Discovery of the catalytic function of a putative 2-oxoacid dehydrogenase multienzyme complex in the thermophilic archaeon *Thermoplasma acidophilum*

Caroline Heath, Alex C. Jeffries, David W. Hough, Michael J. Danson*

Centre for Extremophile Research, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

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Abstract Those aerobic archaea whose genomes have been sequenced possess a single 4-gene operon that, by sequence comparisons with Bacteria and Eukarya, appears to encode the three component enzymes of a 2-oxoacid dehydrogenase multienzyme complex. However, no catalytic activity of any such complex has ever been detected in the Archaea. In the current paper, we have cloned and expressed the first two genes of this operon from the thermophilic archaeon, *Thermoplasma acidophilum*. We demonstrate that the protein products form an $\alpha_2\beta_2$ hetero-tetramer possessing the decarboxylase catalytic activity characteristic of the first component enzyme of a branched-chain 2-oxoacid dehydrogenase multienzyme complex. This represents the first report of the catalytic function of these putative archaeal multienzyme complexes.

Keywords: Archaea; 2-Oxoacid dehydrogenase; Multienzyme complex; Thermophile; Metabolism

1. Introduction

In aerobic bacteria and eukaryotes, a family of 2-oxoacid dehydrogenase multienzyme complexes (OADHCs) functions in the pathways of central metabolism. The complexes are responsible for the oxidative decarboxylation of 2-oxoacids to their corresponding acyl-CoAs (Fig. 1). Members of the family include the pyruvate dehydrogenase complex (PDHC), which catalyses the conversion of pyruvate to acetyl-CoA and so links glycolysis and the citric acid cycle; the 2-oxoglutarate dehydrogenase complex (OGDHC), which catalyses the conversion of 2-oxoglutarate to succinyl-CoA within the citric acid cycle; and the branched-chain 2-oxoacid dehydrogenase complex (BCOADHC), which oxidatively decarboxylates the branched-chain 2-oxoacids produced by the transamination of valine, leucine and isoleucine.

The complexes comprise multiple copies of three component enzymes: 2-oxoacid decarboxylase (E1), dihydrolipoyl acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) [1–3]. E2 forms the structural core of the complex, with multiple polypeptide chains associating into octahedral (24-mer) or icosahedral (60-mer) configurations, depending on the particular complex and the source organism [2,4]. E1 and E3 bind non-covalently to the E2 core. E1 may occur as a homodimer or as an $\alpha_2\beta_2$ hetero-tetramer, depending upon the source and the type of complex, although in all cases E3 is a dimer of identical subunits.

E2 also forms the catalytic core of the complex (Fig. 1): a lipoyl moiety, covalently attached to a lysine residue in the lipoyl domain, serves as a swinging arm, connecting the active sites of each enzyme and channelling substrate through the complex [3]. Thus, E1 catalyses the thiamine pyrophosphate (TPP)-dependent oxidative decarboxylation of the 2-oxoacid and the transfer of the resulting acyl group to the lipoic acid of E2. E2 then transfers the acyl-group to coenzyme-A, after which E3 serves to reoxidise the dihydrolipoyl moiety. It does so by the reduction of the non-covalently bound co-factor FAD, in conjunction with a protein disulfide bond and an amino acid base, which are themselves then reoxidised by NAD^+, forming NADH.

No OADHC activity has ever been detected in the Archaea [5]; instead, the oxidation of 2-oxoacids is catalysed by an unrelated and structurally more simple family of 2-oxoacid ferredoxin oxidoreductases (FORs). This comprises the pyruvate FOR, the 2-oxoglutarate FOR and the 2-oxoisovalerate FOR, which catabolise pyruvate, 2-oxoglutarate and the branched-chain 2-oxoacids, respectively [6–8]. The pyruvate FOR from *Halobacterium halobium* has an $\alpha_2\beta_2$ structure [9], but in the thermophilic archaea these enzymes generally contain four different subunits and occur as octamers ($\alpha_2\beta_2\gamma_2\delta_2$) [8]. Importantly in the current context, the FOR's catalytic reaction does not involve a lipoic acid moiety or NAD^+; rather, the acyl-moiety formed on decarboxylation of the 2-oxoacid is handed on direct to coenzyme-A, and the reducing equivalents to ferredoxin via the enzyme's iron-sulfur centre [6].

