Reconstitution, morphology and crystallization of a fatty acid β -oxidation multienzyme complex from *Pseudomonas fragi*

Momoyo ISHIKAWA*, Yuriko MIKAMI*, Jiro USUKURA†, Hiroshi IWASAKI‡, Hideo SHINAGAWA‡ and Kosuke MORIKAWA*¹

*Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan, †Department of Anatomy, School of Medicine, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan, and ‡Department of Molecular Microbiology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan

The fatty acid β -oxidation multienzyme complex from *Pseudomonas fragi*, HDT, exhibits predominantly the three enzymic activities of 2-enoyl-CoA hydratase (EC 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and 3-oxoacyl-CoA thiolase (EC 2.3.1.16). The HDT complex is encoded by the *faoAB* operon, consisting of the *faoA* and *faoB* genes that encode two individual constituents, the α -subunit and the β -subunit. We have constructed *Escherichia coli* overexpression systems for the *faoAB* gene product (coexpression of the α - and β -subunits), the α -subunit alone and the β -subunit alone, and have purified the three respective products. Gel-filtration analysis revealed that the *faoAB* gene product forms a heterotetrameric structure, $\alpha_2\beta_2$, identical with the native HDT oligomeric state from *P. fragi*, whereas the α -subunit and β -subunit individually form dimers. Electron microscopy demonstrated that each protein morphologically adopts the above oligomeric structures. The HDT

complex, reconstituted *in vitro* from the isolated α - and β -subunits, exhibits the three original enzymic activities and yields the same crystal as those from the native enzyme. CD measurements indicated that the α - and β -dimers hardly alter their global conformations upon the formation of the HDT complex. Interestingly, the β -dimer alone does not exhibit 3-oxoacyl-CoA thiolase activity, whereas the α -dimer alone exhibits both the 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. These results suggest that the contact between the α - and β -subunits is essential for the thiolase activity. We have identified several structurally important proteolytic sites within each subunit, which are protected in the intact heterotetrameric molecule. These findings allow the possible location of the interface between the two subunits, which should be crucial for the exhibition of thiolase activity.

INTRODUCTION

Enzymes involved in fatty acid β -oxidation form a multienzyme complex in bacteria, such as *Escherichia coli* [1] and *Pseudomonas fragi* [2]. Peroxisomes and glyoxisomes in some eukaryotes were found to contain multienzyme complexes for fatty acid β -oxidation, whereas their detailed architectures are different from the bacterial multienzyme complexes. It remains unclear why these enzymic activities in some cases are unified in the form of a multienzyme complex, while the same activities in others are separately carried on individual enzymes. It has been suggested that some enzymes, which participate in sequential metabolic reactions other than fatty acid β -oxidation, also tend to become integrated as a multifunctional enzyme or a multienzyme complex in the evolutionary process [3].

Comparative studies between multienzyme complexes and individual enzyme molecules, particularly in terms of their three-dimensional structure, should facilitate an understanding of the physiological significance of complex formation and how this type of organization has been acquired, from the viewpoint of molecular evolution. The bacterial fatty acid β -oxidation cycle involves four enzymic activities, acyl-CoA dehydrogenase (EC 1.3.99.3), 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-CoA thiolase. The multienzyme complex from *P. fragi* is known to predominantly exhibit the last three of these four enzymic activities, and additionally *cis*-3-*trans*-2-enoyl-CoA isomerase (5.3.3.8) and 3-hydroxyacyl-CoA epimerase (5.1.2.3) activities for unsaturated fatty acid β -oxidation. This

enzyme complex has been designated as HDT, according to the 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-oxoacyl-CoA thiolase activities, and was characterized by Imamura et al. [4]. HDT is composed of an α -subunit and a β -subunit of 73 kDa and 42 kDa respectively, as estimated by SDS/PAGE. Sato et al. [2] have previously reported that, among the three major enzymic activities, the hydratase and dehydrogenase activities reside within the α -subunit, whereas the thiolase activity is harboured in the β -subunit, although it remains unknown whether the isolated β -subunit itself possesses the activity *in vitro*.

