Biochem. J. (1991) 280, 51–55 (Printed in Great Britain)

Comparison of the domain-level organization of starch hydrolases and related enzymes

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Structure-prediction and hydrophobic-cluster analysis of several starch hydrolases and related enzymes indicated the organization of eleven domain types. Most enzymes possess a catalytic $(\beta/\alpha)_8$-barrel and a smaller *C*-terminal domain as seen in crystal structures of α-amylase and cyclodextrin glucanotransferase. Some also have a starch-granule-binding domain. Enzymes breaking or forming endo-α-1,6 linkages contain domains *N*-terminal to the $(\beta/\alpha)_8$-barrel.

INTRODUCTION

The structures of two α-amylases (EC 3.2.1.1) and two cyclodextrin glucanotransferases (CGTase, EC 2.4.1.19) are known from X-ray crystallography [1–5]. All four enzymes contain an *N*-terminal $(\beta/\alpha)_8$-barrel catalytic domain (domain A) followed by a domain consisting of β-strands folded in a Greek-key motif (domain C). A smaller (domain B) is inserted as a loop between the third β-strand and third helix of the $(\beta/\alpha)_8$-barrel. The CGTases have in addition a domain D with β-strands arranged in an immunoglobulin-type fold and a domain E of a different β-strand fold motif. Sequence comparison [6,7] and prediction of secondary structure [8–11] have recently led to the conclusion that other α-amylases and CGTases, as well as a maltase (EC 3.2.1.20) and an oligo-1,6-glucosidase (EC 3.2.1.10), also contain an A- and a B-domain. Furthermore, we have used regional sequence homology to predict the presence of an E-domain in most CGTases, a maltotetraohydrolase (EC 3.2.1.60), a maltogenic α-amylase (EC 3.2.1.-), two microbial α-amylases of high sequence identity, a bacterial β-amylase (EC 3.2.1.2) and two fungal glucoamylases (EC 3.2.1.3) [12]. It has been demonstrated for the glucoamylases that domain E is involved in binding to raw starch [13,14]. Although the β-amylases show no sequence similarity to the rest of the α-amylase superfamily members, new crystallographic data on soybean β-amylase reveal that it is in fact a $(\beta/\alpha)_8$-barrel protein, but this barrel differs from that in α-amlyases and CGTases [15].

Here we have used suitable sequence-comparison and structure-prediction procedures to investigate starch hydrolases and related enzymes representing nine enzyme classes not hitherto examined, and find new examples of domain types A–D. These enzymes are also shown to share other types of structural domains.

METHODS

The present investigations explore published amino acid sequences for *Streptomyces limosus* α-amylase [16], *Pseudomonas saccharophila* maltotetraohydrolase [17], *Bacillus stearothermophilus* maltogenic α-amylase [18], a *Bacillus* species CGTase [19], *Streptococcus mutans* dextran glucosidase (EC 3.2.1.70) [20], *Pseudomonas amyloderamosa* isoamylase (EC 3.2.1.68) [21], *Escherichia coli* branching enzyme (EC 2.4.1.18) [22], pullulanases (EC 3.2.1.41) from *B. stearothermophilus* [23] and *Klebsiella aerogenes* [24], *B. stearothermophilus* neopullulanase (EC 3.2.1.-) [25], an α-amylase–pullulanase from *Clostridium thermohydrosulfuricum* (EC 3.2.1.1/41) [26], a maltopentaose-producing α-amylase from an alkaliphilic Gram-positive bacterium (EC 3.2.1.-) [27] and β-amylases from *Bacillus polymyxa* [28,29] and *Clostridium thermosulfurogenes* [30]. Comparisons were made with the α-amylases of *Aspergillus oryzae* and porcine pancreas, the CGTase of *Bacillus circulans*, and soybean β-amylase, for which the crystal structures have been determined [1–4,15] and with a *Saccharomyces cerevisiae* maltase and *Bacillus cereus* oligo-1,6-glucosidase, for which the secondary structures have been reported [9,11].

