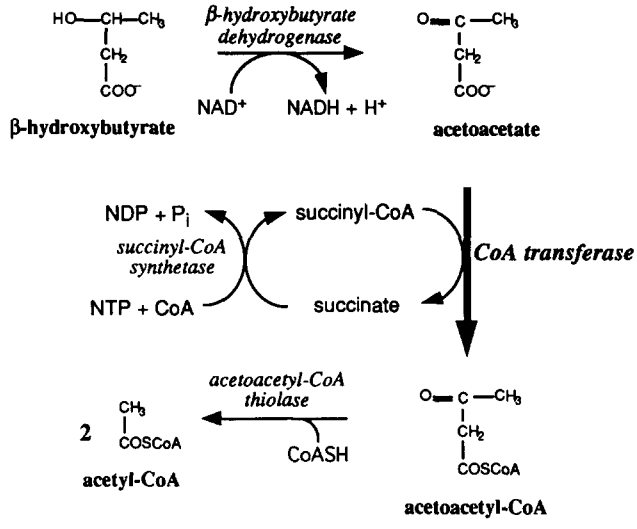

Fig. 1. Role of CoA transferase in ketone body metabolism.

cysteine are known to be autolytically cleaved when subjected to heat (Howard et al., 1986). This autolysis reaction, involving cyclization to form an oxyproline (Khan & Erickson, 1984), was also found to occur at the active site of CoA transferase when the enzyme-CoA intermediate is heated (Howard et al., 1986). On the basis of the size of the autolytic products, the location of the catalytically active glutamate residue was estimated to be approximately two-thirds of the distance from the enzyme's N-terminus. The deduced amino acid sequence reveals 3 glutamates in this vicinity of the CoA transferase primary structure (Fig. 2). The site of autolysis could not, however, be identified more precisely by Edman sequencing of the C-terminal fragment because the latter is blocked at the N-terminus by a pyroglutamate moiety. Attempts to remove this group either enzymatically with pyroglutaminase (Howard et al., 1986) or chemically by methanolysis (procedure of Kawasaki & Itano, 1972) have proven unsuccessful.

In this study, we provide further evidence for the autolytic nature of the heat-dependent fragmentation of CoA transferase by demonstrating its occurrence in the presence of a number of protease inhibitors. In addition, the proteolytically nicked form of the enzyme is also shown to be susceptible to autolysis. This

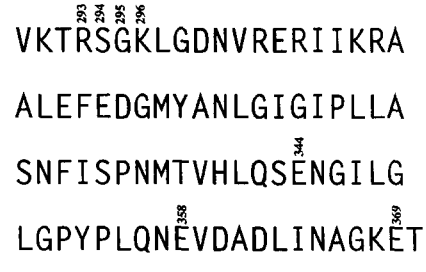

Fig. 2. Primary structure of pig heart CoA transferase in the vicinity of the active-site glutamate. Numbers based on the sequence of the precursor form of the enzyme (Lin & Bridger, 1992) indicate amino acids referred to in the text.

reaction generates a small protein segment whose N-terminus corresponds to the site of proteolysis and whose C-terminus is determined by the location of the autolytic fragmentation. Finally, we have determined the molecular weight of this peptide using electrospray mass spectrometry (Fenn et al., 1989; Smith et al., 1990). The molecular weight value obtained, when compared with predicted values deduced from the amino acid sequence, allows us to identify Ser 343 as the C-terminus of the peptide and therefore Glu 344 as the residue that participates covalently in catalysis.

Results

SDS-PAGE analysis of autolytically fragmented CoA transferase

The extent of autolysis of CoA transferase under different conditions was monitored by SDS-PAGE analysis. In an initial experiment, pure (>95%), unnicked enzyme isolated from pig myocardium was incubated, with or without 3 mM succinyl-CoA, for 75 min at 25 °C, or for 15 min at 25 °C and subsequently for 60 min at 70 °C. Samples were then diluted in an appropriate volume of sample buffer and analyzed by SDS-PAGE (Fig. 3A). Two bands representing fragments of molecular weights approximately equal to 40 kDa and 20 kDa are diagnostic for the autolytic fragmentation of CoA transferase; these fragments are only produced by samples in which the enzyme-CoA intermediate has formed (Fig. 3A, lanes 1 and 3). Furthermore, incubation of the covalent intermediate at 70 °C results in about 50% of the enzyme being fragmented, an efficiency much superior to that achieved by incubating at lower temperatures (Fig. 3A, compare lanes 1 and 3).