The goal of this study is to elucidate the sequential catalytic reaction mechanisms of the multienzyme complex at the atomic level. Taking its relatively small molecular mass and high stability into account, the HDT complex should be the most appropriate target for a structural study of the multienzyme complex, using the combined techniques of protein engineering and X-ray crystallography. This approach requires the establishment of an efficient *in vitro* reconstitution system, which allows us to characterize various mutant complexes and thus to investigate the relationships between the activity and the three-dimensional structure. In this paper, we show that the intact HDT molecule and each of the two subunits were successfully overexpressed in large quantities, using the respective *E. coli* overexpression systems of the cloned *faoAB*, *faoA* and *faoB* genes, which encode the HDT complex and the α -subunit (715 amino acids) and β -subunit (390 amino acids) respectively. We also describe the characterization of the *faoAB*, *faoA* and *faoB* gene products, the

Abbreviations used: DTT, dithiothreitol; HDT, the fatty acid β -oxidation multienzyme complex from *Pseudomonas fragi*.

¹ To whom correspondence should be addressed.

in vitro reconstitution of the intact molecule from the individual subunits and their morphological observation by electron microscopy, in addition to their successful crystallization.

EXPERIMENTAL

Overexpression

The *E. coli* strain MIF100 ($\Delta fadBA::cat$) is a derivative of BL21 (DE3) [5], and was used as a host strain for the T7-mediated overexpression system in this study. The *E. coli fadBA* operon [6] corresponds to the *P. fragi faoAB* operon, and the $\Delta fadBA::cat$ allele was introduced according to the procedure described by Russell et al. [7], to prevent contamination by the *E. coli fadBA* product (the fatty acid β -oxidation multienzyme complex) during protein purification.

The *faoAB* operon is composed of the *faoA* and *faoB* genes. Three kinds of pT7-7-based overexpression plasmids [8] were constructed using the cloned *faoAB* gene from pHDT4 [2]. One of these plasmids, designated pMP110, carried the *faoAB* operon, which allowed the coexpression of the α - and β -subunits to produce the HDT complex in the cells. The other two plasmids, pMP109 and pMP111, carried either the *faoA* gene or *faoB* gene respectively (Figure 1), and thus they separately expressed the α - and β -subunit. An *NdeI* site had been introduced by site-directed mutagenesis [9] into the region including the *faoA* initiation codon, to allow the structural genes in pMP109 and pMP110 to be placed at an ideal position for expression by taking advantage of the pT7-7 vector.

E. coli MIF100 cells harbouring each of these overexpression plasmids were grown at 37 °C in 2 litres of Luria-Bertani medium containing 100 μ g/ml ampicillin and 20 μ g/ml chloramphenicol. In the case of overexpression of the HDT complex, pLysE [5] was transformed, together with pMP110, into the host cells to repress the basal level of its expression. All transformants were grown to an absorbance of about 0.8 at 600 nm and were then induced by 1 mM isopropyl β -D-thiogalactoside for 3 h. Cells were harvested by centrifugation for 15 min at 6000 g.

Purification

All procedures were performed at 0–4 °C. Column chromatography was carried out with an FPLC system (Pharmacia).

Figure 1 Plasmid construction of the HDT complex and the α - and β -subunits

Restriction map of *P. fragi* chromosomal DNA containing the *faoA* and *faoB* genes. The new *NdeI* site was introduced at the initiation codon on the *faoA* gene by site-directed mutagenesis [9]. The α -subunit overproducer, plasmid pMP109, carries the *faoA* gene in a 2.5 kb *NdeI*–*NdeI* fragment. The HDT complex overproducer, pMP110, carries *faoAB* in a 3.7 kb *NdeI*–*EcoRI* fragment, and the β -subunit overproducer, pMP111, carries *faoB* in a 3.5 kb *EcoRI*–*EcoRI* fragment.

