Reaction Mechanism and Structural Model of ADP-forming Acetyl-CoA Synthetase from the Hyperthermophilic Archaeon Pyrococcus furiosus

EVIDENCE FOR A SECOND ACTIVE SITE HISTIDINE RESIDUE

Received for publication, December 14, 2007, and in revised form, March 26, 2008. Published, JBC Papers in Press, March 27, 2008, DOI 10.1074/jbc.M710218200

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In Archaea, acetate formation and ATP synthesis from acetyl-CoA is catalyzed by an unusual ADP-forming acetyl-CoA synthetase (ACD) (acetyl-CoA + ADP + P_i ⇌ acetate + ATP + HS-CoA) catalyzing the formation of acetate from acetyl-CoA and concomitant ATP synthesis by the mechanism of substrate level phosphorylation. ACD belongs to the protein superfamily of nucleoside diphosphate-forming acyl-CoA synthetases, which also include succinyl-CoA synthetases (SCS). ACD differs from SCS in domain organization of subunits and in the presence of a second highly conserved histidine residue in the β-subunit, which is absent in SCS. The influence of these differences on structure and reaction mechanism of ACD was studied with heterotetrameric ACD (αCβD) from the hyperthermophilic archaeon Pyrococcus furiosus in comparison with heterotetrameric SCS. A structural model of P. furiosus ACD was constructed suggesting a novel spatial arrangement of the subunits different from SCS, however, maintaining a similar catalytic site. Furthermore, kinetic and molecular properties and enzyme phosphorylation as well as the ability to couple of acetate formation to ATP synthesis of ACD were studied in wild type ACD and several mutant enzymes. The data indicate that the formation of enzyme-bound acetyl phosphate and enzyme phosphorylation at His-257α, respectively, proceed in analogy to SCS. In contrast to SCS, in ACD the phosphorolysis group is transferred from the His-257α to ADP via transient phosphorylation of a second conserved histidine residue in the β-subunit, His-71β. It is proposed that ACD reaction follows a novel four-step mechanism including transient phosphorylation of two active site histidine residues:

\[
\text{E} + \text{acetyl-CoA} + \text{P}_i \rightleftharpoons \text{E} \cdot \text{acetyl}^\beta \cdot \text{P} + \text{CoA} \quad \text{(Eq. 1)}
\]

\[
\text{E} \cdot \text{acetyl}^\beta \cdot \text{P} \rightleftharpoons \text{acetyl} + \text{E} \cdot \text{His}^{357} \alpha \cdot \text{P} \quad \text{(Eq. 2)}
\]

\[
\text{E} \cdot \text{His}^{357} \alpha \cdot \text{P} \rightleftharpoons \text{E} \cdot \text{His}^{357} \beta \cdot \text{P} \quad \text{(Eq. 3)}
\]

\[
\text{E} \cdot \text{His}^{357} \beta \cdot \text{P} + \text{ADP} \rightleftharpoons \text{ATP} + \text{E} \quad \text{(Eq. 4)}
\]

ADP-forming acetyl-CoA synthetase (ACD)\(^2\) (acetyl-CoA + ADP + P_i ⇌ acetate + ATP + CoA) is a novel enzyme in Archaea that catalyzes the conversion of acetyl-CoA and other acyl-CoA esters to the corresponding acids and couples this reaction with the synthesis of ATP. This unusual synthetase has first been detected in the eukaryotic protists Entamoeba histolytica and Giardia lamblia (1, 2). In our group, ACD activities have been identified in all acetate-forming Archaea tested, including anaerobic hyperthermophiles and aerobic halophiles (3, 4). The conversion of acetyl-CoA to acetate by one enzyme is unusual in prokaryotes and appears to be restricted to Archaea, since all acetate-forming bacteria, including the hyperthermophile Thermotoga maritima, utilize two enzymes, phosphate acetyltransferase and acetate kinase, for acetate formation and ATP synthesis (4, 5).

In anaerobic hyperthermophilic Archaea, e.g. Pyrococcus furiosus, ACD represents the major energy-conserving reaction during peptide, pyruvate, and sugar fermentation to acetate (4, 6, 7). Two ACD isoenzymes, ACD I and ACD II, have been characterized from P. furiosus, which differ in their substrate specificity for CoA esters. ACD I preferentially utilizes acetyl-CoA and is involved in sugar degradation, whereas ACD II is involved in peptide fermentation (8).

Subsequently, ACDs have been purified from various hyperthermophilic Archaea, from the halophilic Archaea Haloarcula marismortui as well as from the eukaryote G. lamblia (2, 9–12), and the encoding genes have been identified. ACDs are either heterotetrameric or homodimeric proteins composed either of two separate subunits, α and β, or of only one subunit representing a fusion of the respective α and β subunits. Using these sequences, numerous putative ACD homologs could be identified in several archaeal and bacterial genomes. A more refined bioinformatic study revealed that ACDs belong to the newly recognized superfamily of nucleoside diphosphate-forming (NDP-forming) acyl-CoA synthetases, which also include the well known succinyl-CoA synthetases (SCSs) of the citric acid cycle, ubiquitous enzymes in all three domains of life (13).

\(^{a}\) This work was supported by grants of the Deutsche Forschungsgemeinschaft (SCHO 316/10-1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^{b}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3 and Table 1.

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2 The abbreviations used are: ACD, ADP-forming acetyl-CoA synthetase; SCS, succinyl-CoA synthetase; NDP, nucleoside diphosphate; MES, 4-morpholineethanesulfonic acid.
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(a) SCS α

1. CoA-binding
2. Ligase CoA

(b) ACD α

1. CoA-binding
2. Ligase CoA
5. Ligase CoA

(c) SCS β

3. ATP-grasp
4. ATP-grasp

ACD β

3. ATP-grasp
4. ATP-grasp

FIGURE 1. Comparative illustration of subunit composition and domain organization in E. coli SCS (a) and ACD from P. furiosus (b). The domains are specified and numbered as described in the introduction. The conservation of the histidine residues in α- and β-subunit are highlighted with the bar, c, detailed sequence alignment of the sequence region of the conserved histidine residues in α- and β-subunit of ACD and SCS. The histidine in the α-subunit conserved in SCS and ACD is highlighted by a red background, the conserved second histidine in the β-subunit of ACD is shown on a green background.