Although this fragmentation clearly depends on the exposure of the enzyme-CoA intermediate to an elevated temperature, the involvement of a protease had not been ruled out prior to this study. CoA transferase is known to undergo a conformational change upon forming the thiolester intermediate (White et al., 1976) and could hence become more susceptible to the effects of a heat-insensitive proteolytic activity. Figure 3B reveals, however, that the autolytic fragmentation occurs to similar yield in the presence and in the absence of a protease inhibitor cocktail consisting of pepstatin A, antipain, PMSF, and EDTA (compare lanes 1 and 2). This result, along with the temperature optimum of the cleavage reaction, confirms that the fragmentation is not likely the result of a proteolytic event.

Finally, although the initial autolysis protocol (Howard et al., 1986) suggested a range of 1-3 mM as the optimal concentration of succinyl-CoA, we found that the ultraviolet absorbance due to this excess of substrate tended to obscure a large number of potentially significant peaks in the analysis of fragmented enzyme by reversed-phase HPLC. Furthermore, we aimed to lower the salt concentration within the autolytically fragmented sample in order that it not interfere with subsequent mass spectrometry measurements. Therefore, the fragmentation protocol described above was repeated using a range of succinyl-CoA concentrations and the samples were analyzed by SDS-PAGE as before. Figure 3C indicates that a concentration of succinyl-CoA as low as 0.5 μM, or 2,000-fold lower than the minimum amount previously recommended (Howard et al., 1986), is sufficient to allow for heat-induced autolysis.

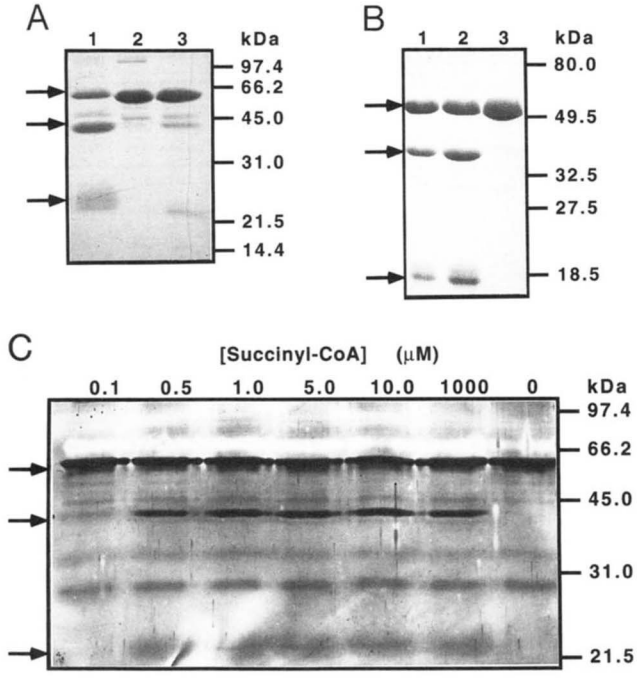

Fig. 3. SDS-PAGE analysis of autolytically fragmented CoA transferase. For all 3 gels, the top arrow identifies the band corresponding to intact enzyme, while the lower 2 arrows indicate bands that represent the fragments generated by autolysis. **A:** Standard autolytic fragmentation of CoA transferase. Unnicked enzyme (1.7 nmol or 90 μg) was incubated at 25 °C for 15 min and then at 70 °C for 60 min in the presence (lane 1) or absence (lane 2) of 3 mM succinyl-CoA. The same amount of enzyme was incubated at 25 °C for 75 min in the presence of 3 mM succinyl-CoA (lane 3). Samples were subjected to electrophoresis on a 12% (w/v) polyacrylamide gel, which was subsequently stained with Coomassie blue. **B:** Effect of protease inhibitors on the autolytic fragmentation of CoA transferase. Unnicked enzyme (1.0 nmol or 50 μg) was subjected to autolysis in the presence (lane 1) or absence (lane 2) of a protease inhibitor cocktail consisting of pepstatin A, antipain, PMSF, and EDTA. The same amount of enzyme was left unfragmented and included as a control (lane 3). Samples were subjected to electrophoresis on a 7–15% (w/v) gradient polyacrylamide gel, which was subsequently stained with Coomassie blue. **C:** Effect of succinyl-CoA concentration on the autolysis of CoA transferase. Unnicked enzyme (0.6 nmol or 30 μg) was autolytically fragmented in the presence of the indicated amounts of succinyl-CoA. Samples were subjected to electrophoresis on a 12% (w/v) polyacrylamide gel, which was subsequently stained with silver.