The cells harvested from the 2 litres of culture medium were suspended in 45 ml of buffer A, which consists of 10 mM potassium phosphate (pH 7.0), 10% (v/v) glycerol and 3 mM dithiothreitol (DTT). PMSF was added to a final concentration of 1 mM and the cell suspension was homogenized by sonication (Branson sonifier model 450). The homogenate was centrifuged to remove the cell debris at 17000 g for 30 min, and the supernatant was subjected to ammonium sulphate precipitation. Solid ammonium sulphate (196 g) was gradually added to 1 litre of the supernatant to achieve 33% saturation and this mixture was centrifuged at 16000 g for 10 min. This supernatant was brought to 55% saturation by adding more solid ammonium sulphate. After centrifugation (16000 g for 10 min), the ammonium sulphate precipitate was resuspended in 20 ml of buffer A and was dialysed against buffer A containing 10 mM KCl overnight. The dialysate was loaded onto a DEAE-Sephacrose column (column volume 80 ml), which was equilibrated with buffer A containing 10 mM KCl. The column was washed with nine column volumes of the initial solution, i.e. buffer A containing 10 mM KCl, and was eluted with 800 ml of a linear KCl gradient (0.01 M to 0.4 M) at a flow rate of 3 ml/min. Fractions exhibiting enzyme activities were collected. To concentrate the enzyme solution pooled from the DEAE-Sephacrose column chromatography, solid ammonium sulphate was added to 60% saturation. The resulting precipitate was collected by centrifugation at 12000 g for 10 min, dissolved in 10 ml of buffer A containing 0.15 M KCl and loaded on a Superdex 200 HiLoad (26/60) column. The column was equilibrated and eluted with buffer A containing 0.15 M KCl at a flow rate of 2 ml/min, and 3 ml fractions were collected. Fractions containing enzyme, as detected by SDS/PAGE, were combined and dialysed against buffer A. The dialysed enzyme solution was applied to a hydroxyapatite (Bio-Gel HT; Bio-Rad) column (column volume 30 ml) equilibrated with buffer A. The column was washed with five column volumes of buffer A and was eluted with 230 ml of a linear potassium phosphate pH 7.0 gradient (0.01 M to 0.3 M). The flow rate was 1 ml/min and 3 ml fractions were collected. The fractions containing enzyme were pooled and concentrated with Centriprep-30 devices (Amicon). To store the purified protein at –80 °C, the concentrated solution was dialysed against the storage buffer, which consists of 50 mM Pipes (pH 6.5), 70% (v/v) glycerol and 3 mM DTT. The purification procedure for the *faoA* and *faoB* gene products did not involve Superdex 200 HiLoad column chromatography.

Some fractions of the purified native HDT complex from *P. fragi* [4] were a gift from Dr. S. Imamura.

Enzyme assays

The enzymic activities of the purified products were measured by spectrophotometric assays after enzyme reactions at 37 °C. The assays for the combined activities of 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase (hydratase–dehydrogenase) and for the 3-oxoacyl-CoA thiolase (thiolase) activity were carried out as previously reported [4]. One unit of enzymic activity is defined as the amount of enzyme that catalyses the conversion of 1 μ mol of substrate to product per minute.

Molecular-mass determination

The molecular masses of the isolated individual subunits, the complex and the reconstituted complex were determined by gel filtration on a Superdex 200 HR (10/30) column. The sample was applied in a volume of 25 μ l and was eluted with buffer B, which consists of 50 mM potassium phosphate (pH 7.0), 0.15 M

KCl, 10% (v/v) glycerol and 1 mM DTT at a flow rate 0.4 ml/min. The molecular masses of the subunits and the proteolytic fragments were determined by SDS/PAGE.

Proteolytic analysis

The three purified gene products were partially digested with subtilisin BPN', trypsin, and chymotrypsin in 100 mM Tris/HCl (pH 8.0) and 1 mM DTT for 1 h at 20 °C. The final concentration of the purified protein was 1 mg/ml and those of the trypsin and subtilisin BPN' were 5 µg/ml (chymotrypsin, 2.5 µg/ml). Reactions were stopped by boiling for 3 min in SDS sample buffer and the reaction products were analysed by SDS/PAGE.

Low-angle rotary-shadowing electron microscopy

The isolated individual subunits and the complex at a concentration of 2.4–3.3 mg/ml were equilibrated with 50% (v/v) glycerol. A 30 µl volume of each of these proteins was sprayed respectively onto a freshly cleaved mica surface by using a painter's air brush, and immediately brought into the evaporator, which had been recently improved for freeze-etching replica (Hitachi FR 7000). The samples were dried for 10 min while cooling from room temperature to –100 °C, and rotary-shadowed with platinum using an electron gun positioned at 2.5° to the mica surface and then coated with a film of carbon, according to the method of Tyler and Branton [10] with slight modification. The replicas were floated on distilled water and collected on a grid covered with formvar film. Each protein replica was observed under a JEOL 1200EX electron microscope operating at 100 kV. Most of the images were originally taken at $\times 100000$, and then printed with enlargements in reverse contrast for improved clarity.