However, there is growing evidence to suggest that some archaea may possess an OADHC (reviewed in [10]). Whilst no whole complex activity has been found, E3 activity and lipoic acid have been detected in halophilic archaea [11,12] and an operon containing four genes, whose predicted protein

* Corresponding author. Fax: +44-1225-386779.
E-mail address: M.J.Danson@bath.ac.uk (M.J. Danson).

Abbreviations: BCOADHC, branched-chain 2-oxoacid dehydrogenase complex; DCPIP, 2,6-dichlorophenolindophenol; E1, 2-oxoacid decarboxylase; E2, dihydrolipoyl acyl-transferase; E3, dihydrolipoamide dehydrogenase; FOR, ferredoxin oxidoreductases; M_r, relative molecular mass; OADHC, 2-oxoacid dehydrogenase complexes; OGDHC, 2-oxoglutarate dehydrogenase complex; PDHC, pyruvate dehydrogenase complex; TPP, thiamine pyrophosphate

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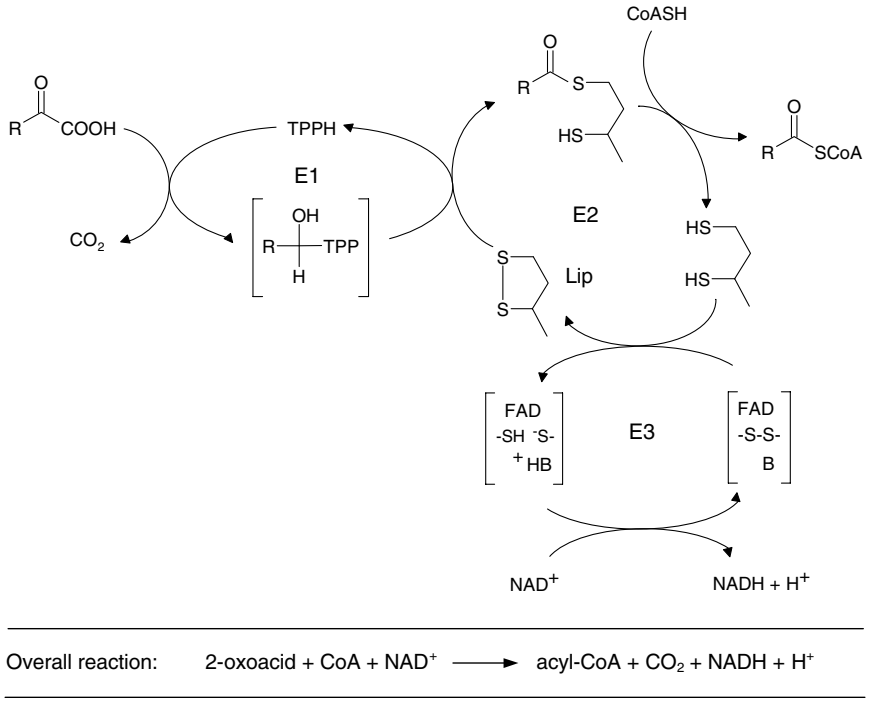

Fig. 1. The general reaction catabolised by OADHCs. The complex mechanism is described in Section 1. E1 (2-oxoacid decarboxylase); E2 (dihydrolipoyl acyl-transferase); E3 (dihydrolipoamide dehydrogenase). Symbols: B (a histidine base on E3); Lip (enzyme-bound lipoic acid); CoASH (coenzyme-A); TPP-H (thiamine pyrophosphate); FAD (flavin adenine dinucleotide); NAD (nicotinamide adenine dinucleotide).

sequences show significant identity to OADHC components E1α, E1β, E2 and E3 of bacteria and eukaryotes, was subsequently found in *Haloferax volcanii* [13]. E3 activity has also been detected in cell extracts of *Thermoplasma acidophilum* [14]. The recent sequencing of a number of archaeal genomes has revealed the presence of putative OADHC-encoding operons in the aerobic archaea: *H. halobium*, *T. acidophilum*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, and *Sulfolobus solfataricus*. To date, there is no experimental evidence to suggest that a functional complex is assembled from these operons, and E3-knockout studies in *Hfx. volcanii* failed to elucidate any metabolic function [15].

