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Monomeric and dimeric forms of cholesterol esterase from *Candida cylindracea*

Primary structure, identity in peptide patterns, and additional microheterogeneity

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Abstract

Cholesterol esterase from *Candida cylindracea* was separated into two fractions, corresponding to a dimeric and a monomeric form. Fingerprint analysis after lysine cleavages shows identical patterns, suggesting lack of primary differences. Crystals obtained from the two proteins differ and suggest the possibility of an equilibrium between the two forms, influenced by the substrate cholesterol linoleate, which appears to stabilize the more active, dimeric form. All crystals have dimers as the asymmetric unit. The primary structure of the enzyme was determined at the peptide level and shows only one difference, Leu-350 instead of Ile, from a DNA-deduced amino acid sequence, and conservation of features typical for cholesterol esterases characterized.

Key words: Cholesterol esterase; Primary structure; Quaternary structure; Fingerprint analysis; Microheterogeneity

1. Introduction

Cholesterol and cholesterol esters are major constituents of intimal atherosclerotic lesions [1], and their ratio is influenced by cholesterol esterases. These enzymes occur in multiple forms in many organisms, including apparent dimers and monomers, reported not only in mammals [2] but also in the enzymes from completely different sources, e.g. yeasts [3]. In addition, the enzyme is represented by multiple genomic sequences, and five of these have recently been determined for the asporogenic yeast *Candida cylindracea* [4–6]. These enzymes are members of a large enzyme family of serine hydrolases, including acetylcholine esterases, neutral lipases, many other esterases, and also non-hydrolytic proteins (e.g. [7–10]). Three-dimensional structures are known for several of these enzymes, including a *Torpedo* acetylcholine esterase [11] and four lipases with highly different origins, from human pancreas [12], a *Rhizomucor* [13], a *Geotrichum* [14,15] and a *Candida* [16]. Comparisons of sequences and structures support similarities and suggest functional mechanisms for the catalysis at the water/lipid interface of these enzymes, which often have the catalytic triad Ser-His-Glu although the common serine hydrolase triad Ser-His-Asp also occurs [7–18].

The *Candida cylindracea* enzymes constitute forms much studied [3–6]. One of these cholesterol esterases has also been crystallized [3] and electron density maps are now available [19], soon allowing further analyses of the relationships within this divergent enzyme family. For interpretation of the crystallographic data, knowledge of the primary structure and of the relationships between the dimeric and monomeric forms of the enzyme is essential.

In this study, we have fingerprinted the dimeric and monomeric forms of *Candida cylindracea* cholesterol esterase which crystallize in different crystal classes [3] and have also analyzed the entire primary structure of the enzyme. After completion of the work, a corresponding DNA structure appeared [6], which has a minor difference, suggesting microheterogeneity of the enzyme. Analysis at the protein level is of additional interest in this case, since *Candida* utilizes one codon (CTG, normally for Leu) in a different manner (for Ser) [4]. The monomeric and dimeric forms of the enzyme appear

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identical in primary structure. The presence of the substrate cholesterol linoleate appears to promote a shift toward the more active, dimeric form of the enzyme [3].

2. Materials and methods

2.1. Protein

Commercially available enzyme (Boehringer-Mannheim) with an initial specific activity of 46 U/mg was passed through a polyacrylamide gel P-6DG (Biorad; 6000 Da exclusion limit) desalting column (2.5 × 20 cm) to eliminate NaCl (pooled fraction, 25 ml). It was then equilibrated with 50 mM phosphate buffer, pH 7.3, and separated from contaminating bovine albumin (in the commercial preparation) by gel filtration on Bio-Gel P-100 (2.5 × 100 cm) in 50 mM phosphate buffer, pH 7.3. Cholesterol esterase eluted as two baseline-resolved peaks, a dimer and a monomer, at volumes 131 and 165 ml, respectively (fractions 1 and 2; Fig. 1). Each active fraction was pooled and concentrated (Amicon concentrator) to 10–15 mg/ml. The entire purification was performed at 4°C. Enzymatic activities were assayed at 500 nm in approximately 10 mM cholic acid with cholesterol linoleate as described [20]. The substrate solution was prepared by dissolving 70 mg of the linoleate in 2.5 ml Thesit (Boehringer-Mannheim) and addition of 23 ml of 1 M NaCl.