Both, archaenal ACDs and SCSs catalyze the conversion of acyl-CoA esters (acetyl-CoA or succinyl-CoA, respectively) to the corresponding acids and couple these reactions with the synthesis of ATP (GTP) by substrate level phosphorylation,

\[ R-Co-S-CoA + NDP + P \rightleftharpoons R-CoO^- + HS-CoA + NTP \]  

(Eq. 5)

Furthermore, few other enzymes of more specialized metabolic functions belong to this protein family. These include, e.g., ATP citrate lyase (citrate + CoA + ATP \rightleftharpoons acetyl-CoA + ADP + P, + oxaloacetate), involved in autotrophic CO2 fixation via the reductive citric acid cycle, and in eukaryotes in acetyl-CoA supply for fatty acid synthesis as well as malyl-CoA synthetase, pimelyl-CoA synthetase, and feruloyl-CoA synthetases, which are involved in formaldehyde fixation, biotin biosynthesis, and furalic acid degradation, respectively (14–17). In contrast to ACD and SCS, these enzymes function in acid activation to the corresponding CoA esters.

So far the reaction mechanism and the structure of ACD are not known, whereas detailed structural and mechanistic studies on SCS have previously been reported, e.g., of the Escherichia coli enzyme (18–20). The overall reaction of SCS was assumed to proceed in three partial reactions including a transient phosphorylation of the enzyme at an essential histidine residue (see Fig. 1) located in the α-subunit (His-246α),

\[ E + \text{succinyl-CoA} + P_\text{i} \rightleftharpoons E \cdot \text{succinyl} - P + \text{CoA} \]  

(Eq. 6)

\[ E \cdot \text{succinyl} - P \rightleftharpoons \text{succinate} + E \cdot \text{His}^{246}_\alpha - P \]  

(Eq. 7)

\[ E \cdot \text{His}^{246}_\alpha - P + \text{ADP} \rightleftharpoons \text{ATP} + E \]  

(Eq. 8)

Sequence comparisons of ACD with SCS indicated that many amino acid residues that are known to be important for catalysis in SCS are also conserved in ACDs, e.g., the histidine residue, His-246α, shown to be transiently phosphorylated in the course of the SCS-catalyzed reaction, is also conserved in all ACDs. Moreover, the glutamate residue, Glu-208α, proposed to stabilize the His-246 during transient phosphorylation of the enzyme by succinyl-CoA and P, (Equations 7 and 8), is also well conserved in ACDs. However, the second glutamate residue, Glu-197β, interacting with His-246 during the third partial reaction, the phosphoryl transfer between the phosphohistidine residue and the nucleotide, is substituted by aspartate in ACDs. This probably represents a functional conserved substitution since the mutation of Glu-197β to aspartate in the E. coli SCS had almost no effect on activity, indicating that the charge of the residue and not the length of the side chain is critical for SCS activity (19). The observation that these essential residues of the SCS are also well conserved in ACDs suggests a similar reaction mechanism for both enzymes (13).

However, ACD shows remarkable differences to SCS with respect to domain organization of subunits (13). Furthermore, we identified a second highly conserved histidine residue in the β-subunit, His-71β, which is absent in SCS (Fig. 1). As deduced from the crystal structure of SCSs and sequence comparisons as well as conserved domain searches (21), SCS and ACD are composed of five domains (Fig. 1). Domain 1 constitutes the CoA-binding site and is in all members of the family C-terminal connected to domain 2, i.e., a CoA-ligase domain. These two domains constitute the α-subunit of SCS (SucD) (red in Figs. 1 and 2). The domains 3 and 4 comprise the ATP-binding site, the so-called ATP-grasp fold (green in Fig. 1 and 2). In SCS this ATP-grasp domain together with the C-terminal extended domain 5, a second CoA-ligase domain (cyan in Fig. 1 and 2), form the β-subunit (SucC). Conversely, in ACDs this second CoA-ligase domain is attached to the C terminus of the α-sub-
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Construction, Expression, and Purification of ACD Mutants—The acdla and acdlb genes encoding α- and β-subunit of ACD from P. furiosus have been cloned into the pET17b and pET14b plasmids (10). Mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene). The primers used to introduce the mutations are listed in supplemental Table 1. The sequences of the mutated acdla and acdlb genes were confirmed by sequencing according to the Sanger method (27). For expression of acdla and acdlb and their variants, E. coli BL21 codon plus(DE3)-RII cells transformed with the respective plasmid were grown in Luria-Bertani medium at 37 °C. Expression was initiated by induction with 0.4 mM isopropyl 1-thio-β-d-galactopyranoside. After 4 h of further growth, cells were harvested by centrifugation. Cell pellets were suspended in Tris-HCl, pH 8.4, and disrupted by passing through a French pressure cell 4 times at 1.3 × 10⁸ Pa followed by centrifugation. Supernatant was heat-precipitated at 90 °C for 30 min, and precipitated proteins were removed by centrifugation. For reconstitution of holoenzyme, equimolar amounts of the >90% homogenous α and β subunits were mixed and incubated on ice (1 h) followed by hydrophobic interaction chromatography on a phenyl-Sepharose column equilibrated with 50 mM Tris-HCl, pH 7.5, containing 1.0 M ammonium sulfate. Protein was eluted with a linear gradient from 1.0 to 0 M ammonium sulfate. After concentration via ultrafiltration, fractions with the highest ACD content were applied to a Superdex 200 16/60 column equilibrated with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl. Protein was eluted at a flow rate of 1 ml/min, and the fractions with the highest ACD content were pooled. To remove residual DNA for CD spectroscopy, volumes of the respective preparations containing about 15 mg of protein were supplemented with 1000 units of DNase I (Roche Applied Science) and incubated at 37 °C for 2 h followed by heat precipitation at 75 °C for 30 min and centrifugation. Wild type and mutant ACD proteins were further purified by two additional chromatographic steps on phenyl-Sepharose and Superdex 200 as described above. Finally, the purified proteins were dialyzed against 50 mM Tris-HCl, pH 7.5.