Autolysis of proteolytically nicked CoA transferase

The above results allowed us to predict that the proteolytically cleaved form of CoA transferase should also be autolytically fragmented when heated in the presence of micromolar concentrations of succinyl-CoA. This reaction was expected to generate a peptide bordered at its N-terminus by the nicking site and at its C-terminus by the site of autolysis (Fig. 4, fragment D). Our ultimate goal consisted of obtaining a value for this fragment's molecular weight. Because the location of proteolytic cleavage has been identified in the sequence of CoA transferase (Lin & Bridger, 1992), accurate determination of the mass of peptide D should lead to the identification of the active-site glutamate residue (Table 1). Prior to developing this approach, we had attempted to purify the C-terminal autolytic fragment (Fig. 4, fragment C) by RP-HPLC, with little success. This was likely due to the poor solubility of the autolytically fragmented sample prior to application to the HPLC column, although it could not be ruled out that successfully injected fragment C was not eluted from the column. We therefore predicted that the much smaller fragment D, by virtue of its lower hydrophobicity, might be more easily isolated prior to analysis by electrospray mass spectrometry.

In order to verify that fragment D can indeed be generated, nicked CoA transferase was incubated in the presence or absence of 10 μM succinyl-CoA and subsequently heated as described above. The reaction was then terminated by dilution in an appropriate volume of sample buffer and analyzed by tricine SDS-PAGE (Fig. 5). Two bands, representing fragments of molecular weights approximately equal to 20 kDa and 5 kDa, are again associated only with the sample in which the nicked enzyme–CoA intermediate has formed (Fig. 5, lane 1). The deduced molecular weight of the smaller fragment (possibly fragment D) is 5,900 Da, which suggests that Glu 344 or possibly Glu 358 could be the site of autolysis. Curiously, however, the apparent molecular weights of CoA transferase and its proteolytic fragments, as measured by SDS-PAGE analysis, are generally greater than the values calculated from the sequence. For this reason, we aimed to obtain a more accurate value for the mass of the smaller peptide.

RP-HPLC and electrospray mass spectrometry

Nicked CoA transferase was autolytically fragmented as described above and subsequently analyzed by RP-HPLC. Nicked but unfragmented enzyme was similarly analyzed as a control. A comparison of their corresponding elution profiles (Fig. 6) indicates that 3 peptides, represented by peaks denoted X, Y, and Z, are specifically associated with the autolytically fragmented sample. Conversely, peaks labeled 1–5 are present in both plots

Table 1. *Predicted molecular weights for fragment D*[a]

Potential active-site residue	Fragment[b]	Predicted mass $M + H^+$ (Da)[c]
E344	S294–S343	5,491.3
	G295–S343	5,404.2
	K296–S343	5,347.2
E358	S294–N357	6,958.0
	G295–N357	6,870.9
	K296–N357	6,813.8
E369	S294–K368	8,084.2
	G295–K368	7,997.1
	K296–K368	7,940.1

[a] The size of this peptide is related to the site of autolysis, which could be located at 1 of the 3 glutamate residues listed. Masses for the corresponding hypothetical fragments were calculated using the program MacProMass.

[b] Three alternative peptides are listed in each case because protease and/or aminopeptidase activities apparently generate N-terminal heterogeneity in the 24-kDa fragment (see text for further discussion).

[c] Calculation of predicted mass assumes the oxidation of both methionine residues to methionine sulfoxide. The mass of the corresponding unoxidized fragments is obtained by subtracting 32.0 Da.

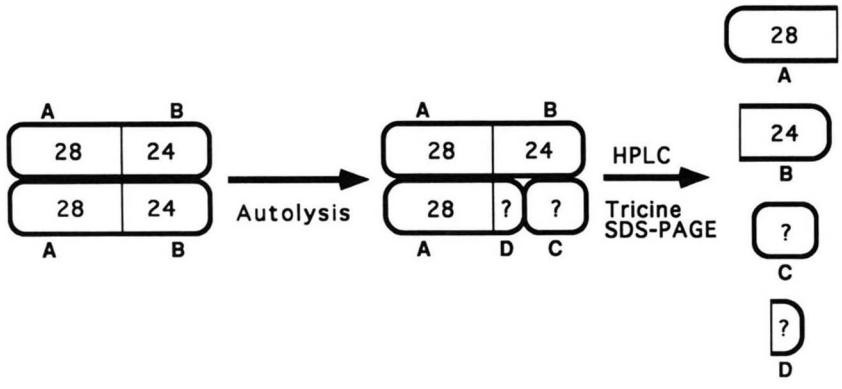

Fig. 4. Schematic representation of the nicked form of CoA transferase, both prior and subsequent to autolysis. Proteolysis alone generates segments A and B, while the autolytic fragmentation releases peptides C and D. Numbers refer to previously determined molecular weight values in kDa (Lin & Bridger, 1992). Fragments C and D are shown to be released from only 1 of the 2 subunits to reflect the observed 50% efficiency of the autolytic fragmentation. Significantly, peptide D is bordered at its N-terminus by the previously identified proteolytic cleavage site and at its C-terminus by the site of autolysis (i.e., the active site).

and are therefore not likely to represent the peptide of interest, fragment D.