Crystals of the two active enzyme fractions, corresponding to fractions 1 and 2 in Fig. 1, were grown by vapour diffusion of the hanging drop technique at 22°C [3]. The droplets of protein solution were stabilized with PEG-3350 and 50 mM phosphate buffer, pH 7.3, and left in vapour diffusion against sealed wells containing reservoirs of 8–25% PEG solution in the same buffer. Crystals appeared within 4–7 days and continued to grow for two weeks. Before the conditions were fully optimized, thin plates of types II and III crystals tended to cluster together, but adjustment of the protein concentration made plates thicker and largely eliminated the clustering problem.

2.2. Structural analysis

Redissolved crystals of type II and III were transferred into 6 M guanidine hydrochloride, 0.4 M Tris, pH 8.15, 2 mM EDTA, using a centricon filter (cut-off 1000), and were carboxymethylated with labelled iodoacetate after reduction with dithiothreitol [21]. The carboxymethylated proteins were cleaved in separate batches with proteolytic enzymes, *Achromobacter* Lys-specific protease (Waco Chemicals, Neuss, Germany), *Staphylococcal* Glu-C-specific and *Pseudemonas* N-Asp-specific proteases (from Boehringer-Mannheim), all in 0.1 M ammonium bicarbonate, 1.5 M urea, 37°C, for 4–20 h. Soluble peptides obtained were separated by HPLC on Vydac C-8 or on UltroPac TSK ODS-120T C-18. Samples were also digested with CNBr in 70% formic acid (24 h at room temperature) and resultant peptides separated by FPLC on Sepharose 12 (LKB/Pharmacia) in 70% formic acid.

Pure peptides were analyzed for total composition in an LKB/Pharmacia Alpha Plus analyzer after acid hydrolysis with 6 M HCl/0.5% phenol for 24 h at 110°C. Sequence degradations were performed with a Milligen Prosequencer 6600 and an Applied Biosystems 120A sequencer, both equipped with an on-line analyzer.

3. Results

Commercially available cholesterol esterase from *Candida cylindracea* was purified as described in section 2, resulting in two active fractions corresponding to a dimer and a monomer (Fig. 1), respectively. Enzymes from these two fractions were treated separately to give three types of native crystals: triclinic (type II crystals; Table I) from the dimeric form (fraction 1 in Fig. 1), monoclinic (type I crystals, in the absence of substrate; Table I), and another triclinic (type III crystals; in the presence of 0.05 mM cholesterol linoleate; Table I) from the originally monomeric form, fraction 2 in Fig. 1. In all crystal forms, the dimer is a basic entity of the enzyme. Apparently, the presence of the substrate added to the monomeric enzyme shifts the equilibrium toward the dimeric state, the most active form of the enzyme, which then crystallizes as type III.

The material corresponding to the type II and III crystals, grown from enzymes originally eluting as dimeric (fraction 1 in Fig. 1) and monomeric (fraction 2 in

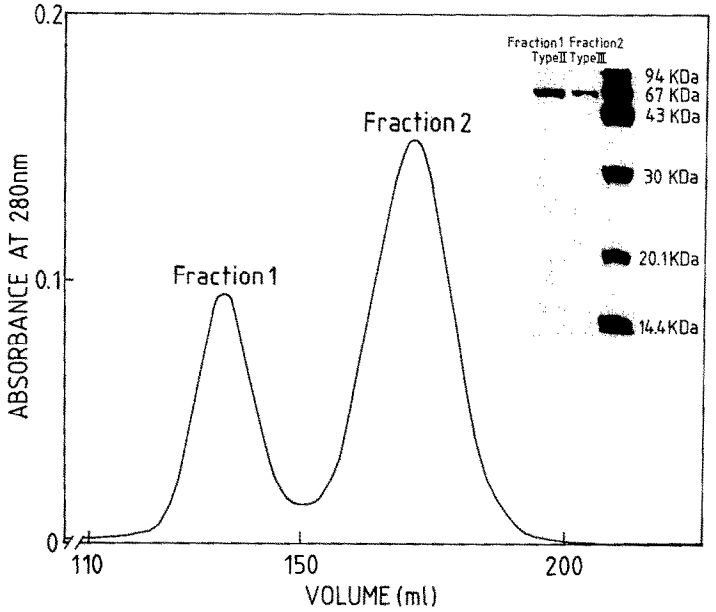

Fig. 1. Exclusion chromatography on P-100 in 50 mM phosphate, pH 7.3, of cholesterol esterase from *Candida cylindracea*. Fractions 1 and 2 differ in size and correspond to a dimer–monomer interrelationship. The insert shows SDS/polyacrylamide gel electrophoresis of redissolved crystals (according to Table I) of the material of the two fractions.