Determination of Kinetic Parameters—Vₘₕ values and Kₘ values of wild type and mutant ACDs were measured, if possible, in both directions at 55 °C to be able to compare the results directly with the phosphorylation experiments which had to be carried out at this temperature (see below). The following assay systems were used. (i) In the direction of acetate formation the ADP and Pₐ-dependent HS-CoA release from acetyl-CoA was assayed according to Srere et al. (28) with Ellman’s thiol reagent (5’5’-dithiobis(2-nitrobenzoic acid)) by measuring the formation of thiophenolane anion at 412 nm (ε = 13.6 mM⁻¹ cm⁻¹). The assay mixture contained 100 mM MES, pH 6.5, 0.1 mM 5’5'-dithiobis(2-nitrobenzoic acid, 0.1 mM acetyl-CoA, 1 mM ADP, 5 mM KH₂PO₄, 5 mM MgCl₂, and 0.4–4 μM of the respective ACD protein. This assay system was also used to measure the kinetic parameters of the wild type ACD at 80 °C. (ii) In the direction of acetyl-CoA formation the kinetic constants were measured at 365 nm as HS-CoA and acetate-dependent ADP formation from ATP by coupling the reaction with the oxidation of NADH (ε = 3.4 mM⁻¹ cm⁻¹) via pyruvate kinase and lactate dehydrogenase. The assay mixture contained 100 mM

EXPERIMENTAL PROCEDURES

Modeling of P. furiosus ACD—The heterotetrameric ACD from P. furiosus consists of two different subunits α and β (α₂β₂) encoded by open reading frame PF1540 α, PF1787 β from P. furiosus in comparison to heterotetrameric SCS from E. coli (19, 22). Mutant enzymes were constructed in which the amino acid residues homologous to catalytically essential His-246, Glu-208α, and Glu-197β in SCS as well as the conserved histidine in the β-subunit, His-71β, were mutated by site-directed mutagenesis. Wild type and mutant enzymes were characterized by kinetic measurements, phosphorylation experiments using either [γ-³²P]ATP or ³²P, together with acetyl-CoA, respectively, as well as by arsenolysis experiments. The results indicate that the ACD reaction follows a novel four-step mechanism in which two active site histidine residues in the α- and β-subunit, respectively, are essential for catalysis via transient phosphorylation. Furthermore, a structural model of ACD from P. furiosus is presented.

Construction, Expression, and Purification of ACD Mutants—The acdla and acdlb genes encoding α- and β-subunit of ACD from P. furiosus have been cloned into the pET17b and pET14b plasmids (10). Mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene). The primers used to introduce the mutations are listed in supplemental Table 1. The sequences of the mutated acdla and acdlb genes were confirmed by sequencing according to the Sanger method (27). For expression of acdla and acdlb and their variants, E. coli BL21 codon plus(DE3)-RII cells transformed with the respective plasmid were grown in Luria-Bertani medium at 37 °C. Expression was initiated by induction with 0.4 mM isopropyl 1-thio-β-d-galactopyranoside. After 4 h of further growth, cells were harvested by centrifugation. Cell pellets were suspended in Tris-HCl, pH 8.4, and disrupted by passing through a French pressure cell 4 times at 1.3 × 10⁸ Pa followed by centrifugation. Supernatant was heat-precipitated at 90 °C for 30 min, and precipitated proteins were removed by centrifugation. For reconstitution of holoenzyme, equimolar amounts of the >90% homogenous α and β subunits were mixed and incubated on ice (1 h) followed by hydrophobic interaction chromatography on a phenyl-Sepharose column equilibrated with 50 mM Tris-HCl, pH 7.5, containing 1.0 M ammonium sulfate. Protein was eluted with a linear gradient from 1.0 to 0 M ammonium sulfate. After concentration via ultrafiltration, fractions with the highest ACD content were applied to a Superdex 200 16/60 column equilibrated with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl. Protein was eluted at a flow rate of 1 ml/min, and the fractions with the highest ACD content were pooled. To remove residual DNA for CD spectroscopy, volumes of the respective preparations containing about 15 mg of protein were supplemented with 1000 units of DNase I (Roche Applied Science) and incubated at 37 °C for 2 h followed by heat precipitation at 75 °C for 30 min and centrifugation. Wild type and mutant ACD proteins were further purified by two additional chromatographic steps on phenyl-Sepharose and Superdex 200 as described above. Finally, the purified proteins were dialyzed against 50 mM Tris-HCl, pH 7.5.

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To generate the heterotetrameric ACD complex, consisting of two α and two β subunits, the structure of heterotetrameric succinyl-CoA synthetase from E. coli was used as template. The C-terminal domain of the β-subunit from the SCS was superimposed onto the C-terminal domains of each of the two α subunits in the dimer of ACD. Thereby the spatial arrangement of the α and β subunits in heterotetrameric ACD could be defined. The structural representations were generated with the RIBBON (26) program.
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\[
\begin{align*}
E + \text{Ac-CoA} + \text{As}_i &\rightarrow E(\text{Ac-As}) + \text{CoA} + H_2O \\
\text{REACTION 1}
\end{align*}
\]

MES, pH 6.5, 5 mM MgCl₂, 10 mM sodium acetate, 2 mM ATP, 1 mM HS-CoA, 2.5 mM phosphoenolpyruvate, 0.3 mM NADH, 5 units of lactate dehydrogenase, 4 units of pyruvate kinase, and 0.4–4 μg of the respective ACD protein.

Phosphorylation Experiments—The ability of wild type and mutant ACD enzymes to be phosphorylated was tested in either direction using \[^{32}P\]Pi, together with acetyl-CoA and \[^{32}P\]ATP, respectively. The assay mixture in a total volume of 100 μl contained 100 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.005 mM acetyl-CoA, 0.01 μCi of \[^{32}P\]Pi, and 12 μg of protein. In the opposite direction buffer was used except that acetyl-CoA and \[^{32}P\]Pi were replaced by 2.5 μCi of \[^{32}P\]ATP and 2 μl unlabeled ATP. Because the radioactive label of the proteins was not stable at elevated temperatures, incubation was carried out at 55 °C. At the times indicated 10 μl were taken from the mixture, and reaction was stopped by mixing with 2.5 μl of SDS-PAGE loading buffer (63 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.002% bromphenol blue, and 250 mM dithiothreitol). Immediately upon completion of the respective experiments, samples were applied on 12% SDS polyacrylamide gels without heat or trichloroacetic acid treatment. After electrophoresis gels were dried on a piece of Whatman paper and exposed to a phosphorimaging screen overnight. Results were visualized using a Fuji-film FLA-5000 (Fuji Medical System, Stamford, CT).

