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New conserved amino acid region of α-amylases in the third loop of their $(\beta/\alpha)_8$-barrel domains

The basic structural features of α-amylase (EC 3.2.1.1) are known [1,2] together with the three-dimensional structures of the enzyme from *Aspergillus oryzae* [3], porcine pancreas [4] and *Aspergillus niger* [5,6]. Probably each α-amylase, independently of its origin, contains an eightfolded $(\beta/\alpha)_8$-supersecondary structure [1], i.e. a barrel of eight parallel β-strands surrounded by eight α-helices. Although all α-amylases possess the same catalytic function, their amino acid sequences are quite varying. A search for similarity among them may reveal structural dependencies of activity or stability as well as an evolutionary tree. These investigations are promoted by the availability of about 50 known amino acid sequences [7].

Based on comparison of eleven different α-amylases [8] generally four stretches with high degree of similarity are accepted as conserved regions [9]. Three of them have been demonstrated earlier [10]. The present communication brings the new conserved region identified by multiple alignment of amino acid sequences of α-amylases from microbial, plant and animal origins (Fig. 1). The sequences (taken from the literature) have been either determined by direct amino acid sequencing for *Aspergillus oryzae* [11] and porcine pancreatic [12] α-amylases or deduced from nucleotide sequences for α-amylases from barley [13], *Bacillus subtilis* [14], *Bacillus stearothermophilus* [15], *Drosophila melanogaster* [16], *Streptomyces limosus* [17], and *Clostridium thermosulfurogenes* [18]. This set of amino acid sequences was constructed on the basis of low sequence similarities, i.e. only one example of each of the groups with high degree of similarity (*Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus stearothermophilus* [19], mammalian α-amylases [8], *Saccharomycopsis fibuligera* and *Aspergillus oryzae* [20], *Aspergillus niger* and *Aspergillus oryzae* [6]) was considered here. Amino acid alignment was made using the program CLUSTAL V [21] involving manual adjustments in order to get the four conserved regions already demonstrated [1,8] and, if possible, further sequence similarities. Since X-ray crystallography [3,4] and secondary structure predictions [1,2] have indicated that approximately 400 amino acid residues of the *N*-terminal regions of α-amylases are folded into the $(\beta/\alpha)_8$-barrel domain containing the active site, only the first 420 amino acids from the *N*-terminal ends were aligned in this study.

Single amino acids (except for those from conserved regions) common to all aligned α-amylases (positions 24, 42, 56, 64, 82, 95, 125, 242, 350, and 369 in the enzyme of *A. oryzae*) are not conserved in all available α-amylase sequences but at these points some sequence similarity could be found most easily. Based on this assumption, four such stretches were recognized (56–64, 173–177, 350–353, and 367–371 in *A. oryzae* α-amylase), the region 173–177 (LPDLD in *A. oryzae*, Fig. 1) being identified as very similar in the amino acid sequences of α-amylases of various origins. This stretch of amino acids is located near the *C*-terminal end of the remarkably long third loop region joining the third β-strand of the α-amylase $(\beta/\alpha)_8$-barrel domain to the third α-helix [1,2,4]. It has been shown for all $(\beta/\alpha)_8$-barrel enzymes [22] that most of the amino acid residues involved in different functions are localized in loops of their $(\beta/\alpha)_8$-barrels. Indeed, the aspartate residue common to all α-amylases in this region is involved in the co-ordination of the Ca^{2+} ion in all three-dimensional α-amylase structures solved to date [4,5,23]. For these reasons the region with high degree of similarity in Fig. 1 is proposed to be the fifth conserved region in all α-amylases.

It should be pointed out, however, that only further studies on α-amylase amino acid sequences, in conjunction with determination of their three-dimensional structures [24–26], and with elucidation of the other $(\beta/\alpha)_8$-barrel enzymes [22,27], can shed more light on the relationships between structure and activity or stability as well as on evolution of the α-amylase enzyme family.

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AOR	*152*	IQNYEDQTQVEDCWLGD---NTVS	LPDLD	TTK-DVV---KNEWYDW	*190*
BAR	*128*	DTKYSDGTA----NLDTGA-DFAA	APDID	HLNDRVQRELKE-WLLW	*167*
BST	*177*	KRFGIGKAWDWEVDTENGNYDYLM	YADLD	MDHPEVVTELKS-WGKW	*221*
BSU	*127*	IKNWSDRWDVTQN--------SLLG	LYDWN	TQNTQVQSYLKRFLDRA	*165*
CTH	*182*	FSSYEDG--------IYR---NLFD	LADLN	QQNSTIDSYLKSAIKVW	*217*
DME	*137*	ISNYNDANEVRNC--------ELVG	LRDLN	QGN----SYVQDKVVEF	*171*
PPA	*148*	IESYNDPYQVRDC--------QLVG	LLDLA	LEK----DYVRSMIADY	*182*
SLI	*128*	INDYGNRANVQNC--------ELVG	LADLD	TGE----SYVRDRIAAY	*162*
			*		

Fig. 1. The fifth conserved region in amino acid sequences of α-amylases

Amino acids are shown in the single-letter code. Enzyme sources are abbreviated as: *AOR*, *Aspergillus oryzae*; *BAR*, barley; *BST*, *Bacillus stearothermophilus*; *BSU*, *Bacillus subtilis*; *CTH*, *Clostridium thermosulfurogenes*; *DME*, *Drosophila melanogaster*; *PPA*, porcine pancrease; *SLI*, *Streptomyces limosus*. Conserved region is boxed. Gaps are indicated by dashes. The asterisk signifies the conserved aspartate residue involved in the binding of calcium ion.