CD measurement

The CD spectra of the purified proteins were measured on a JASCO J-720 spectropolarimeter over the range 194–250 nm using a cell with an optical path-length of 1 mm at 10 °C. Solutions contained 0.2 mg/ml of protein in 5 mM Pipes (pH 6.5) and 1 mM DTT.

Crystallization

For crystallization, protein solutions were concentrated to 2–5 mg/ml using a Centricon-30 (Amicon) after dialysis against buffer consisting of 50 mM Pipes (pH 6.5), 10% (v/v) glycerol and 3 mM DTT. Crystallization was performed using the hanging-drop mode of vapour diffusion and the microdialysis method [11]. X-Ray diffraction patterns were taken at 20 °C with a Huber camera on a rotating anode X-ray generator (Rigaku RU-300).

Other procedures

SDS/PAGE was carried out as described by Laemmli [12]. The low-molecular-mass electrophoresis calibration kit (Pharmacia) was used for the marker proteins. The protein concentration was determined by the method of Bradford [13], using a Bio-Rad protein assay kit with BSA as the standard. N-Terminal amino acid sequences of proteins were analysed with an Applied Biosystems model 492 protein sequencer.

RESULTS AND DISCUSSION

Overexpression in *E. coli* and purification of the intact HDT complex and its constitutive subunits

The HDT enzyme complex is encoded by the *faoAB* operon, which consists of the *faoA* and *faoB* genes. The *faoAB*, *faoA*, and *faoB* gene products, encoding the HDT complex, the α -subunit and the β -subunit respectively, were expressed in large quantities. The production efficiencies for these three proteins were roughly estimated to be 7, 20 and 3 mg/l of culture respectively, from the intensities of the bands revealed with Coomassie Brilliant Blue staining after SDS/PAGE of whole cell extracts (Figure 2A). Thus, the expression systems should be efficient enough to facilitate structural studies using the combined techniques of X-ray crystallography and mutational analyses. The expressed proteins were confirmed to be HDT and the α - and β -subunits through cross-reaction with anti- α - and β -subunit sera and N-terminal sequence analyses (results not shown). Most of the expressed proteins were obtained as soluble fractions after sonication of the cells. The recombinant HDT complex and the individual subunits from the *E. coli* overexpression strain were purified through a combination of ion-exchange and hydroxyapatite chromatography, and occasionally by gel filtration. All of these proteins were electrophoretically homogeneous and the molecular mass of each single band was estimated to be 73 kDa and 42 kDa by SDS/PAGE (Figure 2B). The HDT expressed in

Figure 2 Overexpression of the HDT complex, and the α - and β -subunits in *E. coli*: SDS/PAGE of cell extracts (A) and purified proteins (B)

An aliquot (1 ml) was taken from cultures at 3 h after the induction of T7 polymerase. Crude extracts (5 µl) and the purified proteins (3 µg) were electrophoresed on 15% (A) and 10% (B) SDS polyacrylamide gels respectively, and were stained with Coomassie Brilliant Blue R250. The arrows indicate the 73 kDa α -subunit and the 42 kDa β -subunit. (A) M, molecular-mass markers. Lanes 1 and 2, non-induced and induced cell extracts from *E. coli* MIF100 pLysE transformants containing the pT7-7 vector; lanes 3 and 4, non-induced and induced cell extracts respectively from *E. coli* MIF100 pLysE transformants containing pMP110; lanes 5 and 6, non-induced and induced cell extracts respectively from *E. coli* MIF100 transformants containing the pT7-7 vector; lanes 7 and 8, non-induced and induced cell extracts (pMP109); lanes 9 and 10, non-induced and induced cell extracts (pMP111). (B) M, molecular-mass markers. Lane 1, the *faoAB* gene product; lane 2, the *faoA* gene product; lane 3, the *faoB* gene product. The marker proteins are α -lactalbumin (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase (94 kDa).

Table 1 Activities of the HDT complex and subunits

Enzymic activities of the native HDT complex from *P. fragi*, the expressed HDT complex (*faoAB* gene product), the complex reconstituted from the isolated α - and β -subunits, and the individual α - and β -subunits were measured spectrophotometrically [4]. The combined activities of 2-oxoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase (HD) and the activity of 3-oxoacyl-CoA thiolase (T) are given.