Arsenolysis Reaction—Arsenolysis experiments were performed to test whether wild type and mutant ACD enzymes are able to catalyze the presumed first part of the overall reaction, i.e. the phosphorolysis of acetyl-CoA by Pᵢ to enzyme-bound acetyl phosphate. This phosphorolysis reaction can be effectively isolated from subsequent phosphoryl transfer to ADP by substitution of Pᵢ for the substrate analog arsenate taking advantage of the known instability of mixed anhydrides of arsenate (Reaction 1). Thus, the first partial reaction can be assayed as arsenate-dependent but ADP-independent release of free CoA from acetyl-CoA according to the method of Srere et al. (28) with 5’-dithiobis(2-nitrobenzoic acid) (see above). The arsenolysis experiments were performed at 80 °C because measurements further below the temperature optimum of the enzyme, e.g. at 55 °C, yielded ambiguous results. The assay mixture contained 100 mM MES, pH 6.5, 0.1 mM 5’-dithiobis(2-nitrobenzoic acid, 0.1 mM acetyl-CoA, 10 mM K₂AsO₄, 5 mM MgCl₂, and 0.4 μg of the respective ACD protein. To compare the arsenolysis experiments with the overall reaction, the kinetic parameters of the wild type P. furiosus ACD were also determined at 80 °C as described above.

Circular Dichroism Spectroscopy—CD measurements were carried out on a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) calibrated according to Chen et al. (29). The spectral bandwidth was 2 nm. Three scans were recorded between 190 and 250 nm with 1-nm acquisition steps, and the spectrum was base-line-corrected. Measurements were carried out at room temperature, and cuvettes of 0.01-mm optical path length were used. The protein concentration was 15 μM in 50 mM Tris-HCl, pH 7.5.

RESULTS

Structural Model of P. furiosus ACD—To investigate the structural consequences of the domain shuffling in ACD compared with SCS, homology models of the α- and β-subunit of P. furiosus ACD were constructed using the crystal structures of the dimeric α-subunit homolog PH0766 and the monomeric β-subunit homolog PH1788 from P. horikoshii available in the Protein Data Bank data base. The α and β subunits of P. horikoshii and P. furiosus share 49 and 54% identity, respectively. The spatial arrangement of the heterotetramer was constructed by superimposing the C-terminal domain of the β-subunit from the SCS, the second Coa-ligase domain, onto the C-terminal domains of each of the two α subunits in the dimer of ACD. In the P. furiosus ACD model presented in Fig. 2 and supplemental Fig. 2, the core of the heterotetrameric enzyme is made up by the α dimer. Each of the two ATP-grasp domains, i.e. the β subunits, is attached laterally to the α dimer, forming an interaction with the N-terminal domain of each of the two α subunits. Thus, the different domain organization in heterotetrameric ACD results in a different spatial arrangement of α and β subunits compared with heterotetrameric E. coli SCS. However, the different domains in ACD are similarly oriented to each other, forming a similar active site as in SCS (Fig. 2, Fig. 3). Thus, domain shuffling in ACD does not result in a significant change of active site architecture.

The loop in the α-subunit containing the catalytically essential histidine residue, which was not resolved in the structure of PH0766 (2CSU) from P. horikoshii, was built in the P. furiosus ACD model using the loop from the E. coli SCS as template. Accordingly, the modeled phosphohistidine loop in P. furiosus ACD is shortened by three amino acid residues compared with SCS.

Expression, Purification, CD Spectra of Wild Type and Mutant P. furiosus ACD—In analogy to experiments carried out with the E. coli SCS, mutant ACDs were constructed in which H²⁵⁷α was mutated to aspartate, changing the positive charge to a negative one. Furthermore, Glu-218 was substituted for aspartate to shorten the side chain or for glutamine to eliminate the charge but maintaining the length of the side chain. Asp-212β was mutated to glutamate to lengthen the side chain or to asparagine to eliminate the charge. Finally, His-71β, which is highly conserved in ACDs but not in SCSs, was mutated to alanine. All mutations were confirmed by sequencing. Wild type and mutant α and β subunits were expressed separately in E. coli BL21(DE3) codon plus RIL and reconstituted to the respective holoenzymes after heat precipitation. The wild type and mutant holoACD proteins were further purified to homogeneity by hydrophilic interaction chromatography on phenyl-Sepharose and by gel filtration. The native wild type and mutant ACD all constitute 150-kDa αβ₂ heterotetrameric proteins composed of 47-kDa α and 27-kDa β subunits. Additionally, CD spectra of H257Δα and H71Δβ were recorded and compared with that obtained for the wild type P. furiosus ACD. The CD spectrum of the H257Δα mutant showed no significant differences compared with the wild type (supplemental Fig. 3). Also, the spectrum of the H71Δβ exhibited nearly the same shape as the wild type spectrum even
though the magnitudes of the signals were somewhat stronger (supplemental Fig. 3). However, the shape of the spectrum and also the activity of the protein in arsenolysis experiments (see below) indicate that the protein is still conformationally intact. From the CD spectra a content of 35% \( \alpha \)-helices and 20% \( \beta \)-sheets was calculated. From the model 35% \( \alpha \)-helices and 25% \( \beta \)-sheets were estimated.

**Kinetic Properties of Wild Type and Mutant ACDs**—Kinetic parameters were determined in both directions of the reaction for wild type and, if measurable, mutant ACDs at 55 °C. In all cases, Michaelis-Menten kinetic behavior was observed. The apparent \( V_{\text{max}} \) and \( K_m \) values are listed in Table 1. Additionally, the wild type enzyme was also measured at 80 °C in the direction of acetate formation to compare the results with those obtained for the arsenolysis reaction (see below). The \( V_{\text{max}} \) and \( K_m \) values at 80 °C were 30 units mg\(^{-1}\) and 8.4 \( \mu \text{m} \) (acetyl-CoA), 93 \( \mu \text{m} \) (ADP), and 230 \( \mu \text{m} \) (Pi), respectively. The H257Da mutant showed no activity in either direction of the reaction. Also, the H71A\( \beta \) mutant did not catalyze the overall reaction. Thus, both histidine residues are essential for ACD catalysis.