Electrospray mass spectrometry of a pool of HPLC fractions representing peaks X and Y indicated the presence of a single peptide of molecular weight 2,510.0 Da (data not shown). This value does not match any of the masses predicted for fragment D (Table 1). On the other hand, a similar analysis of a pool of fractions representing peak Z revealed that the mass of its major component is 5,404.3 Da (Fig. 7, peak A1). This value is virtually equal to the mass predicted for fragment D (with N-terminal Gly 295) if the site of autolysis is Glu 344 (Table 1). The minor component of mass 5,347.0 Da (Fig. 7, peak B1) most likely corresponds to the main component minus the N-terminal glycine. Similarly, the minor component of mass 5,491.0 Da (Fig. 7, peak C1) represents the major component with an additional serine at its N-terminus. Thus, although Lin and Bridger (1992) had shown Lys 296 to be the N-terminal residue of the 24-kDa fragment, it is clear that this sample of nicked CoA transferase contains a population of C-terminal proteolytic segments that are heterogeneous in their N-terminal extensions. The major component A1, then, is likely to have Gly 295 at its N-terminus, whereas the minor components B1 and C1 have Lys 296 and Ser 294 at their N-termini, respectively. Furthermore, the measured masses are equal to those calculated with the assumption that both methionine residues are oxidized to methionine sulfoxide. The oxidation of methionine to methionine sulfoxide on peptide D is a likely outcome of the elevated temperature used in the autolytic fragmentation reaction.

Table 2 summarizes the identification of these fragments and other more minor components predicted to be sodium or potas-

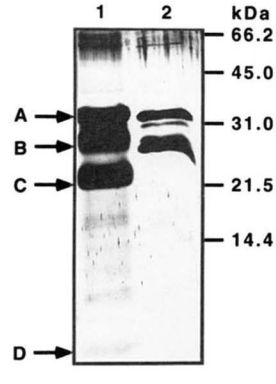

Fig. 5. Tricine SDS-PAGE analysis of nicked, autolytically fragmented CoA transferase. Nicked enzyme (0.6 nmol or 30 μg) was incubated at 25 °C for 15 min and then at 70 °C for 60 min in the presence (lane 1) or absence (lane 2) of 10 μM succinyl-CoA. Samples were subjected to electrophoresis on a 16.5% T, 3% C polyacrylamide gel, which was subsequently stained with silver. Letters A, B, C, and D identify the fragments represented by each band indicated.

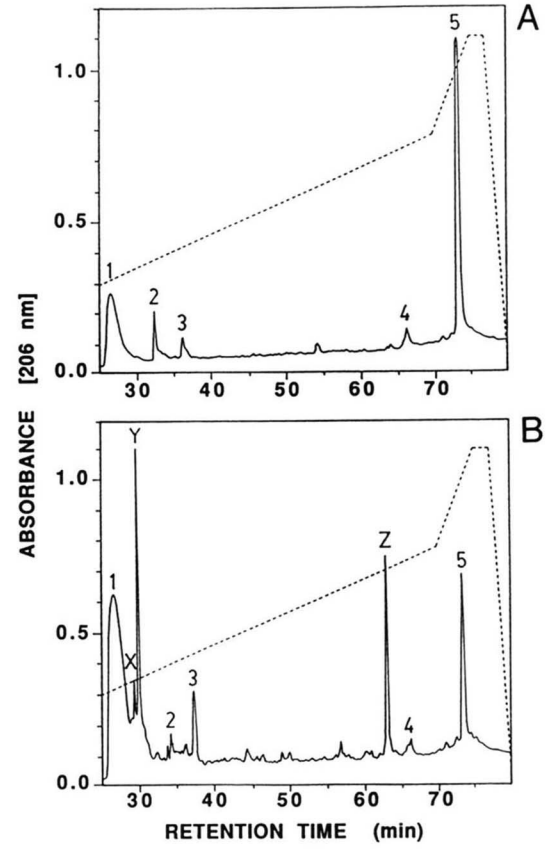

Fig. 6. Elution profiles for RP-HPLC of CoA transferase, either (**A**) nicked or (**B**) nicked/autolytically fragmented. Separations were performed as described in the Materials and methods. The gradient curve indicates 25% B → 70% B → 100% B → 100% A, where A is 0.1% TFA in H_2O and B is 0.1% TFA in CH_3CN. Peaks appearing in both plots are labeled 1–5, whereas those unique to the lower plot are labeled X, Y, or Z. Adapted from Rochet and Bridger (1994).