Secondary-structure prediction was performed using a combination of the procedures of Garnier *et al.* [31] and Cid *et al.* [32] as described by MacGregor [8] specifically for the α-amylase barrel-domain supersecondary structure. Hydrophobic-cluster analysis (HCA) was carried out as described previously [33,34]. The HCA patterns were compared with each other and with those previously reported for selected α-amylases [10]. For pairwise alignments of amino acid sequences the program ALIGN version 2.1 of the Protein Identification Resource [35] was applied. Alignment of more than two sequences involved manual adjustment of pairwise aligned sequences. Use of these procedures in conjunction with comparison to two α-amylases of known crystal structure [1,2] allowed definition of the $(\beta/\alpha)_8$-barrel domain, including its structural elements, in the sequences examined. Additional domains were defined on the basis of (i) pattern similarity to one of the five structural domains identified by protein crystallography in the α-amylases [1,2] or the CGTases [3–5], (ii) the existence of either general or regional sequence similarity or (iii) the occurrence of sequence repeats. Spacer regions connecting domains were apparent either from the nature of the amino acid sequence or from features of the HCA plot.

RESULTS AND DISCUSSION

The $(\beta/\alpha)_8$-barrel domain

In nine types of starch- or dextran-processing enzymes not previously examined, i.e. the maltogenic α-amylase, maltotetraohydrolase, dextran glucosidase, isoamylase, branching enzyme, pullulanase, neopullulanase, α-amylase–pullulanase and maltopentaose-producing α-amylase, the combination employed here of secondary-structure prediction [8] and HCA indicates the presence of an A-type $(\beta/\alpha)_8$-barrel catalytic domain. The locations of these domains are outlined in Fig. 1. In spite of the low sequence similarity, these $(\beta/\alpha)_8$-barrels are believed to be

Abbreviations used: CGTase, cyclodextrin glucanotransferase; HCA, hydrophobic-cluster analysis.

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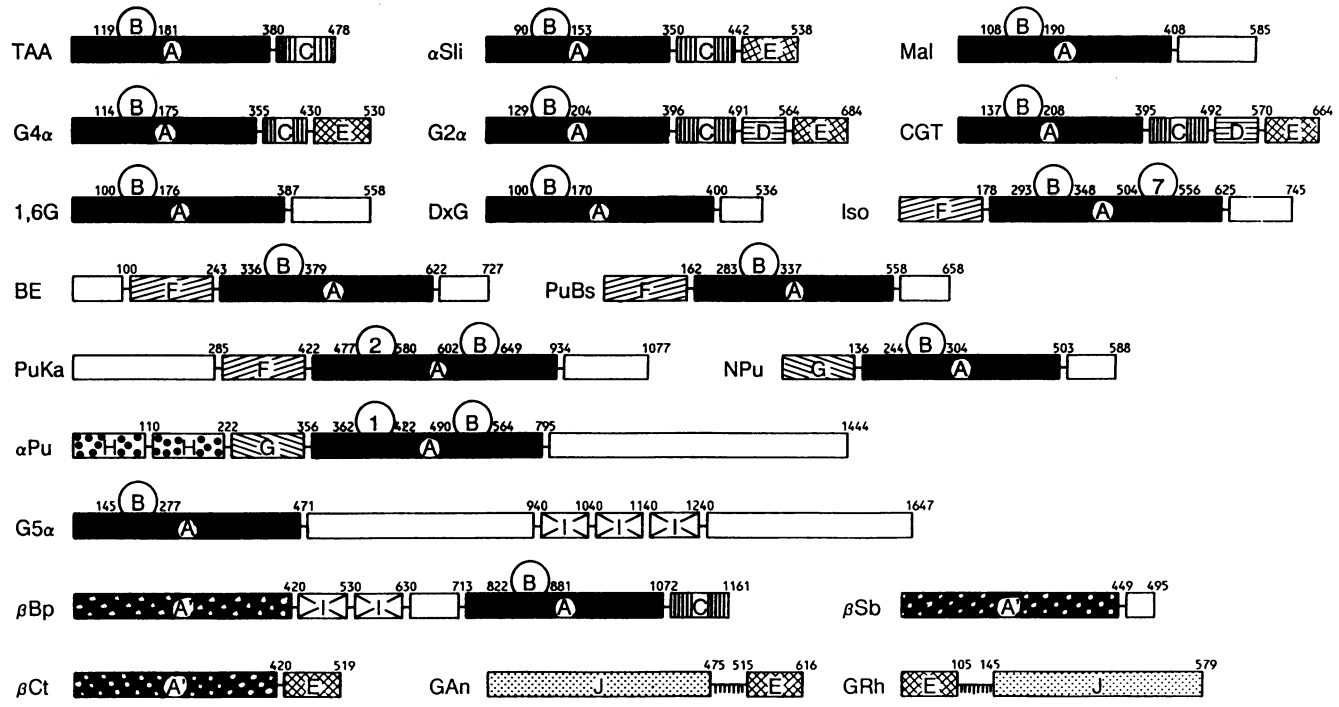