The mutation of the conserved glutamate residue in the \( \alpha \)-subunit significantly effects \( P.\) furiosus ACD. The E218D\( \alpha \) mutant maintaining the charge but shortening the side chain showed only 1–10% activity of the wild type enzyme. However, the \( K_m \) values for substrates were similar to those in the wild type except for phosphate and CoA, respectively, indicating a role of Glu-218\( \alpha \) in binding of these substrates. The mutation of Glu-218\( \alpha \) to glutamine, maintaining the length of the side chain but eliminating the charge, completely abolished the activity. Similar results were obtained for the mutations of the Asp-212\( \beta \) residue. Lengthening the side chain but maintaining the charge by the mutation to glutamate resulted in a significant reduction of activity (\(~2–4\%\) that of wild type activity). The elimination of the charge by the mutation to asparagine caused a complete loss of activity. Thus, the mutations of the Glu-218\( \alpha \) and Asp-212\( \beta \), respectively, indicate that the charge in these positions is essential for activity and also the length of the side chain is important for full activity.

**Characterization of the First Partial Reaction Using Arsenolysis Experiments**—The first partial reaction was assayed at 80 °C as arsenate-dependent but ADP-independent release of free CoA from acetyl-CoA. Michaelis-Menten kinetics were obeyed in the arsenolysis reaction catalyzed by wild type and mutant ACDs as well as by the \( \alpha \)-subunit alone with apparent \( V_{\text{max}} \) and \( K_m \) values given in Table 2.

The wild type \( P.\) furiosus ACD catalyzed arsenolysis with a specific activity of 44 units mg\(^{-1}\). The \( K_m \) value for acetyl-CoA
was in the same range as determined for the overall reaction, whereas the $K_m$ value for arsenate was 50-fold higher than that observed for phosphate. The $\alpha$-subunit alone also catalyzed arsenolysis with similar $K_m$ values but with somewhat reduced activity (11 units mg$^{-1}$).

All mutations in the $\alpha$-subunit had dramatic effects on arsenolysis activity; the H257D as well as the E218Q$\alpha$ mutations completely abolished arsenolysis. The E218D$\alpha$ mutant exhibited only less than 1% but still measurable activity compared with the wild type. However, the mutations in the $\beta$-subunit, i.e., H71A$\beta$, D212E$\beta$, and D212N$\beta$, caused only a moderate loss of activity to the level observed for the $\alpha$-subunit alone but with slightly higher $K_m$ values. The results obtained from the arsenolysis experiments indicate that (i) the first partial reaction of ACD is the phosphorolysis of acetyl-CoA to form enzyme-bound acetyl phosphate, (ii) the phosphorolysis is catalyzed by the $\alpha$-subunit alone, independent of the $\beta$-subunit, (iii) both the His-257$\alpha$ and Glu-218$\alpha$ are crucial for phosphorolysis. In case of Glu-218$\alpha$ the charge is essential for activity and also the length of the side chain is important.

**Phosphorylation Experiments of P. furiosus ACD**—Phosphorylation of P. furiosus ACD using acetyl-CoA and 32P (Fig. 4). Both $\alpha$ and $\beta$ subunits of heterotetrameric wild type ACD were phosphorylated in a time-dependent manner. Phosphorylation of the $\alpha$-subunit was almost complete after 1 min, whereas labeling of the $\beta$-subunit was delayed, being maximal after 10–20 min. Phosphorylation was also observed using ACD $\alpha$-subunit alone. His-257$\alpha$ was identified as site of transient phosphorylation since mutation of this residue inhibited labeling and overall ACD activity, indicating that the second partial reaction in ACD, the phosphor transfer from acetyl phosphate to His-257$\alpha$, proceeds in analogy to SCS.

In contrast to SCS, the $\beta$-subunit was also phosphorylated in ACD. The $\beta$-subunit could be specifically prevented from phosphorylation by mutation of His-71$\beta$ to alanine. This mutation abolished the overall reaction but maintained phosphorylation of the $\alpha$-subunit as well as arsenolysis activity. These findings demonstrate His-71$\beta$ to be a second active site histidine residue in ACD involved in a novel third partial reaction, i.e., the transfer of the phosphoryl group between His-257$\alpha$ and His-71$\beta$, which is not observed in SCS. Interestingly, the $\alpha$-subunit alone catalyzed both the first and the second partial reaction (see also arsenolysis experiments) in the absence of the $\beta$-subunit, indicating that the binding sites for acetyl-CoA and phosphate in ACD are solely located in the $\alpha$-subunit dimer.

Mutation of Glu-218$\alpha$ to aspartate reduced the degree of phosphorylation of both $\alpha$- and $\beta$-subunit, whereas in the E218Q$\alpha$ mutant phosphorylation was completely inhibited. Thus, the effect of Glu-218 mutations on enzyme phosphorylation is in accordance with data from the kinetic characterization and arsenolysis experiments. The results indicate that the charge in the position of Glu-218$\alpha$ is essential for the first and the second partial reaction, and the length of the side chain is also important. However, the mutations of Asp-212$\beta$ to glutamate and asparagine, respectively, did not significantly affect the phosphorylation pattern of ACD, supporting the finding.

**TABLE 1**

| Kinetic parameters of wild type and mutant ACD from *P. furiosus* at 55 °C |
|-----------------------------------------------|
|                                         | Apparent $V_{\text{max}}$ $\mu$mol mg$^{-1}$ | Apparent $K_m$ $\mu$mol |
|                                            | Acetate-forming reaction$^a$ | Acetyl-CoA-forming reaction$^a$ | Ac-CoA | ADP | P$_i$ | Acetate | ATP | HS-CoA |
| Wild type                                  | 11.4 ± 0.4 | 15.5 ± 0.5 | 3.9 ± 0.3 | 94 ± 14 | 272 ± 19 | 625 ± 86 | 221 ± 15 | 13.9 ± 2.1 |
| H257D$\alpha$                               | ND | ND | ND | ND | ND | ND | ND | ND |
| H71A$\beta$                                 | 0.060 ± 0.003 | 1.2 ± 0.04 | 6.2 ± 0.4 | 74.1 ± 5.5 | 919 ± 41 | 783 ± 30 | 388 ± 17 | 77.1 ± 2.8 |
| E218Q$\alpha$                               | 0.219 ± 0.02 | 8.2 ± 0.4 | 283 ± 45 | ND | ND | ND | ND | ND |
| E218Q$\beta$                                | 0.429 ± 0.06 | 12.2 ± 0.04 | 6.2 ± 0.4 | 74.1 ± 5.5 | 919 ± 41 | 783 ± 30 | 388 ± 17 | 77.1 ± 2.8 |
| D212E$\beta$                                | 0.219 ± 0.02 | 8.2 ± 0.4 | 283 ± 45 | ND | ND | ND | ND | ND |
| D212N$\beta$                                | 0.219 ± 0.02 | 8.2 ± 0.4 | 283 ± 45 | ND | ND | ND | ND | ND |

$^a$ Activity was measured as acetate and CoA-dependent release of ADP from ATP via pyruvate kinase and lactate dehydrogenase.