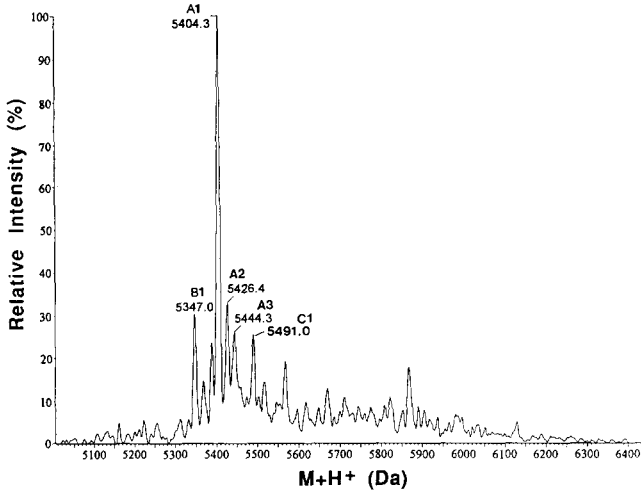

Fig. 7. Electrospray mass spectrum of peptide Z (approximately 10 pmol). The symbols A1, A2, A3, B1, and C1 indicate peaks referred to in the text. See also Table 2 for a summary of relevant peaks.

sium adducts. Clearly, the molecular weight values obtained by electrospray mass spectrometry point to Ser 343 as the C-terminus of peptide D, and therefore to Glu 344 as the site of the autolytic fragmentation.

Discussion

The fragmentation of CoA transferase at 70 °C was proposed to be autolytic in nature because it only occurs under conditions allowing for the formation of the enzyme-CoA intermediate. Furthermore, the release of an N-terminally blocked C-terminal fragment supported the predicted mechanism for autolysis (Howard et al., 1986). This mechanism, which suggests that the enzyme's catalytic glutamate may be identified by locating the site of autolysis, is further substantiated by our demonstration that the degree of fragmentation is not altered by the presence of a mixture of protease inhibitors. In order to determine where the fragmentation occurs in the CoA transferase sequence, we have applied RP-HPLC in combination with mass spectrometry, techniques that are generally susceptible to interference by a high concentration of salt. For this reason, determining that micromolar amounts of succinyl-CoA are sufficient for obtaining a high yield of autolysis contributed significantly to the success of our approach.

Table 2. *Summary of significant components detected by electrospray mass spectrometry*

Peak identity[a]	M + H$^+$ (Da)	Predicted peptide[b]	Associated ion(s)
A1	5,404.3	G295–S343	–
A2	5,426.4	G295–S343	1 Na^+
A3	5,444.3	G295–S343	1 K^+
B1	5,347.0	K296–S343	–
C1	5,491.0	S294–S343	–

[a] Peaks are identified according to the lettering scheme used in Figure 7.

[b] The single-letter amino acid code is used to indicate the N-terminal and C-terminal residues of each peptide. Numbering is based on the sequence of the precursor form of the enzyme (Lin & Bridger, 1992). Note that the 2 methionine residues in each of the 3 peptides listed are predicted to be oxidized to methionine sulfoxide.

A peptide (fragment D) bordered N-terminally by the previously identified proteolytic cleavage site (Lin & Bridger, 1992) and C-terminally by the autolytic fragmentation site is released upon subjecting the nicked form of CoA transferase to autolysis. RP-HPLC analysis of a sample of nicked, fragmented enzyme revealed the presence of 2 species absent from the control. The first of these, represented by 2 peaks eluting at very similar retention times, was assigned a molecular weight of 2,510.0 Da by electrospray mass spectrometry. This species may correspond to a degradation product of fragment C, given that its mass is significantly less than the minimum predicted for peptide D, and consistent with our inability to determine its N-terminal sequence by the Edman degradation method. The second eluted species, similarly analyzed by electrospray mass spectrometry, consists predominantly of a 5,404.3-Da peptide. This mass value is nearly equivalent to that predicted for fragment D (with N-terminal Gly 295) if Glu 344 is the site of autolysis. The fact that a peptide of this size is specifically associated with the nicked, autolytically fragmented form of the enzyme was further confirmed by MALDI mass spectrometry (data not shown).

The electrospray mass spectrum also reveals peaks at 5,491.0 Da and 5,347.0 Da (i.e., respectively, 86.7 Da greater than and 57.3 Da less than the mass of the 5,404.3-Da species). These mass increments most likely represent the addition of a serine and the loss of a glycine, respectively, at the N-terminus of the 5,404.3-Da species, assuming that the C-terminus of all peptides in this fraction must be strictly defined by the autolytic fragmentation. Based on our knowledge of the sequence of CoA transferase, we predict that this identification of terminal Ser-Gly- and Gly- reflects the N-terminal sequence of the 24-kDa fragment. Thus, we have successfully identified the species represented by peak Z as peptide D, both by its mass and by its N-terminal sequence.

