Conversion of glutaconate CoA-transferase from *Acidaminococcus fermentans* into an acyl-CoA hydrolase by site-directed mutagenesis

Matthias Mack, Wolfgang Buckel*

Laboratorium für Mikrobiologie des Fachbereichs Biologie der Philipps-Universität Marburg, Karl-von-Frisch-Str., D-35032 Marburg, Germany

Received 3 February 1997

Abstract The heterooctameric $(\alpha\beta)_4$ glutaconate CoA-transferase (EC 2.8.3.12) from the anaerobic bacterium *Acidaminococcus fermentans* catalyses the transfer of CoASH from acetyl-CoA to the 1-carboxylate of glutaconate. During this reaction the glutamate residue 54 of the β-subunit (βE54) forms a CoA-ester. The single amino acid replacement βE54D resulted in a drastic change of enzymatic function. The CoA-transferase activity decreased from 140 to less than 0.01 s^{-1}, whereas the acyl-CoA hydrolase activity increased from less than 0.01 to 16 s^{-1}. The new enzyme was able to catalyse the hydrolysis of glutaryl-CoA, acetyl-CoA and 3-butenoyl-CoA. Since the mutants βE54A and βE54N showed neither acyl-CoA hydrolase nor CoA-transferase activity, it was concluded that the aspartate carboxylate of the mutant βE54D acted as a general base which facilitated the attack of water at the thiolester carbonyl. Surprisingly, K_m for glutaryl-CoA hydrolysis by the mutant (0.7 μM) as compared to CoA-transfer by the wild-type (28 μM) was 40 times lower. A 65 kDa protein, obtained by fusing the genes, *gctA–gctB*, coding for glutaconate CoA-transferase, retained 30% of the wild-type activity. Comparison of the amino acid sequences of 13 related enzymes demonstrated that Nature already has applied gene fusion in the case of pig heart CoA-transferase and has been using the E → D mutation for catalysis by a yeast acetyl-CoA hydrolase.

Key words: CoA-transferase; CoA-ester hydrolase; Mutation; Active site glutamate; Active site aspartate; Gene fusion; Enzyme mechanism; *Acidaminococcus fermentans*

1. Introduction

Glutaconate CoA-transferase (EC 2.8.3.12) from the strict anaerobic bacterium *Acidaminococcus fermentans* participates in the fermentation of glutamate to ammonia, CO_2, acetate, butyrate and H_2. The enzyme is responsible for the reversible transfer of the $CoAS^-$ moiety from acetyl-CoA to the characteristic intermediate of this pathway, (*R*)-2-hydroxyglutarate, to yield (*R*)-2-hydroxyglutaryl-CoA, the substrate for the subsequent dehydration to glutaconyl-CoA followed by decarboxylation to crotonyl-CoA. Propionyl-CoA serves as an even better CoA-donor than acetyl-CoA and acceptors superior to (*R*)-2-hydroxyglutarate are glutaconate and glutarate. Glutaconate CoA-transferase consists of two different subunits, GctA (α, 36 kDa) and GctB (β, 29 kDa), which form a heterooctameric enzyme $(\alpha\beta)_4$. The two corresponding genes, *gctA* and *gctB,* which are located at the beginning of the hydroxyglutarate operon of *A. fermentans*, have been cloned and overexpressed together in *Escherichia coli* [1,2]. With pig heart succinyl-CoA:3-ketoacid CoA-transferase (EC 2.8.3.5) Jencks and coworkers [3,4] showed that a glutamate residue participated in catalysis, forming a transient thiolester with CoASH (Scheme 1). Rochet and Bridger tentatively identified this residue as E344 by the ability of the CoA-ester intermediate to cleave the peptide bond to the preceding amino acid S343 at 70°C [5]. The enzyme from *A. fermentans* contains a comparable glutamate residue on the β-subunit, which was identified as βE54 by reduction of the CoA-ester intermediate with $NaB[^3H]_4$ followed by sequencing of the ^3H-labelled tryptic peptide [1,2]. Site-directed mutagenesis proved the importance of this glutamate residue for catalysis, since βE54A was almost completely inactive (0.02% activity of that of the wild-type), whereas mutation of the next glutamate residue in the sequence, βE64A, did not affect the activity very much (30%). Another mutant, βE54D, also showed significant residual CoA-transferase activity (13%), although the thiolester intermediate could not be detected by reduction with $NaB[^3H]_4$ [6]. A more thorough analysis revealed, however, that this mutant was indeed an acyl-CoA *hydrolase* and not CoA-*transferase*. This work describes the purification and characterisation of this new enzyme.