Fig. 1. Domain-level architecture in starch-degrading and related enzymes

The domain organizations of *A. oryzae* α-amylase, *B. circulans* CGTase and soybean β-amylase are taken from the crystal structures [1,3,4,15]. Structures for maltase, oligo-1,6-glucosidase and glucoamylases have been reported previously [9,11,12], but are included here for comparison. The organization proposed here for other enzymes is based on structure predictions performed in the present study and sequence similarity. Loops longer than 50 residues within the $(\beta/\alpha)_8$-barrel are indicated by B (loop 3) or loop number (see the text). White areas indicate segments in which no similarity to other sequences has been found; ⊓⊓⊓⊓⊓⊓⊓⊓ indicates heavily glycosylated 'hinge' regions in the glucoamylases. Numbering is from the *N*-terminus of the mature enzyme. See the text for definition of domains A–I. Domain J represents the catalytic domain of glucoamylases. Key to enzyme abbreviations: TAA, α-amylase, *A. oryzae* (Taka-amylase A) [1,46]; αSli, α-amylase, *Strep. limosus* [16]; Mal, maltase, *S. cerevisiae* [47]; G4α, maltotetraohydrolase, *Ps. saccharophila* [17]; G2a, maltogenic α-amylase, *B. stearothermophilus* [18]; CGT, cyclodextrin glucanotransferase, *Bacillus* species [3,4,19]; 1,6G, oligo-1,6-glucosidase, *B. cereus* [11]; DxG, dextran glucosidase, *Strep. mutans* [20]; Iso, isoamylase, *Ps. amyloderamosa* [21]; BE, branching enzyme, *E. coli* [22]; PuBs, pullulanase, *B. stearothermophilus* [23]; PuKa, pullulanase, *K. aerogenes* [24]; NPu, neopullulanase, *B. stearothermophilus* [25]; αPu, α-amylase–pullulanase, *C. thermohydrosulfuricum* [26]; G5α, maltopentaose-producing amylase, alkaliphilic Gram-positive bacterium [27]; βBp, β-amylase, *B. polymyxa* [28,29]; βSb, β-amylase, soybean [48]; βCt, β-amylase, *C. thermosulfurogenes* [30]; GAn, glucoamylase, *A. niger* [49]; GRh, glucoamylase, *R. oryzae* [50].

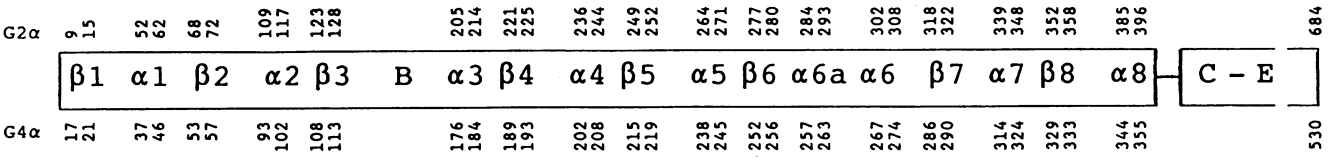

Fig. 2. Details of the $(\beta/\alpha)_8$-barrel predictions for two exo-α-amylases