In the current paper, to elucidate the function of this putative OADHC in the Archaea, we have cloned and expressed in *Escherichia coli* the E1α and E1β genes from the archaeon, *T. acidophilum*, which is a thermoacidophile that grows optimally at 59 °C and pH 2.0 [16]. Enzyme assays of the purified recombinant E1 enzyme show it to be an active branched-chain 2-oxoacid decarboxylase component, suggesting that the operon in this archaeon encodes a functional BCOADHC.

2. Materials and methods

2.1. Bioinformatics

The putative OADHC operon was identified in the *T. acidophilum* DSM1728 genome from the ENTREZ Nucleotides database (http://www3.ncbi.nlm.nih.gov). The features of this operon and the arrangement of the four genes [E1α: Ta1438; E1β: Ta1437; E2: Ta1436; and E3: Ta1435] are illustrated diagrammatically in Fig. 2.

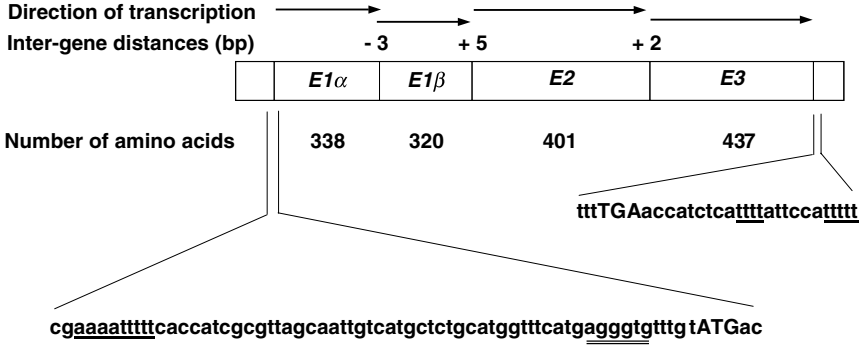

Fig. 2. The arrangement, inter-gene distances (base pairs, bp) and proposed direction of transcription (→) of the four open reading frames constituting the proposed *T. acidophilum* OADHC operon. The corresponding number of amino acids of the protein products is given. The DNA sequence at the 5′ end of the E1α gene, plus the upstream region, is shown to illustrate a potential promoter sequence (underlined), a potential Shine–Dalgarno sequence (doubly underlined) and the ATG start codon. The 3′ TGA stop codon and the proposed transcriptional stop signals (underlined) are also shown.

2.2. Gene cloning

T. acidophilum DSM 1728 was grown at 59 °C in DSM medium 158 and, after cell lysis in 50 mM Tris buffer (pH 7.5), genomic DNA was extracted according to [17]. The genes encoding E1α and E1β were individually PCR-amplified from this *T. acidophilum* genomic DNA, using primers that introduced restriction sites to the 5′ and 3′ ends of the gene products (*Xho*I and *Bam*HI for E1α, *Nhe*I and *Bam*HI for E1β). These primers also permitted the introduction of an N-terminal His tag into the protein products when expressed in the pET vector system (Novagen). PCR-amplification was carried out using *Vent* polymerase (New England Biolabs), followed by A-tailing with *Taq* polymerase (Promega). Amplified DNA fragments were purified by electrophoresis in a 0.8% (w/v) agarose gel and extracted using the Qiaex II Gel Extraction kit (Qiagen). Fragments were separately ligated into the pGEM-T (Promega) intermediate cloning vector, from which the E1α gene fragment was excised and cloned into pET-19b (Amp^r), and the E1β fragment into pET-28a (Kan^r), using T4 DNA ligase, generating the recombinant expression vectors pET-19b-E1α and pET-28a-E1β.

2.3. Expression of gene products

E. coli BL21(DE3)pLysS cells were separately heat-shock transformed with the pET-19b-E1α and pET-28a-E1β constructs. For co-transformation with both plasmids, cells were first transformed with plasmid pET-19b-E1α and selected by growth on LB agar containing carbenicillin (50 μg/ml); transformants were then made competent by treatment with calcium chloride and subjected to a second transformation, resulting in pET-19b-E1α-pET-28a-E1β co-transformants, selected for by growth on media containing both carbenicillin (50 μg/ml) and kanamycin (30 μg/ml).