2. Materials and methods

2.1. Materials

5,5′-Dithiobis(2-nitrobenzoate) and coenzyme A were obtained from Serva (Heidelberg, Germany); all other chemicals were purchased from Merck (Darmstadt, Germany). CoA-esters of glutaric, acetic and 3-butanoic acid were prepared from the corresponding anhydrides and CoASH in 1 M $KHCO_3$ (pH 8) at ambient temperature [7]. Enzymes for DNA manipulations were purchased from Amersham (Braunschweig, Germany).

2.2. Mutant construction and expression

For the newly constructed mutant in this work, βE54N, the same procedure was applied as in the replacements of βE54 by A, D or Q [6] using the mismatch oligonucleotide primer 5′-CATCGTGAACAGCGGTCTG-3′. The fusion protein GctF was generated with 5′-CGAAGGAGGACAAAGGAGCAATGGCTGATTAC-3′. The mutant genes were proof-sequenced and no secondary mutations were detected. Cell-free extracts of *E. coli* strains containing the wild-type and mutant enzymes were prepared by sonication as in [2] in the presence of additional 1 mM phenylmethylsulfonyl fluoride. The mutant enzyme βE54D was purified by ammonium sulfate fractionation and crystallisation as described for the wild-type enzyme [2]. The fusion protein GctF was purified by chromatography on Q-Sepharose HP HiLoad (26/10, Pharmacia) in 20 mM potassium phosphate (pH 6.5) and elution with a linear gradient around 0.4 M NaCl, followed by chromatography on Superdex 200 (35/600, Pharmacia) in the same buffer containing 0.1 M NaCl. Protein was determined using the BioRad-assay (BioRad, Munich, Germany). The purity of the proteins was monitored by SDS-PAGE with Coomassie Brilliant Blue R-250 stain.

*Corresponding author. Fax: (49) 6421-288979.
E-mail: buckel@mailer.uni.marburg.de