The predictions were performed the using methods described previously [8]. β1–β8 and α1–α8 denote β-strands 1–8 and α-helices 1–8 respectively. α6a denotes the extra helix located between β6 and α6. Domain B is located between β3 and α3. Uncertainty in positions is ± two residues.

the same type as has previously been either found experimentally or predicted in CGTases [3–5,9], maltase [9], endo-acting α-amylases [1,2,8,10] and oligo-1,6-glucosidase [11]. As examples from the present study the secondary-structural elements of the A-domains are shown for two exo-α-amylases (Fig. 2), and the HCA analysis for the entire sequence of maltotetraohydrolase is provided in Fig. 3.

Recently, a soybean β-amylase was shown by crystallography to fold as a $(\beta/\alpha)_8$-barrel, different from the α-amylase-type $(\beta/\alpha)_8$-barrel [15]. This β-amylase-type barrel (A^1-domain) should also exist in the β-amylases from other higher plants as well as from *C. thermosulfurogenes* and *B. polymyxa* (Fig. 1), given the high sequence similarity [36]. The gene product from *B. polymyxa* β-amylase is unusual in that the translated protein contains both an α-amylase and a β-amylase. The α-amylase moiety is found here to have the characteristic A-, B- and C-domains (Fig. 1).

The loop domains of the $(\beta/\alpha)_8$-barrel

A smaller structural domain (designated B in Figs. 1–3) is inserted between β-strand 3 and helix 3 in the structural subfamily of α/β-barrel proteins that contains α-amylase and CGTase [8,37]. The B-domains predicted here for all of the enzymes examined range in length from 44 to 133 amino acid residues (Fig. 1), compared with 60, 63 and 75 residues in the three reported crystal structures [1,2,4]. In the α-amylases, the B-

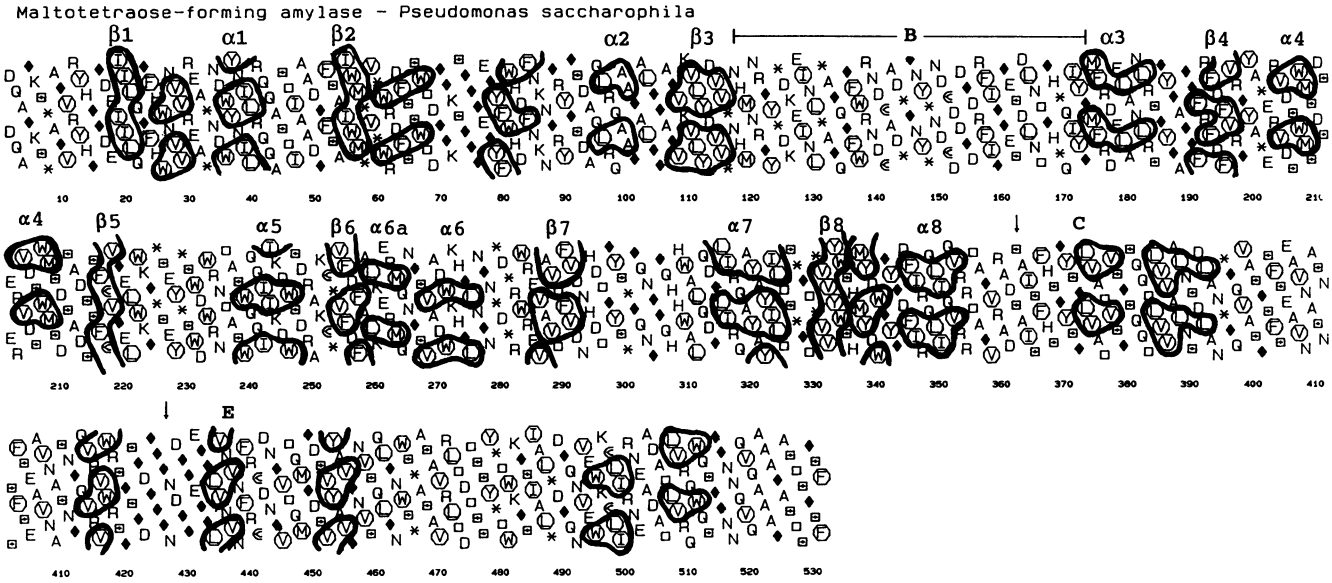

Fig. 3. Hydrophobic cluster analysis of the maltotetraohydrolase from *Ps. saccharophila*

The secondary-structural elements of the $(\beta/\alpha)_8$-barrel, the position of the B-domain and the domain transitions A–C and C–E (arrows) are indicated.