Enzyme	Specific activity (units/mg of protein)	
	HD	T
HDT complex		
Purified from <i>P. fragi</i>	130	286
<i>faoAB</i> gene product	146	216
Reconstituted	135	201
α -subunit	90	< 0.1
β -subunit	< 0.1	< 0.1

E. coli, the *faoAB* gene product, exhibited the hydratase–dehydrogenase and thiolase activities to the same extent as those of native *P. fragi* HDT (Table 1). The HDT, the α -subunit, and the β -subunit could be stored in storage buffer pH 6.5, at -80°C for 1 year without any detectable loss of the enzymic activities, underscoring their stability for *in vitro* reconstitution.

Subunit and secondary structures

Each protein isolated from the *E. coli* overexpression strain was subjected to Superdex 200 HR column chromatography to determine the molecular mass. They were calculated from molecular-size markers to be 240 kDa, 148 kDa and 62 kDa for HDT and the α - and β -subunits respectively (results not shown). On the other hand, the molecular masses of the α - and β -subunits were estimated by SDS/PAGE to be 73 kDa and 42 kDa respectively. These values indicate that the intact HDT complex forms an $\alpha_2\beta_2$ structure, while the α -subunit constitutes a dimer. The value for the β -subunit, which largely exceeds 42 kDa, also suggests dimer formation. This finding implies that the α_2 and β_2

subunit structures are the protomers preceding intact $\alpha_2\beta_2$ complex formation *in vitro*.

Furthermore, we measured the CD spectra to examine whether the individual dimeric subunits retain stable and native conformations that are globally similar to those within the intact $\alpha_2\beta_2$ structure. The summation of the two spectra, corresponding to the α - and β -subunits, provides a spectrum nearly identical with that of the intact HDT complex (results not shown). These results demonstrate that the individual dimeric subunits overall retain a similar architecture to those in the intact HDT complex. In addition, the crystal structure of the *Saccharomyces cerevisiae* 3-ketoacyl-CoA thiolase [14] exhibits secondary structure comparable with that of the β -subunit obtained by CD measurement.

Reconstitution of HDT

The purified α - and β -subunits, in storage buffer, were mixed in equimolar amounts and were incubated on ice for 30 min. Subsequently, the mixture was subjected to gel filtration on a 200 HR (10/30) column. The protein eluted at the same position as that of the *E. coli*-expressed HDT on the Superdex 200 HR column (results not shown), demonstrating that the intact heterotetrameric $\alpha_2\beta_2$ molecule can be reconstituted by mixing the α - and β -subunits, which were separately expressed and purified.

The hydratase–dehydrogenase and thiolase activities of the reconstituted HDT complex were measured separately by spectrophotometric assays. Its specific activities for both the hydratase–dehydrogenase and the thiolase were almost the same as those of the *E. coli*-expressed HDT (Table 1). Together with the estimation of the molecular masses from gel filtration, the complete recovery of the activities demonstrates that the HDT complex can be reconstituted from the dimeric α - and β -subunits *in vitro*. Interestingly, the α -subunit still retains the hydratase–dehydrogenase activity. On the other hand, the β -subunit alone did not show any thiolase activity. Consistent with the previous result from a different molecular genetic approach [2], this finding proves that the emergence of the thiolase activity, which belongs to the β -subunit, strongly depends upon its contact with the α -subunit. It is interesting to investigate *in vitro* the mechanism

Figure 3 Rotary-shadowed HDT complex, α - and β -subunits

(A) Electron micrographs of the three isolated proteins, the HDT complex, α - and β -subunits, were taken as described in the Experimental section. Scale bars represent 20 nm. Each protein molecule is indicated by arrowheads. (B) Schematic drawing of the HDT complex subunit structure deduced from electron microscopic observation and proteolytic analyses. The α -subunit (the thicker shadowed sphere with the larger size) is mounted on the loop domains of the β -subunit, which are denoted by bold lines. Note the two-fold axis shared by the α - and β -dimers.