PII S0014-5793(97)00187-7

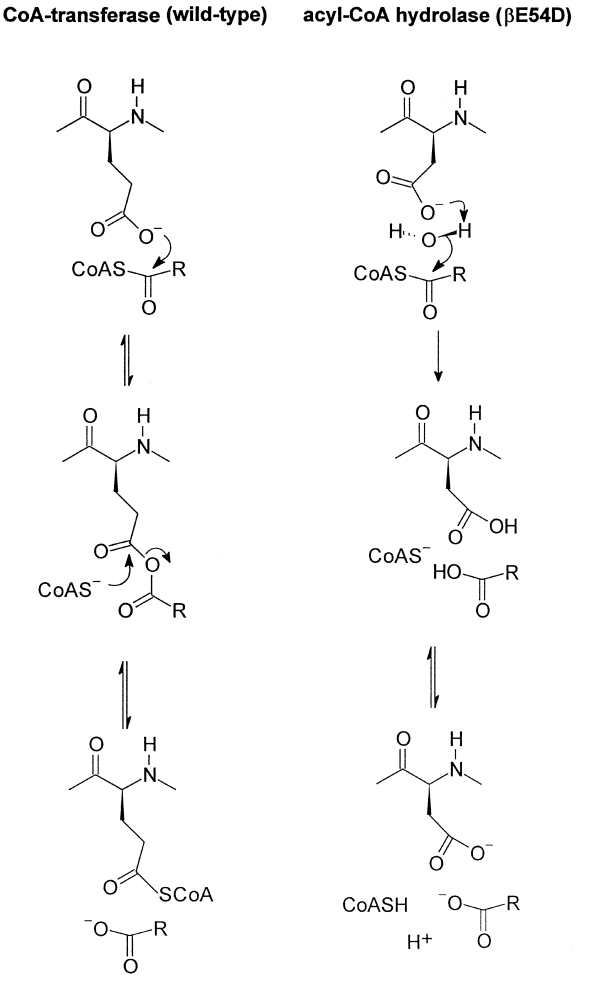
2.3. Enzyme assays

The activity of glutaconate CoA-transferase was measured spectrophotometrically in a coupled assay in 100 mM potassium phosphate (pH 7.0) via the formation of acetyl-CoA from 0.1 mM glutaryl-CoA and 200 mM sodium acetate. CoASH, which was liberated from acetyl-CoA by condensation with 1 mM oxaloacetate to citrate catalysed by citrate synthase from pig heart (20 μg/ml), was determined at 412 nm using 1.0 mM 5,5′-dithiobis(2-nitrobenzoate) [1]. Acyl-CoA hydrolase activity was measured with the same assay, from which acetate, oxaloacetate and citrate synthase were omitted. The turnover numbers (k_{cat}, s^{-1}) are based on the molecular mass of the heterodimer (αβ; 65 kDa), thus 1.00 U/mg = 1.08 s^{-1}.

3. Results

Overproducing *Escherichia coli* strains harbouring the pMM2 derivatives, which contained the genes coding for the wild-type or the mutant glutaconate CoA-transferases [2,6], were routinely induced with 300 μM isopropyl-1-thio-β-D-galactoside after growth reached $OD_{578} \approx 1.0$. The cells were harvested after growth for further 5 h up to $OD_{578} \approx 4$. Only the βE54D mutant strain reduced its growth rate immediately after induction and ceased to grow already at $OD_{578} \leq 1.5$. Despite the difference in growth behaviour, the same purification factor with wild-type and βE54D mutant strain indicated the same amount of gene expression per cell mass. About 10 mg pure βE54D mutant enzyme was isolated from a 2 l culture.

Wild-type glutaconate CoA-transferase activity was routinely measured with glutaryl-CoA (0.1 mM) and acetate (200 mM) as substrates. The produced acetyl-CoA was condensed with oxaloacetate catalysed by citrate synthase to give (*S*)-citryl-CoA followed by hydrolysis to citrate and CoASH. Finally CoASH reacted with 5,5′-dithiobis(2-nitrobenzoate) to form the yellow anion of 2-nitro-5-mercaptobenzoate, which causes an increase in absorbance at 412 nm. Since citrate synthase was applied as a specific acetyl-CoA hydrolase, this assay was unable to distinguish between the activities of hydrolysis and CoA-transfer to acetate. This fact misled the authors to conclude that the βE54D mutant had still 13% transferase activity [6]. The hydrolase activity of the mutant was demonstrated by omitting acetate, citrate synthase and oxaloacetate from the CoA-transferase assay (hydrolase assay). Whereas the activity of the pure wild-type enzyme apparently decreased under these conditions from 140 s^{-1} to less than 0.01 s^{-1}, that of the βE54D mutant remained unchanged at 4.0 U/mg, when measured in the cell extract, and at 15 U/mg (16 s^{-1}) after purification. In another assay, which monitors the formation of the α,β-enoyl-thiolester moiety in glutaconyl-CoA at 263 nm ($\Delta\varepsilon = 6.0$ mM^{-1} cm^{-1}) with glutaconate and acetyl-CoA as substrates, the mutant was inactive (< 0.01 s^{-1}), whereas with the wild-type an activity of 17 s^{-1} was obtained [1]. Finally, the hydrolysis of acetyl-CoA was also measured at the absorbance maximum of unconjugated thiolesters (232 nm, $\Delta\varepsilon = 4.5$ mM^{-1} cm^{-1}) [8]. A surprising result with this pure new hydrolase was the 40-times lower apparent K_m of glutaryl-CoA (0.7 μM) as compared to that in the CoA-transferase reaction (28 μM at 200 mM acetate). Thus, the specificity constant k_{cat}/K_m of the new hydrolase was even 3-times higher (23 s^{-1} μM^{-1}) than that of the wild-type CoA-transferase (7.6 s^{-1} μM^{-1}) (Table 1). The hydrolase activity with glutaryl-CoA as substrate remained unchanged in various buffer systems with different pH values (potassium phosphate, pH 5.8–7.8; Tris-HCl, pH 7.7–9.5; sodium borate, pH 8.9–9.8; 100 mM each). The specific activity of glutaryl-CoA hydrolysis increased, however, by adding KCl, KNO_3 or NaCl to the assay; at the optimum concentration of 0.8 M salt it was almost twice as high. In addition, K_m for glutaryl-CoA increased 40-fold under these conditions. On the other hand, the specific activities of acetyl-CoA and 3-



Scheme 1.