$^b$ Activity was monitored as ADP and P$_i$-dependent HS-CoA release from acetyl-CoA using DTNB.
that phosphorolysis is catalyzed by the α-subunit alone, i.e. in the absence of the β-subunit.

**Phosphorylation of P. furiosus ACD Using [γ-32P]ATP (Fig. 4)—**
A time-dependent phosphorylation of both subunits of the wild type ACD was also observed in the reverse direction using [γ-32P]ATP with a delayed labeling of the β-subunit. The α-subunit of ACD wild type alone as well as the α-subunit in the reconstituted (αβββ) H71Aβ mutant were also phosphorylated by [γ-32P]ATP. The H257Dα mutant was not phosphorylated in both α- and β-subunit. Thus, this residue is crucial for both ATP cleavage and phosphorylation of the β-subunit as well as for the transfer of the phosphoryl group between the α- and β-subunit. This is also supported by the fact that the β-subunit alone, which comprises the nucleotide binding site, is not able to catalyze its own phosphorylation by ATP (data not shown).

The H71Aβ mutant is impaired in the phosphorylation of the β-subunit by [γ-32P]ATP. This result together with the inability to catalyze the overall reaction indicates the presence of a second active site histidine residue in ACD located in the β-subunit. The mutations of Glu-218α and Asp-212β, respectively, all resulted in a considerably lower degree of phosphorylation by ATP, especially of the α-subunit compared with the wild type. The labeling of the β-subunit is almost unaffected by these mutations. This indicates that both residues are important for the phosphoryl transfer between the subunits.

**DISCUSSION**

In this study we analyzed the reaction mechanism of ACD from *P. furiosus* by kinetic measurements, by phosphorylation studies, and arsenolysis experiments of wild type and mutant enzymes in comparison with SCS. The data indicate that the ACD reaction (acetyl-CoA + ADP + P ↔ acetate + CoA + ATP), in contrast to SCS, proceeds via a novel four-step mechanism involving the transient phosphorylation of two active site histidines. Furthermore, a structural model of ACD is presented.

**Structural Model**—Although in a previous study we reported successful crystallization of the heterotetrameric ACD from *P. furiosus* (30), the structure could not be solved due to twinning phenomenon. Here, structural models of the *P. furiosus* ACD α and β subunits were generated based on the x-ray structures of the homologous proteins from *P. horikoshii* (2CSU and 1WR2) available at the Protein Data Bank data base, which share about 50% sequence identity. The α-subunit homolog of *P. horikoshii* is dimeric, which is in good agreement with the molecular mass of 90 kDa reported for the *P. furiosus* α-subunit determined by gel filtration (10). However, the crystal structure of the *P. horikoshii* β-subunit homolog revealed a monomeric state, whereas the molecular weight determined for the β-subunit from *P. furiosus* indicated a dimeric state (10), suggesting the tendency of the *P. furiosus* β-subunit to aggregate under the respective conditions.

ACD and SCS are composed of five homologous domains each showing very similar folds as can be seen from the *P. horikoshii* structures as well as from the α- and β-subunit models from *P. furiosus* presented here. Furthermore, many of the amino acid residues shown to be involved in substrate binding and catalysis in SCS are highly con-

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**TABLE 2**

Kinetic parameters of wild type and mutant ACDI from *P. furiosus* for catalysis of arsenolysis of acetyl-CoA (measured at 80 °C)

|                  | Apparent $V_{max}$ | Apparent $K_m$ for acetyl-CoA | Apparent $K_m$ for arsenate |
|------------------|--------------------|-------------------------------|----------------------------|
|                  | Units mg⁻¹ min⁻¹  | μM                            | μM                         |
| Wild type        | 44.1 ± 1.5         | 4.2 ± 0.3                     | 12,000 ± 1,200             |
| α-Subunit        | 11.3 ± 0.3         | 3.7 ± 0.8                     | 15,190 ± 918               |
| H257Dα           | NM                 | ND                            | ND                         |
| H71Aβ            | 17.4 ± 0.6         | 9.8 ± 0.4                     | ND                         |
| E218Qα           | NM                 | ND                            | ND                         |
| E218Dα           | 0.15 ± 0.002       | ND                            | ND                         |
| D212Eβ           | 15.8 ± 0.9         | 9.5 ± 0.6                     | ND                         |
| D212Nβ           | 30.5 ± 1.2         | 7.1 ± 0.4                     | ND                         |

**FIGURE 4.** Time course for the phosphorylation of wild type (wt) ACD and the wild type α-subunit alone as well as of the His257D and His71βA mutant proteins as well as the Glu218α and the Asp212β mutants. The proteins were incubated in the presence of succinyl-CoA and 32P, (left panel) and of [γ-32P]ATP (right panel). At the times indicated samples were taken and applied to SDS-PAGE. Gels were dried on Whatman paper and analyzed by phosphorimaging (see “Experimental Procedures”).
Mechanism and Model of ADP-forming ACD from *P. furiosus*

erved in ACD, suggesting similar catalytic sites. However, the individual domains are differently arranged in the subunits of both enzymes ("domain shuffling") (13). Despite this domain shuffling the model of the αβ, heterotetrameric complex of the *P. furiosus* ACD presented here clearly indicates a similar catalytic site; in ACD, domains 1 and 2 from one α-subunit (red) and domain 5 from the second α-subunit (cyan) as well as domains 3 and 4 from the β-subunit (green) contribute to the catalytic site. A similar domain arrangement as in the *P. furiosus* ACD model was recently suggested for ACD homologs from *Thermococcus kodakaraensis* (31). However, a structural model was not given. The model of *P. furiosus* ACD also explains why functional αβ heterodimeric ACD enzymes were not reported so far, since in an αβ dimer the domains 1, 2, and 5 would not adopt the right orientation to each other to form a functional active site. In contrast to ACD, one α-subunit (domains 1 and 2) and one β-subunit (domains 3, 4, and 5) in SCS make up one catalytic site. This explains the occurrence of functional SCS enzymes as both αβ, heterotetramers (Gram-negative bacteria) and as αβ heterodimers (Gram-positive bacteria, eukaryotes) (18, 32–34).