Single-plasmid transformants were grown in LB media containing chloramphenicol (34 μg/ml) and either carbenicillin (50 μg/ml) or kanamycin (30 μg/ml). Following induction with 1 mM isopropyl-β-D-thiogalactose at $OD_{600\ nm} \approx 0.6$, cells were grown for a further 4 h at 37 °C with shaking. Co-transformants were grown in media containing all three antibiotics and the proteins expressed similarly.

2.4. Purification of recombinant protein

Co-expressed recombinant E1 proteins were purified by His-Bind Resin (Novagen) chromatography and dialysed into 20 mM Tris buffer (pH 9.3), containing 10% (v/v) glycerol. After 24 h, precipitated protein was isolated by centrifugation ($16\,000 \times g$ for 5 min), resuspended in the same dialysis buffer and heated for 20 min at 55 °C. After this treatment, remaining insoluble precipitate was removed by a similar centrifugation and discarded. Purity of E1 in the retained soluble fraction was examined by SDS–PAGE on a 10% (w/v) polyacrylamide gel.

2.5. Gel filtration

The size of the recombinant E1αβ protein was estimated by analytical gel filtration on an Amersham Biosciences Äkta FPLC system, using a Superdex 200 10/300GL column equilibrated with 20 mM sodium phosphate (pH 7.0), 2 mM $MgCl_2$ and 100 mM NaCl. Protein standards were: β-amylase ($M_r = 200\,000$), alcohol dehydrogenase (150 000), bovine serum albumin (66 000), carbonic anhydrase (29 000) and cytochrome *C* (12 400).

2.6. Densitometry

The E1α and E1β protein bands on SDS–PAGE gel were analysed densitometrically. The Coomassie blue stained gel was scanned using a Fujifilm FLA-5000 phosphorimager at 473 nm, with an LPG filter (for general-purpose excitation fluorescent digitising), and the scanned image was then analysed using the AIDA 2D Densitometry programme (Raytest, Straubenhardt, Germany).

2.7. Enzyme assay

E1 enzymic activity was assayed spectrophotometrically by following the 2-oxoacid dependent reduction of 2,6-dichlorophenolindophenol (DCPIP) at 595 nm [18]. Assays were carried out at 55 °C in 20 mM potassium phosphate (pH 7.0), 2 mM $MgCl_2$ and 0.2 mM TPP. Buffer and recombinant E1α enzyme were pre-incubated at 55 °C for 10 min; 50 μM DCPIP was then added and the assay started by the addition of the 2-oxoacid substrate [either pyruvate, 2-oxoglutarate, 4-methyl-2-oxopentanoate, 3-methyl-2-oxopentanoate or 3-methyl-2-oxobutanoate (Sigma–Aldrich)]. Kinetic parameters were determined by the direct linear method of Eisenthal and Cornish-Bowden [19].

3. Results

3.1. E1 expression and purification

All expression experiments used the *E. coli* BL21(DE3)-pLysS cell line and were performed at 37 °C. Two different methods were used in the attempt to obtain assembled E1 protein. The first was the individual expression of E1α and E1β, to be followed by in vitro mixing of the purified subunits, whereas the second method involved co-expression, which offered the potential of in vivo assembly of the subunits in the same cellular compartment. In the latter, cells were co-transformed with the recombinant pET-19b-E1α and pET-28a-E1β plasmids, resulting in *E. coli* pET-19b-E1α/pET-28a-E1β co-transformants.

High levels of soluble E1α were obtained by individual expression of this subunit, although E1β expression was not detectable under the same conditions (results not shown). As co-expression experiments were successful in yielding soluble E1α and E1β subunits, further manipulations to the individual expression of E1β were not attempted.

Following dialysis of the His-purified E1αβ co-expressed protein into 20 mM Tris buffer (pH 9.3) containing 10% (v/v) glycerol to remove the imidazole, the majority of protein precipitated. Resuspension of precipitated protein at 55 °C in the dialysis buffer resulted in a highly purified E1 sample with an approximately equal ratio of α to β polypeptides. The M_r values of the α and β polypeptides were determined by SDS–PAGE to be 43 000 and 37 000, respectively (Fig. 3), which compares favourably with predicted values of 41 000 and 37 000 from the published gene sequences. Analytical gel filtration revealed a single peak of around $M_r = 165\,000$ that was enzymically active in the E1 assay. SDS–PAGE of the peak fraction after gel filtration and subsequent densitometric analysis showed a 1:1 stoichiometry of the α and β polypeptides. Taken together, these data suggest an $\alpha_2\beta_2$ structure for the active E1 enzyme.