Fig. 4. Regional alignment of segments *N*-terminal to the $(\beta/\alpha)_8$-barrel

(*a*) Four enzymes acting on branched substrates, i.e. isoamylase (Iso), *Klebsiella* pullulanase (PuKa), *B. stearothermophilus* pullulanase (PuBs) and branching enzyme (BE). The definition of domain F in Fig. 1 is based on this alignment. (*b*) Segments from neopullulanase (NPu) and α-amylase–pullulanase (αPu). These constitute domain G of Fig. 1. (*c*) Internal repeats of α-amylase–pullulanase (αPu). Each repeat is domain H of Fig. 1. Amino acid sequences are given in the single-letter code. Residues from dominant exchange groups [35] are shaded. Residues in the last line of each alignment are conserved in all sequences shown.

domain contributes several residues binding to substrate [1,2] or structural Ca^{2+} [2,38]. It is not known, however, whether all of the enzymes analysed in the present study are Ca^{2+}-binding proteins. The $(\beta/\alpha)_8$-barrels predicted here for the enzymes that cleave interior α-1,6-glucosidic linkages contain characteristically a second long loop (> 50 amino acid residues) that may constitute an additional structural domain (Fig. 1). This protrudes from the barrel scaffold after the first β-strand in α-amylase–pullulanase, the second β-strand in the pullulanase from *K. aerogenes* and the seventh β-strand in isoamylase. Among the 17 α/β-barrel enzyme classes seen by crystallography, structural inserts have been identified after β-strands, 1,2,3 and 4, but not yet 7 [37].

The C-, D- and E-domains

The available crystal structures of α-amylases and CGTases show that the $(\beta/\alpha)_8$-barrel is succeeded by a C-domain, approx. 100 amino acid residues long and folded as an antiparallel β-sandwich [1–5]. Although all sequences investigated here have a *C*-terminal extension to the predicted $(\alpha/\alpha)_8$-barrel (Fig. 1) with some pairwise resemblance between then (not shown), a con-

sensus could not be defined for these regions. Sequence comparison and HCA analysis can clearly identify C-type domains in maltogenic α-amylase (Fig. 3), maltotetraohydrolase and α-amylases from *Strep. limosus* and *B. polymyxa* (Fig. 1), but not definitively in the enzymes that cleave or form α-1,6 linkages. The function of the C-domain is unknown, although inactivation caused by mutations in domain C of *B. stearothermophilus* α-amylase suggests it is necessary for enzyme activity [39].

In the model of CGTase as determined by X-ray crystallography, a domain with an immunoglobulin fold (domain D) follows the C-domain [3–5]. Sequence similarity (not shown) between residues 491–564 in the maltogenic α-amylase and residues 492–570 in CGTase from *Bacillus* sp. strain 1011 supports the idea that a D-domain also exists in the maltogenic α-amylase. However, it has not been recognized in any other enzyme class examined here, and no function has been suggested for this domain.

A putative raw-starch-binding C-terminal sequence (domain E) was found in the tertiary structure of CGTases [3–5], and several side chains have been demonstrated by crystallography to interact with maltose [5]. Domain E has been predicted in a number of other enzymes [12] (Fig. 1). In glucoamylase this domain has been shown to expendable as far as catalytic activity is concerned, but to be essential for binding to starch granules [13,14]. No new examples of domain E have been found here.