**Reaction Mechanism**—The studies on the reaction mechanism of ACD revealed that the first two partial reactions, the phosphorolysis of acetyl-CoA and the transfer of the phosphorolysis product from acetyl phosphate to His-257α, proceed in analogy to SCS (22). In addition to this role as phosphorolysis site and phosphoryl donor, established in the enzymes of the NDP-forming acyl-CoA synthetase superfamily, the conserved α-histidine seems to have more diverse functions, e.g. in stabilization of conformational states during phosphorylolation. This is indicated by the observation that arsenolysis (Equation 2) and the phosphoryl transfer between nucleotide and His-71β (Equation 4) is abolished in the HisαD mutants of ACD, although the α-histidine is not directly involved in these reactions.

**Arsenolysis**—The ability of ACD to catalyze arsenolysis indicates that enzyme-bound acetyl phosphate anhydride is the first intermediate of the overall reaction. In previous studies it was shown that SCS also catalyzes arsenolysis, however, with significantly higher efficiency. Although the *V* max of SCS was only 3 units mg⁻¹ (44 units mg⁻¹ in ACD), the *Kₘ* for arsenate was 30 μM compared with 12 mM in ACD (22).

**Phosphorylation Experiments**—The α-subunit of ACD was shown to be phosphorylated at His-257α. Mutation of this residue to aspartate completely abolished phosphorylation as well as the overall activity. The data indicate that the phosphoryl transfer from acetyl phosphate to His-257α represents the second partial reaction as shown for SCS and for ATP citrate lyase, another member of the NDP forming acyl-CoA synthetase superfamily. In both enzymes mutation of the homologous histidine residues had the same effect as in ACD (22, 35).

The most remarkable result of the phosphorylation experiments was that both α and β subunits of ACD were labeled in both directions of the reaction. In contrast, in SCS and ATP citrate lyase only the α-subunit is subject for phosphorylation (22, 35). By mutating His-71β to alanine we could show that this residue is the specific phosphorylation site in the β-subunit of *P. furiosus* ACD. Thus, His-71β is a second active site histidine residue in the ACD β-subunit causing a novel third partial reaction to be operative in ACD catalysis, *i.e.* the transfer of the phosphoryl group between His-257α and His-71β, which is absent in SCS or ATP citrate lyase. The addition of ADP to the phosphorylation assay containing acetyl-CoA and 32P, resulted in unlabeled α and β subunits (data not shown), indicating the fourth partial reaction to be the phosphoryl transfer from His-71β to ADP with concomitant release of ATP. From these results a novel four-step mechanism for the ACD reaction is proposed involving two active site histidine residues in the α- and β-subunit, respectively (Equations 1–4).

A close-up illustration of the active site of ACD showing both catalytically essential histidine residues in the α- and β-subunits is depicted in Fig. 3. Because His-71β is conserved in all ACD homologs, the proposed four-step mechanism is likely to be operative in all ACDs described so far. In contrast, in SCS and ATP citrate lyase, exhibiting SCS-like domain organization, this histidine residue is absent and, thus, catalysis in these enzymes proceeds via a three-step mechanism (13).

This three-step mechanism includes the phosphoryl transfer from phosphohistidine at the phosphate and CoA ester binding site in the α-subunit (site I) to ADP bound in the β-subunit (site II). From the crystal structure of SCS, the distance between site I and site II was estimated to be 35 Å, which is too large to allow a direct phosphoryl transfer. Thus, a conformational change including “swinging” of the phosphohistidine loop has been postulated for SCS (33, 36). In the ACD model the distance from the α-histidine to both, the second active site histidine in the β-subunit and ADP is also too large for a direct phosphoryl transfer from site I to site II. Thus, a similar conformational change including the swinging of the phosphohistidine loop has to be postulated. However, in both SCS and ACD, the postulated conformational change causing phosphoryl transfer has not been experimentally verified so far.

The three-step mechanism of SCS reaction involves only one active site histidine residue, whereas in the four-step mechanism in ACD two active site histidines in the α- and β-subunit are transiently phosphorylated during catalysis. The requirement of a second active site histidine in the β-subunit of ACD might be explained by slight structural differences in the spatial orientation and by the distances of the amino acids involved in phosphoryl transfer from His-257α~P to ADP. This assumption is supported by the following findings, which differ between ACD and SCS. (i) In the *P. furiosus* ACD the loop region containing the conserved His-257α residue is three amino acids shorter than the loop observed in SCS. The shortened loop in ACD might not be able to transfer the phosphoryl group directly to the β-phosphate of ADP and, thus, would necessitate the phosphorylation of a second binding site, *i.e.* His-71β, in close proximity to the β-phosphate of ADP in the β-subunit. (ii) In ACD, both the charge and also the length of the side chains in the position of Glu-218α and Asp-212β are critical for activity. Conversely, in SCS, a similar exchange of the homologous residues Glu-208α and Glu-197β show nearly no effect on activity. This indicates different charge distances in ACD compared with SCS. Furthermore, these residues in SCS have been suggested to stabilize the phosphohistidine loop at site I and site II, respectively (33, 36). In ACD, interaction of Glu-218α with His-257α and, thus, a similar function as Glu-
by acetyl-CoA and $P_v$, i.e. the first two partial reactions. In contrast, the $\alpha$-subunit of SCS, which is made up only by domain 1 and 2, cannot catalyze arsenolysis or the phosphorylation of His-246$\alpha$ by succinyl-CoA and $^{32}$P$_v$ (38). Thus, from the data obtained for ACD we can conclude that, in addition to the domains 1 and 2, domain 5 is essential for the first two partial reactions. In SCS the binding site for the phosphate molecule is positioned at the interface between the domains 2 and 5 (36). Because the orientation of both domains is very similar in ACD, domain 5, the second CoA ligase domain, might contribute to phosphate binding and perhaps also in the binding of the acetyl moiety. However, crystal structures of SCS and of the $P. horikoshii$ ACD $\alpha$ homolog have not been analyzed in the presence of acid substrates, and thus, the acid binding site has not been reported so far.