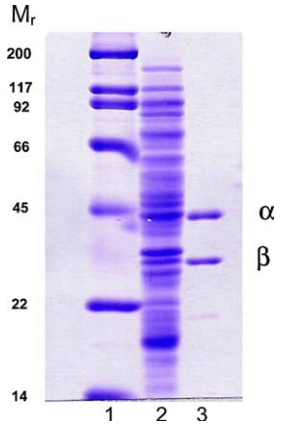

Fig. 3. SDS–PAGE of purified *T. acidophilum* recombinant E1. Lane 1, standard protein markers (M_r values given in kDa); lane 2, soluble cell extract; lane 3, enzyme after His-Bind Resin chromatography (E1α and E1β polypeptides are indicated).

Table 1
Kinetic parameters determined for purified recombinant E1 from *T. acidophilum* at 55 °C with branched-chain 2-oxoacids, pyruvate and 2-oxoglutarate as potential substrates

2-Oxoacid substrate	V_{max} (U mg^{-1} protein)	K_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
3-Methyl-2-oxopentanoate	0.38	0.50	0.1	5
4-Methyl-2-oxopentanoate	0.13	0.17	0.042	4
3-Methyl-2-oxobutanoate	0.23	0.30	0.027	11
Pyruvate	0.07	0.09	0.6	0.15
2-Oxoglutarate	0	0	0	0

1 Unit of enzyme activity (U) is defined as 1 μmol DCPIP reduced per min.
Values of k_{cat} are calculated per αβ dimer of the $\alpha_2\beta_2$ active enzyme.

3.2. E1 activity

The recombinant E1 enzyme was incubated with TPP for 10 min prior to assay, a lag in the production of product being observed if the assay was initiated without prior incubation. However, increasing the pre-incubation period to 4 h resulted in no increased E1 activity over that obtained with the 10 min incubation. E1 enzymic activity was detected with the 2-oxoacids 3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoate and pyruvate, but not with 2-oxoglutarate. The calculated kinetic parameters with these substrates are given in Table 1.

4. Discussion

The discovery in the aerobic archaea of an operon that would appear to encode the components of a OADHC was unexpected because the catalytic activity of such multienzyme systems has never been detected in any archaeon [10]. Also, the presence throughout the Archaea of catalytically active 2-oxoacid FORs that catalyse the same chemical conversions as the OADHCs would argue against a need for the latter multienzyme systems in these organisms.

However, the detection of enzyme activity for the E3 component, dihydrolipoamide dehydrogenase, in cell extracts of the extreme halophiles [11] and in *T. acidophilum* [14], suggests that at least this gene within the operon is functional. Furthermore, sequence analyses and secondary-structural predictions of the putative E1 and E2 enzymes (see [10,13] for programmes used) support the possibility that these are also functional proteins. Hawkins et al. [20] describe a common sequence motif found on the E1α of TPP-binding enzymes, and propose that this element is associated with TPP binding. The sequence is generally around 30 amino acids in length, with highly conserved GDG and NN residues at the N- and C-termini, respectively. Internally, there is an E or D residue that is usually conserved, and A and P residues that are generally conserved. A cluster of hydrophobic residues immediately precedes the NN [20]. As shown in Fig. 4, the motif has been located in the E1α of *T. acidophilum*. In all the bacterial and eukaryotic OADHCs, the E2 polypeptide chain has three different domains: one to three copies of an N-terminal lipoyl domain, an E1–E3 subunit-binding domain, and a catalytic domain. Structural predictions of the proposed protein product of the E2 gene in *Hfx. volcanii* and *T. acidophilum* show that this characteristic domain structure and catalytically important residues, including the lysine to which the lipoic acid is covalently bound, are conserved [10].