Other domains

A variety of α/β-barrels are known to occur in proteins, and in crystal structures of α/β-barrel enzymes from six out of 17 enzyme classes, another domain precedes the barrel [37]. Of the enzymes investigated here for the first time, only those acting in endo-fashion on pullulan and the α-1,6 linkages of branched substrates are predicted to possess domains located *N*-terminal to the $(\beta/\alpha)_8$-barrel (Fig. 1). The local sequence similarity between such *N*-terminal regions of *Pseudomonas* isoamylase and residues 300–325 in *Klebsiella* pullulanase reported previously [21] is here both highly extended in length and expanded to include *N*-terminal regions of *B. stearothermophilus* pullulanase and branching enzyme from *Escherichia coli* (Fig. 4*a*). We suggest, therefore, that a common structural domain is conserved in these four enzymes (labelled F in Fig. 1). In neopullulanase, residues 1–135 show 23% identity with the part of α-amylase–pullulanase immediately preceding the barrel (Fig. 4*b*). These regions are labelled G (Fig. 1), and pairwise comparisons between the different F- and G-regions further reveal a distant sequence similarity between *Klebsiella* pullulanase residues 280–376 and neopullulanase residues 2–130. The possibility exists, therefore, that the F- and G-regions are in fact variants of the same domain type. The *N*-terminal region of α-amylase–pullulanase consists of an internal duplication at residues 8–90 and 110–196, with 38% residue identity (Fig. 4*c*). This region (H in Fig. 1) is assumed to fold as two domains.

Enzymes having very long polypeptide chains, including the maltopentaose-producing α-amylase, α-amylase–pullulanase, the *Klebsiella* pullulanase and the *B. polymyxa* β-amylase–α-amylase-gene product, all possess regions not recognized in any of the enzymes by the present techniques. Although three segments of 6 or 7 residues of α-amylase–pullulanase succeeding the $(\alpha/\alpha)_8$-barrel have a reported similarity to sequences of α-amylases or debranching enzymes [26], no overall relationship to any other segment has been detected by us for this region. HCA and structure prediction easily identified the A-, B- and C-domains in the *N*-terminal region of the extremely long maltopentaose-producing amylase. These are followed first by a region of apparently unique structure, and next by three repeats of a 100-residue segment designated 'domain I' (Fig. 1) having 62–67% internal identity. This I-domain recurs in tandem with 92% internal identity in the *B. polymyxa* β-amylase–α-amylase. In this protein the two I-domains link an *N*-terminal β-amylase domain to the α-amylase moiety. Post-translational proteolytic cleavage in the I-domain region yields the separate β- and α-amylase functions [28]. No comparable processing has been reported, however, in the I-domain of the maltopentaose-producing amylase.

Distantly related enzymes

The fungal glucoamylases contain an essential substrate binding sequence [7,40] also found in several members of the α-amylase family in the loop following the second β-strand [1,4]. However, an α-amylase-type $(\beta/\alpha)_8$-barrel catalytic domain is not predicted for the glucoamylases (Fig. 1). In addition, several homologous α-glucosidases from mammals [41,42], yeast [43] and *Aspergillus* [44] and a bacterial amylomaltase [45] contain a short postulated active-site region recognized in the other enzymes [7], apparently without containing any of the A–I-type domains.

Conclusion

The domain-level organization for nine different types of starch hydrolases and related enzymes is determined here. A $(\beta/\alpha)_8$-barrel (A- plus B-domain) is thus presented for the first time for exo-α-amylases and several enzymes acting on α-1,6 glucosidic bonds. In addition to the A–B domain barrel-fold, seven other domain types are proposed here, of which only domain E, a starch-binding region, has a known function. It is unlikely that models of the larger multidomain proteins will be obtained by X-ray crystallography in the near future, so $(\beta/\alpha)_8$-barrel sequence alignments in conjunction with the present proposed domain organizations can provide a basis for further understanding the relationships between structure and function in these enzymes. For example, some of the different domains indicated in Fig. 1 may play a role in the enzyme specificity, and the structural organization outlined here may guide attempts to identify domain function through either protein engineering or limited proteolysis.

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Received 12 April 1991/16 August 1991; accepted 28 August 1991