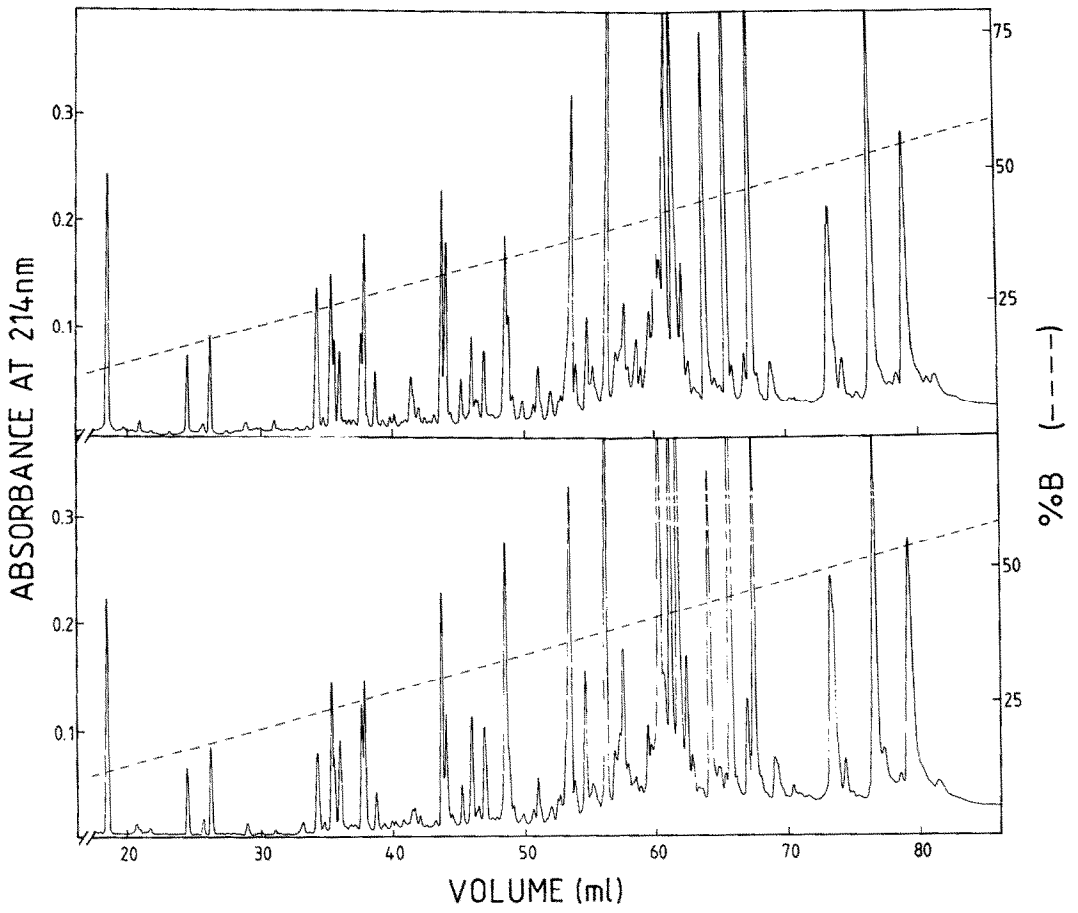

Fig. 2. Fingerprint comparison by HPLC of peptides produced by cleavage with Lys-C specific protease of the monomeric (top) and dimeric (bottom) fractions. As shown, no clearly differing peptides are noticed in either digest.

Table I
Properties of cholesterol esterase forms

Type	I	III	II
Original form	Monomer		Dimer
Fraction (Fig. 1)	2		1
	Substrate −	Substrate +	
Crystal class	Monoclinic	Triclinic	Triclinic
Space group	$P2_1$	P1	P1
a (Å)	122.9	58.84	58.565
b (Å)	101.0	90.93	88.685
c (Å)	95.2	58.74	58.585
α (°)	90.0	93.39	93.29
β (°)	108.3	105.74	113.82
γ (°)	90.0	97.22	95.98
Cell volume (Å^3)	1,121,944	292,211	275,188
Dimer/asymmetric unit	2	1	1
Specific volume (Å^3/Da)	2.2	2.3	2.1
Diffraction limit (Å)	2.5	1.9	1.9

For types I and II, crystal data have also been given in [3].

Fig. 1) fractions, respectively, was used for sequence analysis after carboxymethylation.