Table 1
Kinetic parameters of purified acyl-CoA hydrolase (βE54D)

Substrate	k_{cat} (s^{-1})		K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
Glutaryl-CoA	16	(28)*	0.70 (31)	23 (0.90)
Acetyl-CoA	0.72	(0.64)	33	0.022
3-Butenoyl-CoA	1.4	(0.15)	58	0.024
Glutaryl-CoA in CoA-transfer (wild-type) at 200 mM acetate	214**		28	7.6

*The numbers in brackets were obtained in the presence of additional 0.8 M NaCl.
**Under standard assay conditions the specific activity was 140 s^{-1} (130 U/mg).

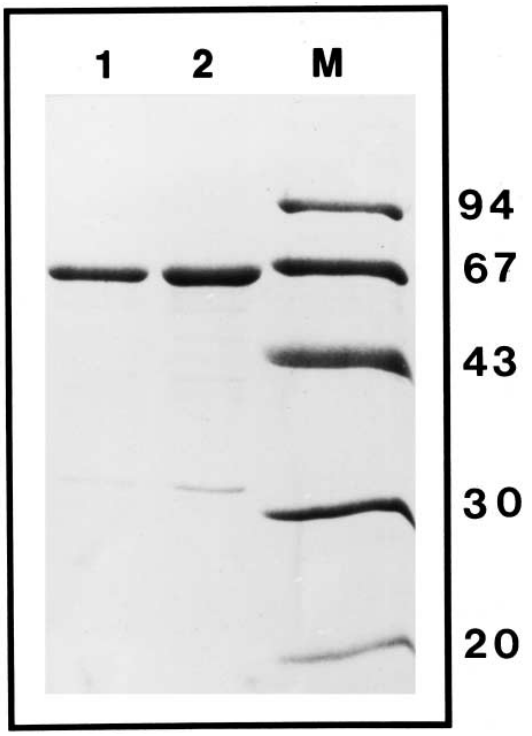


Fig. 1. SDS-PAGE of the fusion protein GctF. Two fractions from the Superdex-200 eluate (see Section 2), 6 and 10 μg protein, were applied on lanes 1 and 2 of a 12% polyacrylamide gel, respectively. Lane M contains a commercial molecular mass standard. The numbers indicate the masses in kDa.

butenoyl-CoA hydrolysis decreased in the presence of salt. Table 1 shows these data for 0 and 0.8 M NaCl in addition to 100 mM potassium phosphate (pH 7.0).

In order to assess the importance of the carboxyl group of the aspartate residue in the βE54D mutant, the βE54A [6] and βE54N mutants were included in this study. The mutant enzymes were overproduced in *E. coli* to the same level as the wild-type as checked by SDS-PAGE [2]. In the hydrolase assay the extracts yielded 0.012 and 0.018 U/mg, and in the transferase assay 0.011 and 0.012 U/mg, respectively. Hence, the transferase activities were about 2000 times lower than that of an extract containing the wild-type enzyme (30 U/mg). Therefore these mutant enzymes were not purified and characterised further.

In another mutant the GctA and B-subunits were fused together to yield GctF (F = fusion of A–B). In the mutant, the stop-codon of *gctA* (TAA) was changed to a codon for glycine (GGA) and a single **G** was added to the short intergenic region to give **G**CA (alanine), whereas the start codon of *gctB* was retained. After expression of *gctF* in *E. coli*, the fusion protein was purified to homogeneity. PAGE in the presence of SDS revealed the calculated molecular mass (35.7+29.2 = 65 kDa; Fig. 1). In lane 2 a trace of an impurity, probably the β-subunit (on SDS-PAGE its apparent molecular mass is 32 instead of 29 kDa), is visible. The ribosome binding site at the 3′-end of the *gctA*-part of the fused gene may have caused additional expression of *gctB* alone. The pure GctF had a CoA-transferase activity of 39 s^{-1} (30% of that of the wild-type) but no hydrolase activity ($< 10^{-3}$ s^{-1}); the apparent K_m for glutaryl-CoA was 64 μM at 200 mM acetate (wild-type 28 μM; Table 1).