Given the above experimental results, and since genome analyses argue strongly against the presence of non-functional ORFs in prokaryotes [21], the work reported in this paper set out to discover the possible catalytic function of the putative OADHCs in Archaea. As the substrate specificity of OADHCs is determined by the E1 component, we chose to express the two genes that, by sequence comparisons, were predicted to code for the α and β polypeptides of the E1 decarboxylase component of the complex from the thermophilic archaeon, *T. acidophilum*. Co-expression of the putative E1α and E1β genes in *E. coli* was necessary to obtain the β protein in soluble form, suggesting that the α and β components associated with each other. This association was supported by gel filtration and SDS–PAGE, an $\alpha_2\beta_2$ enzyme being produced.

The recombinant E1 enzyme was found to possess catalytic activity with the branched-chain 2-oxoacids derived from the transamination of the amino acids, valine, leucine and isoleucine. It also exhibited activity with pyruvate as substrate, but the catalytic efficiency, defined as k_{cat}/K_m, was 25–70 times lower than with the branched-chain 2-oxoacids. No activity could be found with 2-oxoglutarate. Thus, the enzyme may be the first component of a BCOADHC, involved in the catabolism of valine, leucine and isoleucine, the acyl-CoA products then being fed into the citric acid cycle. The E1$\alpha_2\beta_2$ structure and the catalytic properties are similar to those observed in the bacterium *Bacillus subtilis*, where it is proposed that a single multienzyme complex performs an economic 'dual role' of catalysing the oxidative decarboxylation of both branched-chain 2-oxoacids and pyruvate [22]. However, in that case, the activity detected with pyruvate was approximately 50 times greater than with the branched-chain 2-oxoacids.

Finally, it should be noted that the catalytic activity of the recombinant *T. acidophilum* E1 [$k_{cat} = 0.17$–0.50 s^{-1}; Table 1] is similar to that reported for the recombinant E1$\alpha_2\beta_2$

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T. acidophilum  (147)        fgdggtstpdfhaamnfaavfdlpvvflcenng
                              ******  **    ***  *  *  *   **
B. stearothermophilus (172) tgdggtsqgdfyeginfagafkapaifvvqnnr
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Fig. 4. Alignment of a partial amino acid sequence of *T. acidophilum* E1α with the putative TPP-binding motif of the E1α protein of the PDHC of *Bacillus stearothermophilus*. Highly conserved regions associated with a putative TPP-binding motif in TPP-binding enzymes, as described in [20], are indicated in bold type. Hydrophobic amino acid clusters are underlined. Identities are indicated by an asterisk. Bracketed numbers give the first amino acid position in the E1α polypeptide.

[$k_{cat} = 0.47\ s^{-1}$] from the *Bacillus stearothermophilus* PDHC, using the same DCPIP assay but with pyruvate as substrate [18].

In conclusion, our data suggest that *T. acidophilum* possesses a functional E1 that accepts branched-chain 2-oxoacids as substrate. This is the first such activity to be reported in the Archaea and, with E3 activity already detected in cell extracts of this organism, is highly suggestive of a functional BCOADHC in this evolutionary domain of organisms. Thus, it is reasonable to propose that a OADHC was present in the common ancestor to the Bacteria and the Archaea, and that it has been retained in aerobic members of each domain. The 2-oxoacid FORs are also ancient enzymes but, whereas they are found in the anaerobic bacteria, they are functional in both aerobic and anaerobic archaea [7,8]. Furthermore, in the bacterial domain, the ancestral OADHC appears to have diversified to give several complexes of different substrate specificities (PDHC, OGDHC and the BCOADHC). We can find only a single complete OADHC operon in each aerobic archaeon, although a second but partial operon has been discovered in *Hfx. volcanii* [23]. This second operon encodes E1α and E1β proteins, and an unattached lipoyl domain; whilst no enzyme activity could be assigned to these products, mutation-complementation experiments suggest that they are functional during nitrate-respirative growth on Casamino acids.

It remains to be established whether the complexes from the various archaea all have the same substrate specificity, if that particular activity is always duplicated by the corresponding FOR in each organism, and how the respective activities are coordinately regulated. Structurally, the biophysical nature of the archaeal multienzyme complex is a key question, and recombinant E2 and E3 components of *T. acidophilum* must be now generated, and the ambitious goal of complex assembly achieved. The identification of the catalytic activity of the E1 component in this current paper is an important step to enabling these questions to be investigated.

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