Both types of material were treated with the Lys-specific protease and the two digests were separated by reverse phase HPLC. Peptide separations obtained, reveal identical patterns (Fig. 2). All peptide material from the monomeric pool was used for amino acid sequence analysis, and most of the structures obtained were also checked by corresponding analysis of the dimeric pool. In no case were sequence differences found. This fact, together with the identical patterns (Fig. 2) and the crystal conversions (Table I) suggests that the two fractions have identical subunits. By similar separations of additional digests, using Glu-specific and Asp-specific proteases, as well as CNBr, overlapping fragments were recovered from all parts of the protein molecule, establishing an amino acid sequence consisting of 534 residues (Fig. 3). The primary structure is identical with that indirectly deduced from a corresponding nucleotide sequence (Lip 3 in [6]), except for a difference at one position, where we find Leu at position 350 instead of Ile [6]. Apparently, this corresponds to a microheterogeneity of conventional type between different strains.

Fig. 3. Primary structure of cholesterol esterase from *Candida cylindracea*, together with a summary of analytical results for all peptides investigated. Peptide nomenclature: first letter (M or D) denotes origin from the monomeric and dimeric preparations, respectively; second letter denotes cleavage method, K for the Lys-C protease, E for the Glu-C protease, D for the N-Asp protease, and M for CNBr. Parts of peptides analyzed by sequencer degradations are indicated by solid lines, remaining parts, analyzed by total compositions only, by broken lines. The Ser-His-Glu catalytic triad is indicated by arrows, the four highly conserved Cys residues by asterisks, the microheterogeneity toward a DNA report [6] by double underlining, and the residues corresponding to CTG codons in the DNA (usually coding for Leu) but in *Candida* expected to code for Ser [4,7] and now also directly proven to do so at all 15 positions by single underlining.

4. Discussion

4.1. Monomeric and dimeric forms

As shown in Fig. 1, cholesterol esterase of *Candida cylindracea* separates into two fractions upon exclusion chromatography, corresponding to dimeric and monomeric forms respectively. They crystallize differently, the monomer as monoclinic crystals in the absence of substrate, or as triclinic crystals in the presence of 0.05 mM cholesterol linoleate, whereas the dimer gives triclinic crystals (Table I). Although the two triclinic crystal forms as obtained from the dimeric fraction and, in the presence of linoleate, from the monomeric fraction are non-isomorphous with regard to the diffraction intensities (Ghosh, D., unpublished). They differ slightly in unit cell parameters (Table I), the change of the monomer form from the monoclinic to the triclinic crystal type upon addition of substrate, suggests that apparently different quaternary structures constitute reverseable differences, most likely associated with the presence of the substrate, which converts the enzyme into the active, dimeric form. This conclusion is supported further by the fingerprinting analysis (Fig. 2) which shows that the two fractions give identical elusion patterns of their Lys-cleaved products. Furthermore, sequence analysis of most fractions gave identical results. Combined, the data show that the monomeric and dimeric forms are derived from the same primary structure and explained by shifts in the equilibrium caused by the presence of the substrate, which promotes formation of the fully active, dimeric enzyme form.

4.2. Primary structure

In addition to the peptides from the Lys-C protease digests mentioned above, further digests with Glu-C protease, N-Asp protease, and CNBr were similarly analyzed. Combined, they establish the entire structure of the protein by peptide analysis. As stated, it is based on analysis of both enzyme forms, with emphasis on the peptide from originally monomeric enzyme (the redissolved type III crystals). The structure obtained (Fig. 3) has the same size as the other gene products of the multiple genes for lipases from this organism, all giving 534 residue polypeptide chains [6], and confirming at the peptide level, the primary structure deduced from the corresponding cDNA structure. It also proves one residue difference, at position 350 which is now found to be Leu instead of Ile. Such a difference is fully compatible with strain differences, but establishes one further microheterogeneity in this multigene enzyme family. Finally, the present analysis is important in establishing that all the CTG positions in the gene are really occupied by Ser (as expected in *Candida* [4]) and not by Leu (which would be the universal alternative from that codon) as shown in Fig. 3.

The structure obtained is fully compatible with all previous conclusions, showing a Ser-His-Glu catalytic triad [7,11–16] at positions 209, 449, and 341 (Fig. 3), four conserved Cys residues at positions 60, 97, 268, and 277, corresponding to disulphide bridges in the tertiary structures of the model enzymes [11–16], and overall residue similarities toward the four other cholesterol esterases from *Candida cylindracea* at about the 80% level [7].

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