4. Discussion

The expression of the mutated gene *gctB*^{E54D} instead of the wild-type gene in *E. coli* (together with wild-type *gctA*) caused

Table 2
Sequence comparison around the active site glutamate and aspartate residues of CoA-transferases and acyl-CoA hydrolases

D	C	H	**I**	**I**	**V**	**E**	**S**	**G**	**L**	M	**D**	C	S	**P**	GctB, *Acidaminococcus fermentans* (54)
E	V	F	**L**	H	*S*	**E**	N	**G**	**L**	*L*	*G*	*M*	*G*	**P**	PcaJ, *Pseudomonas putida* (50)
D	V	F	**L**	H	**S**	**E**	N	**G**	**L**	*L*	A	F	*G*	**P**	CatJ, *Acinetobacter calcoaceticus* (49)
K	I	T	***F***	Q	*S*	**E**	N	**G**	**I**	*V*	*G*	*M*	*G*	A	CtfB, *Clostridium acetobutylicum* (50)
T	V	H	**L**	Q	*S*	**E**	N	**G**	**I**	*L*	*G*	L	*G*	**P**	Acetoacetate CoA-transferase, pig (344)
V	D	Y	***V***	***V***	*T*	**E**	Y	**G**	***V***	*A*	H	*L*	K	*G*	Orf z, *Clostridium kluyveri* (390)
V	M	V	**I**	***V***	*T*	**E**	*Q*	**G**	***V***	*A*	**D**	*L*	*R*	*G*	Cat1, *Clostridium kluyveri* (429)
A	Q	I	***F***	***V***	*T*	**E**	*Q*	**G**	**L**	*A*	**D**	*L*	*R*	*G*	AarC, *Acetobacter aceti* (471)
V	M	V	***V***	***V***	*T*	**E**	Q	**G**	**L**	*A*	**D**	*L*	*R*	*G*	Cta, *Thermoanaerobacterium thermosaccharolyticum* (421)
V	K	V	**I**	**I**	*T*	**E**	*Q*	**G**	**I**	*A*	**D**	*L*	*R*	*G*	Orf o492, *Escherichia coli* (427)
L	D	V	**I**	***V***	*T*	**E**	N	**G**	**L**	*A*	**D**	*L*	*R*	*G*	Acetate utilization protein, *Neurospora crassa* (450)
L	D	I	**L**	***V***	*T*	**E**	Q	**G**	**L**	*A*	**D**	*L*	*R*	*G*	Orf, *Schizosaccharomyces pombe* (272)
L	D	I	**L**	***V***	*T*	**D**	Q	**G**	**L**	*A*	**D**	*L*	*R*	*G*	Acetyl-CoA hydrolase, *Saccharomyces cerevisiae* (447)
N	P	W	*P*	*N*	**V**	**D**	*A*	*H*	*S*	G	V	*L*	L	Q	Citrate synthase, pig heart (375)
K	L	Y	*P*	*N*	**V**	**D**	F	Y	*S*	G	I	*I*	L	K	Citrate synthase I, *E. coli* (362)
K	M	F	*P*	*N*	*L*	**D**	W	F	*S*	A	V	S	Y	N	Citrate synthase II, *E. coli* (325)

GtcB, β-subunit of glutaconate CoA-transferase $(\alpha\beta)_4$ [2].
PcaJ and CatJ, β-subunits of 3-oxoadipate CoA-transferases $(\alpha\beta)_2$ [16].
CtfB, β-subunit of acetoacetate CoA-transferase $(\alpha\beta)_2$ [17].
Pig heart CoA-transferase α_2 [18].
Orf z, 4-hydroxybutyrate CoA-transferase [19].
Cat1, succinate CoA-transferase [19].
AarC, acetate CoA-transferase [20].
Cta, butyrate CoA-transferase, Staudenbauer, W.L, Technical University, Munich, personal communication, EMBL accession number Z6903.
Orf o492, *E. coli* [21].
Acetate utilization protein [22].
Orf, *S pombe*, SWISS-PROT accession number P32316.
Acetyl-CoA hydrolase, Ach1 [11].
Citrate synthases from pig heart [23] and *E. coli* (I) [24]. The gene coding for citrate synthase II from *E. coli* was identified by S. Textor and W. Buckel (unpublished) in a sequenced region of the *E. coli* genome, GENBANK accession number U73857.
The numbers in parentheses indicate the sequence position of the corresponding *E* or *D* residue.