Figure 4 Limited proteolytic analysis of the purified proteins: SDS/PAGE (A) and the proteolytic cleavage sites on the amino acid sequence (B)

(A) The HDT complex and the α - and β -subunits were digested with each protease for 1 h at 20 °C, and the reaction products were analysed on either 10% or 15% SDS polyacrylamide gels (for details see the Experimental section). M, molecular mass markers are the same as those in Figure 2. Lanes 1 and 2, HDT complex, non-digested and digested respectively; lanes 3 and 4, α -subunit, non-digested and digested respectively; lanes 5 and 6, β -subunit, non-digested and digested respectively. The positions of the intact 73 kDa α - and 42 kDa β -subunits are indicated. A1 and A2 show the tryptic fragments from the α -subunit, and B1 and B2 are the chymotryptic fragments from the β -subunit. These fragments were subjected to N-terminal sequence analyses. (B) The cleavage sites are denoted by arrowheads on the amino acid sequences of the α - and β -subunits. Arabic numerals indicate residue number. The N-terminus of the β -subunit is Ser², because the first methionine is lacking [2]. The shaded regions in the α -subunit indicate 2-enoyl-CoA hydratase (hydratase) and 3-hydroxyacyl-CoA dehydrogenase (dehydrogenase) homologous domains respectively, according to the sequence alignment [2]. In the β -subunit, the black region indicates the sequence of the loop domain, and the asterisks indicate the putative residues important for the thiolase activity, Cys⁹⁵, His³⁴⁷ and Cys³⁷⁷. These hypothetical assignments are made from the homology model, which was built on the basis of the crystal structure of the *S. cerevisiae* 3-ketoacyl-CoA thiolase [14].

by which the inter-subunit contact allows recovery of the thiolase activity.

Morphological observation by electron microscopy

The first attempt to observe the isolated fatty acid β -oxidation multienzyme complex and the individual constituent subunits was carried out by low-angle rotary-shadowing electron microscopy (Figure 3A). Electron micrographs indicate that the isolated HDT complex indeed consists of two dimeric elements of different sizes. It is presumed that the larger dimeric blobs would be the α -subunits stacked onto the smaller dimeric blobs corresponding to the β -subunits. Indeed, the shadowed images of the isolated α - and β -subunits present the dimeric structures with different sizes, as expected. These electron micrographs allow us to see even the extensive interface between the two subunits, suggesting that the large contact within the complex is crucial for the emergence of the three successive catalytic activities required for β -oxidation.

Limited proteolytic analysis

To examine the domain structure of the α - and β -subunits, we analysed fragments produced from each protein, using limited proteolysis by subtilisin BPN', trypsin and chymotrypsin (Figure 4A). The treatments with these proteases, including subtilisin BPN' with low specificity, produced a few similar discrete fragments with respect to the α - and β -subunits, but the HDT complex was hardly susceptible to the digestion by these proteases, except for subtilisin BPN'. These fragments, which migrated as discrete bands on SDS/PAGE, were subjected to N-terminal sequence analyses.

Digestion of the α -subunit with trypsin revealed two fragments of 34 kDa (A1) and 26 kDa (A2) respectively (Figure 4A). The sequences were determined to be MIYEGKAI for the A1 fragment and K⁴⁸⁴MGKNPIVVN for the A2 fragment. The A1 sequence contains the N-terminus of the α -subunit. This cleavage site appears to be located inside the 3-hydroxyacyl-CoA dehydrogenase homologous domain (Figure 4B) [2]. The cleavage at this site appears to be more insensitive in the intact $\alpha_2\beta_2$ complex than in the α -subunit alone. This site, which is protected from proteolysis, may lie in the subunit interface.

Treatment of the β -subunit with chymotrypsin produced two fragments, with molecular masses of 27 kDa (B1) and 16 kDa (B2) (Figure 4A), and with N-terminal sequences of A¹⁴⁴AKASG and SLNPRDVV respectively. The B2 fragment was identical with the N-terminus of the intact β -subunit. Taking the molecular masses of the two fragments into account, the B1 fragment is concluded to correspond to the remaining half at the C-terminus of the β -subunit.