The identification of 3 components with an N-terminal Ser 294, Gly 295, or Lys 296 in the electrospray mass spectrum of fragment D suggests that the 24-kDa C-terminal proteolytic segment of nicked CoA transferase likely consists of a population of polypeptides differing in length at their N-termini. Given that the enzyme is not nicked when purified in the presence of PMSF, a serine proteinase such as trypsin or thrombin is likely responsible for the cleavage when the inhibitor is omitted. We have also observed that native CoA transferase is cleaved by trypsin at a unique site during incubations of 8–12 h (data not shown); this proteolysis generates 2 fragments equivalent in size to those present in a preparation of nicked enzyme (28 kDa and 24 kDa). It is conceivable, then, that CoA transferase purified in the absence of PMSF is cut in its hydrophilic linker between Arg 293 and Ser 294, which corresponds to a trypsin cleavage site. Contaminating aminopeptidases (e.g., cathepsin C) may subsequently remove 1 or 2 amino acids from the N-termini of some of the resulting proteolytic fragments. Significant N-terminal heterogeneity was not detected previously (Lin & Bridger, 1992) because the sequence of the 24-kDa fragment was obtained by the Edman degradation method, which, when applied to a pro-

tein of unknown sequence, is normally used to identify the most abundant amino acid released at each cycle. Furthermore, it is conceivable that the predominant species present in 2 different proteolytically nicked enzyme preparations may not be identical.

Finally, given that the enzyme may bind Na^+ and K^+ by virtue of its exposure to potassium phosphate during the purification and to sodium phosphate and sodium succinyl-CoA upon autolysis, our identification of Na^+ and K^+ adducts present as minor components in the electrospray mass spectrum is reasonable.

Recently, mass spectrometry has been successfully applied to probe mechanism-based events occurring at the active sites of various enzymes. These processes include: covalent modification by a suicide substrate, leading to inactivation of enzymes such as 1-aminocyclopropane-1-carboxylate synthase (Yip et al., 1990) and *S*-adenosylmethionine decarboxylase (Diaz & Anton, 1991); transient covalent-bond formation between a substrate and its cognate enzyme, as demonstrated for α-glucosyltransferase (Mooser et al., 1991); and autolysis of an enzyme such as myeloperoxidase (Taylor et al., 1992). In this report, we demonstrate that the mass spectrometric analysis of autolytic fragments can be used to identify the catalytic residue in an enzyme whose mechanism involves an activated glutamate. Other enzymes potentially sharing this mechanistic feature, and therefore presumably amenable to this approach, include CoA transferases from different species (Fig. 8), tryptophanyl-tRNA synthetase (Kisselev et al., 1979; Kovaleva et al., 1983), long-chain fatty acyl-CoA synthetase (Spector, 1982), and β-galactosidase (Cupples et al., 1990).

The assignment of Glu 344 as the catalytic amino acid of the pig heart enzyme is supported by the previously reported identity within this region of the known CoA transferase sequences (Parales & Harwood, 1992; Fig. 8). Furthermore, following the completion of this work, we have learned that this identification is also entirely consistent with the results of Williams (1990), who isolated and sequenced peptides containing the thioesterified glutamic acid residue. Our results reveal an active-site element suitable for modification by site-directed mutagenesis, thus effectively permitting a more thorough exploration of the structure–function relationships governing the mechanism of this enzyme.

Materials and methods

Enzyme isolation, assay, and purity

Pig heart CoA transferase was isolated from fresh myocardium as previously described (Moore & Jencks, 1982; Lin & Bridger, 1992). Intact enzyme was purified in the presence of 0.1 mM PMSF, whereas the nicked form of CoA transferase was prepared by omission of this serine proteinase inhibitor from all purification buffers. Activity was determined by monitoring the absorbance increase at 310 nm, representative of the initial rate of appearance of acetoacetyl-CoA from the reaction of succinyl-CoA with acetoacetate (Stern et al., 1956). Assays were performed at 22 °C by adding 10 μL of enzyme preparation to 1 mL of assay solution (50 mM Tris-chloride, pH 9.1, 0.3 mM succinyl-CoA, 67 mM acetoacetate, and 15 mM magnesium chloride). The intact and nicked CoA transferase preparations were >95% homogeneous by SDS-PAGE, with typical specific activities ranging from 900 Stern units/mg to 3,000 Stern units/mg.[1]

```
                    344                 358                 369
PigTr   VHLQSENGILGLGPYPLQNEVDADLINAGKETVTV
PcaJ    VFLHSENGLLGMGPSPAPGEEDDDLINAGKQHVTL
CatJ    VFLHSENGLLAFGPPPAAGEEDPELINAGKEYVTM
ClosTr  ITFQSENGIVGMGASPKINEADKDVVNAGGDYTTV
```

Fig. 8. Alignment of deduced CoA transferase sequences in the region of the active-site glutamate. Sequences are from *Pseudomonas putida* (PcaJ), *Acinetobacter calcoaceticus* (CatJ), *Clostridium acetobutylicum* (ClosTr), and pig heart (PigTr). Underlined residues indicate identity among all 4 proteins. Numbers are based on the sequence of the precursor form of the pig heart enzyme (Lin & Bridger, 1992). Adapted from Parales and Harwood (1992).