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What form of cytochrome *c* oxidase reacts with oxygen *in vivo*?

The nature of the immediate donor of reducing equivalents to molecular oxygen has been a puzzle for many years. It had appeared to be resolved in favour of a single haem a species, termed cytochrome a_3 by Keilin & Hartree [1], following their abandonment of the 'copper enzyme' model [2] in favour of a version of Warburg's active haem species [3]. Discovery of more than one prosthetic metal in the terminal oxidase (see Wikström *et al.* [4] for review) led to reappraisals of this simple classical picture. More elaborate models involved several centres in O_2 reduction, working in a concerted way. The most complex [5] required that at least three and probably all four metal groups participate in transferring four electrons to an O_2 molecule initially bound at or close to the cytochrome a_3 haem. But a recent consensus seems to have emerged in which the species reacting with oxygen *in vivo* is the doubly reduced binuclear cytochrome a_3Cu_B centre. Thus Sarti *et al.* [6,7] use a 'bipolar' model in which formation of the oxygen-reactive species involves transfer of two electrons from Cu_A and cytochrome *a* to the binuclear centre, to give a 'mixed valence' form in which the binuclear centre is fully reduced and cytochrome *a* and Cu_A are both oxidized. This is based upon the well-established fact that carbon monoxide binding requires reduction of both cytochrome a_3 and Cu_B [4]. Yet the redox states of the other components in the O_2-sensitive form are not settled by this finding, as CO is not an electron acceptor. Indeed, it acts as a weak electron donor to the binuclear centre. And Babcock & Wikström [8], in their recent lucid and magisterial review of cytochrome oxidase activity in the cell, allowed themselves a similar rather counter-intuitive model of the oxygen-utilizing reaction step, in which the fully reduced enzyme plays no part in the physiological reaction and the role of cytochrome *a*, the first component of the enzyme complex to be discovered [9], is diminished to one of electron transfer to the actual oxygen-reactive centre.

One of the major problems with this enzyme has been to decipher the concerted and co-operative way in which electrons are transferred and coupled to proton movement across the membrane in which it is embedded. Cytochromes *a* and a_3 are located upon the same subunit, contain the same prosthetic group, and interact strongly both spectrophotometrically and in chemical reactivity [4,8]. It is hard to believe that they will not both be implicated in the key oxygen-utilizing and energy-conserving steps.

It is now accepted that a copper atom (Cu_A) located upon the other redox subunit, subunit II, is the immediate electron acceptor in the eukaryotic enzyme from cytochrome *c* and provides the gateway to the centres in subunit I [10]. But the behaviour of cytochrome *a* has long been a puzzle [8]. Its classical distinction from cytochrome a_3 depended upon its reduction under conditions in which cytochrome a_3 is oxidized but liganded and electron transfer to oxygen is slow [1]. When electron transfer is initiated with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine in the absence of cytochrome *c* the flux depends upon the steady state reduction level of cytochrome *a*, once a small fraction of cytochrome *a* associated with unreactive enzyme has been subtracted [11]. Under these conditions the cytochrome, rather than Cu_A, may be the electron entry point. But in the presence of cytochrome *c*, cytochrome *a* behaves differently, both in the isolated enzyme and in cytochrome *c* oxidase-containing vesicles. Its steady state is especially sensitive to ΔpH and rather insensitive to $\Delta\psi$ [12,13], and although turnover is always almost directly proportional to the level of reduced cytochrome *c*, the reduction level of cytochrome *a* does not change significantly as flux changes. This is seen when controlled respiration is released by addition of ionophores [13] or when steady state flux is increased by raising the reductant concentration and hence the percent reduction of cytochrome *c* [14]. Cytochrome *a* can be monitored equally well at 605 nm and at 445 nm [13,14] and its redox changes are thus distinguished from events at the binuclear centre, including apparent spin state and related changes during the steady state. As respiration rates do not track cytochrome *a* reduction, it is doubtful whether the rate-limiting step in the ordinary reaction of the enzyme involves electron transfer from this component. It follows that at least one potentially rate-limiting electron transfer event to the binuclear centre may be from a component that more closely tracks the cytochrome *c* redox level than cytochrome *a*. That component is likely to be Cu_A.

The binuclear centre can be reduced in two steps from the ground (ferric/cupric) state. If this happens before cytochrome *a* itself is reduced, although the O_2 reaction takes place at the same rate as with fully reduced enzyme [15], the following electron transfer steps are much slower than in the presence of reduced cytochrome *a*. The oxygenated complex of the enzyme (Com-