a detrimental effect on cell growth. The finding that the βE54D mutant of glutaconate CoA-transferase acts as a very efficient acyl-CoA hydrolase is consistent with its ability to impair growth of the bacteria. Hence, the acetyl-CoA levels in cells containing the mutant enzyme should be extremely low. Furthermore, if glutaryl-CoA or glutaconyl-CoA with a 40 times lower K_m would play a role in the metabolism of *E. coli*, the expression of the mutant gene in this organism would have probably never occurred.

The conversion of a CoA-transferase into an acyl-CoA hydrolase by the single replacement of the active site glutamate residue βE54 by an aspartate residue immediately shows how thiolester hydrolases might work. We propose a mechanism in which the aspartate residue of the mutant βE54D is too short to attack the carbonyl group of the CoA-ester substrate in order to form the mixed anhydride between substrate and enzyme required for CoA-transfer [4,9]. Instead a water molecule occupies the space of the former methylene group of the wild-type βE54. The aspartate anion of the mutant βD54 acts as a general base and thus increases the nucleophilicity of this water molecule for the attack on the thiolester carbonyl followed by release of CoASH (Scheme 1). Modelling of CoASH into the crystal structure of glutaconate CoA-transferase indeed shows that an aspartate residue cannot reach the thiolester carbonyl [10]. Therefore alternative mechanisms in which a mixed anhydride between βD54 and substrate is still formed are less likely.

Glutaryl-CoA was the best substrate for the new acyl-CoA hydrolase. Both acetyl-CoA and 3-butenoyl-CoA had lower k_{cat} and higher apparent K_m values (Table 1) which demonstrated a large contribution of the 5-carboxylate to binding and rate enhancement. This has also been shown for the wild-type enzyme in the CoA-transfer [1]. Addition of salt increased the glutaryl-CoA hydrolase activity but decreased the rate of hydrolysis of acetyl-CoA or butenoyl-CoA. A possible explanation for this finding could be the ionic interaction between the 5-carboxylate of glutaryl-CoA and a positively charged amino acid side chain of the acyl-CoA hydrolase. The crystal structure revealed, however, an oxyanion hole formed by αT27, αY74, αS78 and βS68 rather than a positively charged residue in the region to which the 5-carboxylate of glutaryl-CoA is proposed to bind [10].

Comparison of the primary structures of several CoA-transferases around their putative active site glutamate residue with that of glutaconate CoA-transferase identified the latter enzyme as the 'odd man out' among this family of enzymes (Table 2). Apparently there are at least three subfamilies of CoA-transferases acting either on C-5 dicarboxylates (1 member), 3-oxoacids (4 members), or short-chain fatty acids (2 real and 5 putative members). Glutaconate CoA-transferase and the three bacterial 3-oxoacid CoA-transferases are composed of two different polypeptides forming $(\alpha\beta)_4$ or $(\alpha\beta)_2$ complexes, respectively. The sequence of the twice as long single polypeptide of pig heart oxoacid CoA-transferase (α_2) indicates an origin common with the bacterial enzymes but in addition suggests a gene fusion as performed deliberately in this work with the genes coding for glutaconate CoA-transferase. Surprisingly, the sequences of the largest subfamily of CoA-transferases are very similar to that of an acetyl-CoA hydrolase (EC 3.1.2.1) from *Saccharomyces cerevisiae* [11] which shows that Nature has already been using the E→D mutation long before this work was performed. Further well-known acyl-CoA hydrolases are the citrate synthases (EC 4.1.3.7) which also contain a catalytic aspartate residue [12]. These enzymes, however, appear not to be related to the CoA-transferases (Table 2). The conserved aspartate residue of citrate synthase is thought to be involved in the enolisation of acetyl-CoA as well as in the hydrolysis of the thiolester intermediate [13]. On the other hand, mutation of the corresponding aspartate (D362) to glutamate in the *E. coli* citrate synthase I abolished the hydrolase activity but the reversible condensation of acetyl-CoA with oxaloacetate to (*S*)-citryl-CoA remained unimpaired [14]. Although model studies of citryl-CoA hydrolysis suggested the initial formation of a mixed anhydride between the aspartate residue and citrate [15], a direct attack of a water molecule at the thiolester assisted by the aspartate carboxylate anion seems to be a much more feasible mechanism. It would be of interest to check whether the D362E mutant forms a CoA-ester as in CoA-transferases.