Autolytic fragmentation

Microgram quantities of intact or nicked CoA transferase were subjected to autolysis as previously described (Howard et al., 1986). Briefly, the enzyme was diluted in 3–4 volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 3 mM or 10 μM succinyl-CoA (see text for details). Where applicable, a protease inhibitor cocktail consisting of pepstatin A (1 μg/mL), antipain (1–2 μg/mL), PMSF (100 μg/mL), and EDTA (1 mM) was included. Samples were incubated at 25 °C for 15 min and then at 70 °C for a further 60 min. The reaction was terminated by adding trifluoroacetic acid (TFA) or formic acid to 0.5% before analysis by RP-HPLC or MALDI mass spectrometry; alternatively, an equivalent volume of sample buffer (consisting of: 50 mM Tris-Cl, pH 6.60; 2.5% SDS; 1% 2-mercaptoethanol; 35% glycerol; and 0.01% bromophenol blue) was added prior to analysis by SDS-PAGE.

Tricine SDS-PAGE

Preparation and electrophoresis of 1.5-mm polyacrylamide gels designed to resolve peptides of low molecular weight were performed as described by Schagger and von Jagow (1987). The overall assembly consisted of a 16.5% T, 3% C separating gel (23 mL), a 10% T, 3% C spacer gel (6 mL), and a 4% T, 3% C stacking gel (4 mL). Electrophoresis was carried out at a constant voltage of 85 V for a period of 20–24 h.

RP-HPLC

Liquid chromatography was performed on a Vydac 218TP C18 column (50 mm × 2.1 mm ID, 5-μm particle size, 300-Å pore size) purchased from The Sep/a/ra/tions Group (Hesperia, California). A gradient defined by a 1%/min increase in solvent B (where A is 0.1% TFA in H_2O and B is 0.1% TFA in CH_3CN) was used to elute peptides at a flow rate of 0.2 mL/min. Elution was monitored by measuring absorbance at 206 nm and 1

[1] Stern units reflect the change in absorbance of a defined volume of solution through a specific path length, and their calculation thus requires the inconvenience of a factor correcting for the dimensions of the cuvette used in the assay. We therefore propose that the specific activity of CoA transferase be expressed in the more conventional units of μmol/min/mg, based on a molar extinction coefficient of 7,800 cm^{-1} M^{-1} for acetoacetyl-CoA (Howard et al., 1986). Thus, for the assay described above, 1 μmol/min = 257 Stern units and our enzyme's specific activity ranged from 3.5 to 11.7 μmol/min/mg.

fraction was collected per min. Beckman System Gold (IBM) was used for analysis and printing of data.

Electrospray ionization mass spectrometry

Molecular weight measurements were performed using a VG Platform electrospray mass spectrometer provided by Fisons Instruments (Manchester, UK). Samples of 10–100 pmol protein diluted in 10 μL of (1) H_2O:CH_3CN (50:50, v/v) + 0.5% formic acid or (2) isopropanol + 0.05% TFA were injected into the ion source at a flow rate of 10 μL/min. The electrospray potential was approximately 4 kV. The quadrupole mass analyzer was set to scan the range from $m/z = 400$ to $m/z = 2{,}400$ at 2 s per scan for a total duration of 10–20 s. The sum of the data acquired over this period constituted the final spectrum. Each molecular weight was determined as the mean value calculated for several multiply charged ions within a coherent series. The instrument was calibrated using the series of ion peaks from either bovine ubiquitin or a synthetic peptide of known mass.

Note added in proof

Subsequent to the completion of this work, we have expressed a mutant form of pig heart CoA transferase in which alanine replaces Glu 344. This form of the enzyme is both completely inactive and incapable of undergoing autolysis, which further confirms our identification of Glu 344 as the site of thioesterification (manuscript in prep.).

Acknowledgments

We are indebted to Edward Brownie for assistance in purifying the pig heart CoA transferase and to Charles Holmes for invaluable advice and for use of his HPLC facility. Paul Semchuk (University of Alberta), Therese Hutton, and Anne Maisey (Fisons Instruments) provided expert assistance with the mass spectrometry. J.-C.R. held a postgraduate studentship from the Natural Sciences and Engineering Research Council of Canada and awards from the Alberta Heritage Scholarship Fund (Ralph Steinhauer Award) and the Alberta Heritage Foundation for Medical Research. This work was supported by a grant (MT-2805) from the Medical Research Council of Canada.