X-Ray analysis of 3-ketoacyl-CoA thiolase from *S. cerevisiae* [14] allowed the identification of the chymotrypsin cleavage site on the β -subunit structure, based on the roughly 30% sequence similarity between this *S. cerevisiae* thiolase and the β -subunit. According to the homology model of the β -subunit [15], interestingly, the chymotrypsin cleavage site is located near a disordered segment between two helices, which belong to a flexible loop domain (Figure 4B) defined by the thiolase crystal structure (see L α 1 and L α 2 in Figure 9 of [14]). This domain is extensively spread perpendicular to the two-fold axis of the dimeric thiolase molecule, corresponding to the β -dimer. It also entirely faces the solvent on one side and is weakly connected to the remaining major domain on the other side, and it partly covers the active site. The cleavage site and the hypothetical catalytic centre, which are about 20 Å apart from each other within the β -subunit

structure made by the homology model, are located at distances of roughly 20 Å and 17 Å respectively, from the two-fold axis.

Most notably, this cleavage site within the loop domain is completely protected in the intact $\alpha_2\beta_2$ complex, implying that in the HDT complex, the interface between the α - and β -subunits would involve this extensive loop domain. Thus, it is likely that, in the complex, the α -subunit is piled on the loop domain of the β -subunit to protect the cleavage site, and shares an identical two-fold axis (Figure 3B). This interpretation is in good agreement with the morphological views from the electron micrographs (Figure 3A). Therefore the disordered segments, which occupy a remarkably large area in the loop domain of the β -subunit, may contribute to the inter-subunit contact.

Crystallization of HDT

With the aim of X-ray structural determination, crystallization was carried out using the three different preparations, i.e. are the native HDT complex isolated from *P. fragi* [4], the recombinantly expressed HDT and the HDT reconstituted from each separately expressed recombinant subunit. We tried various conditions, including different precipitants, such as ammonium sulphate, poly(ethylene glycol) and 2-methyl-2,4-pentanediol. We also tried to add various detergents, such as n-octyl- β -D-glucoside, sucrose monolaurate, lysophosphatidylcholine heptadecanoyl and others, since these detergents generally enhanced the solubility of the complex.

Using the vapour-diffusion and microdialysis techniques, all of the three materials allowed us to obtain crystals of various shapes. It should be noted that the HDT complex, regardless of the different preparations, exhibits remarkable polymorphism, as revealed from the more than ten different crystal forms grown under similar conditions (results not shown). Most of these

crystals yielded too poor diffraction patterns to allow the structural determination at atomic resolution. However, some crystals, which diffract to nearly atomic resolution, are occasionally grown. They were unstable to X-ray exposure and the diffraction patterns deteriorated from higher resolution diffraction spots in a few hours. In addition to more reproducible crystallization conditions, the cryoprotection technique will be required for data collection in the future.

We thank Professor A. Kawaguchi and Dr. S. Imamura for providing the HDT protein from *P. fragi* and the pHDT4 plasmid. We are also grateful to Dr. H. Nakamura for homology model building and to Dr. S. Kanaya for helpful discussions.

REFERENCES

- 1 DiRusso, C. C. (1990) *J. Bacteriol.* **172**, 6459–6488
- 2 Sato, S., Hayashi, M., Imamura, S., Ozeki, Y. and Kawaguchi, A. (1992) *J. Biochem. (Tokyo)* **111**, 8–15
- 3 Srere, P. A. (1987) *Annu. Rev. Biochem.* **56**, 89–124
- 4 Imamura, S., Ueda, S., Mizugaki, M. and Kawaguchi, A. (1990) *J. Biochem. (Tokyo)* **107**, 184–189
- 5 Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89
- 6 Spratt, S. K., Black, P. N., Ragazzino, M. M. and Nunn, W. D. (1984) *J. Bacteriol.* **158**, 535–542
- 7 Russell, C. B., Thaler, D. S. and Dahlquist, F. W. (1989) *J. Bacteriol.* **171**, 2609–2613
- 8 Tabor, S. (1990) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), pp. 16.2.1–16.2.11, Wiley and Sons, New York
- 9 Kunkel, T. A., Roberts, J. and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
- 10 Tyler, J. M. and Branton, D. (1980) *J. Ultrastruct. Res.* **71**, 95–102
- 11 Ollis, D. and White, S. (1990) *Methods Enzymol.* **182**, 646–659
- 12 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 13 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 14 Mathieu, M., Zeelen, J. Ph., Pauptit, R. A., Erdmann, R., Kunau, W.-H. and Wierenga, R. K. (1994) *Structure* **2**, 797–808
- 15 Kabsch, W. and Sander, C. (1983) *Biopolymers* **22**, 2577–2637