Acknowledgements: The authors are indebted to Professor Perry Frey (University of Madison, Madison, WI) for helpful discussion as well as to Professor Walter L. Staudenbauer (Technical University, Munich) for the unpublished sequence from *T. thermosaccharolyticum* and part of the sequence alignments shown in Table 2. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

References

[1] Buckel, W., Dorn, U. and Semmler, R. (1981) Eur. J. Biochem. 118, 315–321.
[2] Mack, M., Bendrat, K., Zelder, O., Eckel, E., Linder, D. and Buckel, W. (1994) Eur. J. Biochem. 226, 41–51.
[3] Solomon, F. and Jencks, W.P. (1969) J. Biol. Chem. 244, 1079–1081.
[4] Jencks, W.P. (1973) The Enzymes (Boyer, P.D., ed.) 3rd edn., Vol. 9B, Academic Press, New York, pp. 483–496.
[5] Rochet, J.-C. and Bridger, W. (1994) Prot. Sci. 3, 975–981.
[6] Mack, M. and Buckel, W. (1995) FEBS Lett. 357, 145–148.
[7] Simon, E. and Shemin, D. (1953) J. Am. Chem. Soc. 75, 2520.
[8] Buckel, W. and Eggerer, H. (1965) Biochem. Z. 343, 29–43.
[9] Buckel, W. (1976) Eur. J. Biochem. 64, 263–267.
[10] Jacob, U., Mack, M., Clausen, T., Huber, R., Buckel, W. and Messerschmidt, A. (1997) Structure, in press.
[11] Lee, F.-J.S., Lin, L.-W. and Smith, J.A. (1990) J. Biol. Chem. 265, 7413–7418.
[12] Remington, S., Wiegand, G. and Huber, R. (1982) J. Mol. Biol. 158, 111–152.
[13] Alter, G.M., Casazza, J.P., Zhi, W., Nemeth, P., Srere, P. and Evans, C.T. (1990) Biochemistry 29, 7557–7563.
[14] Man, W.-J., Li, Y., O'Connor, D. and Wilton, D.C. (1991) Biochem. J. 280, 521–526.
[15] Buckel, W. and Eggerer, H. (1965) Hoppe Seyler's Z. Physiol. Chem. 350, 1367–1376.
[16] Parales, R.E. and Harwood, C.S. (1992) J. Bacteriol. 174, 4657–4666.
[17] Petersen, D.J., Cary, J.W., Vanderleyden, J.G. and Bennett, G.N. (1993) Gene 123, 93–97.
[18] Lin, T. and Bridger, W.A. (1992) J. Biol. Chem. 267, 975–978.
[19] Söhling, B. and Gottschalk, G. (1996) J. Bacteriol. 178, 871–880.
[20] Fukaya, M., Takemura, H., Okumura, H., Kawamura, Y., Horinuchi, S. and Beppu, T. (1993) J. Ferment. Bioeng. 76, 270–275.
[21] Burland, V., Plunkett III, G., Sofia, H.J., Daniels, D.L. and Blattner, F.R. (1995) Nucl. Acids Res. 23, 2105–2119.
[22] Marathe, S., Connerton, I.F. and Finchan, J.R. (1990) Mol. Cell Biol. 10, 2638–2644.
[23] Bloxham, D.P., Parmelee, D.C., Kumar, S., Walsh, K.A. and Titani, K. (1982) Biochemistry 21, 2028–2036.
[24] Bhayana, V. and Duckworth, H. (1984) Biochemistry 23, 2900–2905.