References

Cupples CG, Miller JH, Huber RE. 1990. Determination of the roles of Glu-461 in β-galactosidase (*Escherichia coli*) using site-specific mutagenesis. *J Biol Chem 265*:5512–5518.

Diaz E, Anton DL. 1991. Alkylation of an active-site cysteinyl residue during substrate-dependent inactivation of *Escherichia coli S*-adenosylmethionine decarboxylase. *Biochemistry 30*:4078–4081.

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science 246*:64–71.

Hersh LB, Jencks WP. 1967a. Coenzyme A transferase: Kinetics and exchange reactions. *J Biol Chem 242*:3468–3480.

Hersh LB, Jencks WP. 1967b. Isolation and properties of an enzyme-coenzyme A intermediate. *J Biol Chem 242*:3481–3486.

Howard JB, Zieske L, Clarkson J, Rathe L. 1986. Mechanism-based fragmentation of coenzyme A transferase. *J Biol Chem 261*:60–65.

Kawasaki I, Itano HA. 1972. Methanolysis of the pyrrolidone ring of amino-terminal pyroglutamic acid in model peptides. *Anal Biochem 48*:546–556.

Khan SA, Erickson BW. 1984. Synthesis and regioselective hydrolysis of peptides containing an internal residue of pyroglutamic acid. *J Am Chem Soc 106*:798–799.

Kisselev LL, Favorova OO, Kovaleva GK. 1979. Molecular enzymology of beef pancreas tryptophanyl-tRNA synthetase. In: Schimmel PR, Söll D, Abelson JN, eds. *Transfer RNA: Structure, properties, and recognition*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory. pp 235–246.

Kovaleva GK, Holmuratov EG, Kisselev LL. 1983. Tryptophanyl-tRNA synthetase: Pyrophosphorylation of the enzyme in the course of adenylate formation? *FEBS Lett 151*:79–82.

Lin T, Bridger WA. 1992. Sequence of a cDNA clone encoding pig heart mitochondrial CoA transferase. *J Biol Chem 267*:975–978.

Moore SA, Jencks WP. 1982. Formation of active site thiol esters of CoA transferase and the dependence of catalysis on specific binding interactions. *J Biol Chem 257*:10893–10907.

Mooser G, Hefta SA, Paxton RJ, Shively JE, Lee TD. 1991. Isolation and sequence of an active-site peptide containing a catalytic aspartic acid from two *Streptococcus sobrinus* α-glucosyltransferases. *J Biol Chem 266*: 8916–8922.

Parales RE, Harwood CS. 1992. Characterization of the genes encoding β-ketoadipate:succinyl-coenzyme A transferase in *Pseudomonas putida*. *J Bacteriol 174*:4657–4666.

Rochet JC, Bridger WA. 1994. Identification of the active site of CoA transferase by peptide sequencing. In: Hodges RS, Smith JA, eds. *Peptides: Chemistry, structure, and biology. Proceedings of the Thirteenth American Peptide Symposium*. Forthcoming.

Schagger H, von Jagow G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem 166*:368–379.

Smith RD, Loo JA, Edmonds CG, Baringa CJ, Udseth HR. 1990. New developments in biochemical mass spectrometry: Electrospray ionization. *Anal Chem 62*:882–899.

Solomon F, Jencks WP. 1969. Identification of an enzyme-γ-glutamyl coenzyme A intermediate from coenzyme A transferase. *J Biol Chem 244*:1079–1081.

Spector, L.B. 1982. *Covalent catalysis by enzymes*. New York: Springer-Verlag. pp 212–214.

Stern JR, Coon MJ, Del Campillo A, Schneider MC. 1956. Enzymes of fatty acid metabolism: IV. Preparation and properties of coenzyme A transferase. *J Biol Chem 221*:15–31.

Taylor KL, Pohl J, Kinkade JM Jr. 1992. Unique autolytic cleavage of human myeloperoxidase. *J Biol Chem 267*:25282–25288.

White H, Solomon F, Jencks WP. 1976. Utilization of the inactivation rate of coenzyme A transferase by thiol reagents to determine properties of the enzyme-CoA intermediate. *J Biol Chem 251*:1700–1707.

Williams WA. 1990. Structure/function relationships within pig heart CoA transferase [thesis]. Minneapolis: University of Minnesota.

Yip WK, Dong JG, Kenny JW, Thompson GA, Yang SF. 1990. Characterization and sequencing of the active site of 1-aminocyclopropane-1-carboxylate synthase. *Proc Natl Acad Sci USA 87*:7930–7934.