The ACD $\alpha$-subunit was also phosphorylated in the opposite direction using [$\gamma$-$^{32}$P]ATP (Fig. 4). This was unexpected since the $\beta$-subunit, which harbors the nucleotide binding site (domains 3 and 4) as well as the transiently phosphorylated His-71$\beta$, is essential for the overall reaction. Accordingly, as shown in this work, the ACD $\alpha$-subunit cannot catalyze the overall reaction in either direction despite catalyzing the phosphorylation in both directions, i.e. the first and second as well as the fourth partial reaction. Phosphorylation of the conserved $\alpha$-histidine by the $\alpha$-subunit alone using [$\gamma$-$^{32}$P]ATP was also described for SCS and ATP citrate lyase (35, 38). However, no overall activity was shown for either the SCS or ATP citrate lyase $\alpha$ subunits. An explanation for these results remains highly speculative. It has been discussed that the $\beta$-subunit might not be essential for the overall reaction but assists the catalytic process of nucleotide binding to and phosphorylation of the $\alpha$-subunit (35). However, because the $\beta$-subunit is essential for the overall reaction, the phosphorylation of the $\alpha$-subunit by [$\gamma$-$^{32}$P]ATP is likely to be nonspecific.

**Acknowledgments**—The expert technical assistance of K. Lutter-Mohr and M. Kusche is gratefully acknowledged.

**REFERENCES**

1. Reeves, R. E., Warren, L. G., Susskind, B., and Lo, H. S. (1977) J. Biol. Chem. 252, 726–731
2. Sanchez, L. B., and Müller, M. (1996) FEBS Lett. 378, 240–244
3. Schäfer, A. K., Selig, M., and Schönheit, P. (1993) Arch. Microbiol. 159, 72–83
4. Schönheit, P., and Schäfer, T. (1995) World J. Microbiol. Biotechnol. 11, 26–57
5. Bock, A. K., Glasemacher, J., Schmidt, R., and Schönheit, P. (1999) J. Bacteriol. 181, 1861–1867
6. Schäfer, A. K., and Schönheit, P. (1991) Arch. Microbiol. 155, 368–377
7. Siebers, B., and Schönheit, P. (2005) Curr. Opin. Microbiol. 8, 695–705
8. Mai, X., and Adams, M. W. W. (1996) J. Bacteriol. 178, 5897–5903
9. Sanchez, L. B., Morrison, H. G., Sogin, M. L., and Müller, M. (1999) Gene (Amst.) 233, 225–231
10. Musfeldt, M., Selig, M., and Schönheit, P. (1999) J. Bacteriol. 181, 5885–5888
11. Musfeldt, M., and Schönheit, P. (2002) J. Bacteriol. 184, 636–644
12. Bräsen, C., and Schönheit, P. (2004) Arch. Microbiol. 182, 277–287
13. Sanchez, L. B., Galperin, M. Y., and Müller, M. (2000) J. Biol. Chem. 275, 5794–5804
14. Elshourbagy, N. A., Near, J. C., Kmetz, P. J., Wells, T. N., Groth, P. H.,...
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Saxty, B. A., Hughes, S. A., Franklin, M., and Gloger, I. S. (1992) *Eur. J. Biochem.* **204**, 491–499

15. Chistoserdova, L. V., and Lidstrom, M. E. (1994) *J. Bacteriol.* **176**, 7398–7404

16. Binieda, A., Fuhrmann, M., Lehner, B., Rey-Berthod, C., Frutiger-Hughes, S., Hughes, G., and Shaw, N. M. (1999) *Biochem. J.* **340**, 793–801

17. Plaggenborg, R., Steinbüchel, A., and Priefert, H. (2001) *FEMS Microbiol. Lett.* **205**, 9–16

18. Fraser, M. E., James, M. N., Bridger, W. A., and Wolodko, W. T. (1999) *J. Mol. Biol.* **285**, 1633–1653

19. Fraser, M. E., Joyce, M. A., Ryan, D. G., and Wolodko, W. T. (2002) *Biochemistry* **41**, 537–546

20. Wolodko, W. T., Fraser, M. E., James, M. N., and Bridger, W. A. (1994) *J. Biol. Chem.* **269**, 10883–10890

21. Marchler-Bauer, A., and Bryant, S. H. (2004) *Nucleic Acids Res.* **32**, 327–331

22. Majumdar, R., Guest, J. R., and Bridger, W. A. (1991) *Biochim. Biophys. Acta* **1076**, 86–90

23. Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., Weese-Scott, C., Geer, L. Y., Gwadz, M., He, S., Hurwitz, D. L., Jackson, J. D., Ke, Z., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Marchler, G. H., Mullokandov, M., Shoemaker, B. A., Simonyan, V., Song, J. S., Thiessen, P. A., Yamashita, R. A., Yin, J. J., Zhang, D., and Bryant, S. H. (2005) *Nucleic Acids Res.* **33**, 192–196

24. Thompson, I. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25**, 4876–4882

25. Friendl, G. (1990) *J. Mol. Graph.* **8**, 29 and 52–56

26. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950

27. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467

28. Srere, P. A., Brazil, H., and Gonen, L. (1963) *Acta Chem. Scand.* **17**, 129–134

29. Chen, G. C., and Yang, J. T. (1977) *Anal. Lett.* **10**, 1195–1207

30. Lehtio, L., Fabrichnij, I., Hansen, T., Schönheit, P., and Goldman, A. (2005) *Acta Crystallogr. D Biol. Crystallogr.* **61**, 350–354

31. Shikata, K., Fukui, T., Atomi, H., and Imanaka, T. (2007) *J. Biol. Chem.* **282**, 26963–26970

32. Weitzman, P. D., and Kinghorn, H. A. (1978) *FEBS Lett.* **88**, 255–258

33. Bailey, D. L., Fraser, M. E., Bridger, W. A., James, M. N., and Wolodko, W. T. (1999) *J. Mol. Biol.* **285**, 1655–1666

34. Fraser, M. E., James, M. N., Bridger, W. A., and Wolodko, W. T. (2000) *J. Mol. Biol.* **299**, 1325–1339

35. Kanao, T., Fukui, T., Atomi, H., and Imanaka, T. (2002) *Eur. J. Biochem.* **269**, 3409–3416

36. Joyce, M. A., Fraser, M. E., James, M. N., Bridger, W. A., and Wolodko, W. T. (2000) *Biochemistry* **39**, 17–25

37. Fraser, M. E., Hayakawa, K., Hume, M. S., Ryan, D. G., and Brownie, E. R. (2006) *J. Biol. Chem.* **281**, 11058–11065

38. Pearson, P. H., and Bridger, W. A. (1975) *J. Biol. Chem.* **250**, 8524–8529