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Calcium Binding in α-Amylases: An X-ray Diffraction Study at 2.1-Å Resolution of Two Enzymes from *Aspergillus*[†,‡]

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ABSTRACT: X-ray diffraction analysis (at 2.1-Å resolution) of an acid α-amylase from *Aspergillus niger* allowed a detailed description of the stereochemistry of the calcium-binding sites. The primary site (which is essential in maintaining proper folding around the active site) contains a tightly bound Ca^{2+} with an unusually high number of eight ligands (Oδ1 and Oδ2 of Asp175, Oδ of Asn121, main-chain carbonyl oxygens of Glu162 and Glu210, and three water molecules). A secondary binding site was identified at the bottom of the substrate binding cleft; it involves the residues presumed to play a catalytic role (Asp206 and Glu230). This explains the inhibitory effect of calcium observed at higher concentrations. Neutral *Aspergillus oryzae* (TAKA) α-amylase was also refined in a new crystal at 2.1-Å resolution. The structure of this homologous (over 80%) enzyme and additional kinetic studies support all the structural conclusions regarding both calcium-binding sites.

Calcium (Ca^{2+}) ions are bound by a wide variety of intra- and extracellular proteins, including enzymes and structural, transport, and trigger proteins. Their binding affinities are commonly in the range 10^5-10^8 M^{-1}. No unifying structural basis for this broad range has been established.

Ca^{2+} binding is an important biochemical phenomenon with many widespread consequences. One of the reasons why Ca^{2+} has been so extensively utilized by nature may be the variability of its coordination. In spite of its preference toward octahedral coordination, up to nine ligands can be found in crystalline complexes involving calcium–carboxylate and calcium–water interactions (Einspahr & Bugg, 1980, 1981); this variability makes the regulation of the binding affinity an easier task. A complete structural description of these mechanisms is necessary to understand the ways in which proteins' activities can be modulated by Ca^{2+}. It should also be noted that purely structural Ca^{2+} sites contribute substantially to the stability of the proteins. Recent advances in protein engineering have often been directed at obtaining more stable enzymes suitable for industrial use under demanding conditions. The under-

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standing of how exactly thermostability is achieved through metal binding would very likely help in efforts to design new improved enzymes.

Calcium has been implicated in the regulation of numerous intracellular processes, which necessitates precise control of Ca^{2+} concentrations. A number of functional Ca^{2+}-binding proteins have now been characterized (Kretsinger & Nockolds, 1973; Szebenyi et al., 1981; Szebenyi & Moffat, 1986; Babu et al., 1985, 1988; Herzberg & James, 1988; Satyshur et al., 1988) and the crystallographic structure determinations have revealed a common structural Ca^{2+}-binding motif in these proteins—the EF hand (Kretsinger & Nockolds, 1973; Herzberg & James, 1985a,b). A number of other proteins have also been found to contain bound calcium in a structural role. In concanavalin A, for example, Ca^{2+} binding orients adjacent side chains in a way suitable for Mg^{2+} binding (Derewenda et al., 1989), which in turn seems to organize the structure of the saccharide binding pocket. Ca^{2+} was also found in a bacterial galactose binding protein (Vyas et al., 1987). Although there is no evolutionary relationship between this periplasmic protein and the EF hand type eukaryotic proteins, the stereochemistry of the two binding sites is surprisingly similar.

Ca^{2+} is also found in enzymes. It occurs in a large variety of proteinases—trypsin (Bode & Schwager, 1975; Herzberg & James, 1988), subtilisin (Wright et al., 1969; Drenth et al., 1972), thermolysin (Matthews et al., 1974; Holmes & Matthews, 1982), acid proteinase (Suguna et al., 1987), and proteinase K (Betzel et al., 1988)—and in phospholipase A2 (Dijkstra et al., 1981).

A particularly strongly bound Ca^{2+} ion ($K = 2 \times 10^{11}$ M^{-1}) has been identified in amylases (Vallee et al., 1959); it is essential for the enzyme's tertiary structure and catalytic activity. These ubiquitous enzymes hydrolyze α(1,4) D-glycosidic linkages in various glucose polymers and are commonly found in plants, animals, and bacteria. In spite of their potential industrial significance and important biological role, their structure and catalytic mechanism are not yet known in detail. An α-amylase from *Aspergillus oryzae* (TAKA amylase) was studied at 3.0-Å resolution (Matsuura et al., 1984). Its structure was identified as that of the α_8/β_8 barrel, with an additional smaller C-terminal domain folded into a β sandwich. The general location of a single Ca^{2+}-binding site was also proposed. In porcine pancreatic amylase (PPA), initial heavy-atom substitution experiments seemed to indicate the presence of more than one Ca^{2+}-binding site (Payan et al., 1980). However, in the 3.0-Å electron density map (Buisson et al., 1987), no sites other than that analogous to TAKA were found. Pronounced structural similarity between the fungal and the porcine pancreatic α-amylases, despite the evolutionary gap between the two proteins, has also been noted.

High-resolution (2.1-Å) X-ray crystallographic studies of the acid α-amylase from *Aspergillus niger* allowed us to characterize the stereochemistry of the tightly bound Ca^{2+}. We have also identified and characterized a secondary site, which exhibits a unique inhibitory function. Studies of a new crystal form of the *A. oryzae* amylase (also at 2.1-Å resolution) indicate that this description also extends to the TAKA amylase.

Materials and Methods

Enzymes. The acid α-amylase from *A. niger* is a single-chain protein with 484 residues and four disulfide bridges. The isoelectric point is 3.44 and the stability of the enzyme is highest at pH 4–5. The gene encoding the enzyme has been cloned as cDNA and expressed at high levels in *A. oryzae* (H. F. Woldike et al., in preparation). Table I shows the complete polypeptide sequence (based on the DNA sequence). A crude product was obtained by salt precipitation from the culture liquid; subsequent purification was carried out by chromatography on a QAE-Sephadex column equilibrated with 50 mM Tris-HCl (pH 7.6) buffer. The enzyme was eluted with a gradient of NaCl (0–1 M) in the same buffer. Further purification was performed on a mono Q FPLC column equilibrated with 10 mM Bis-Tris (pH 6.0) buffer containing 2 mM $CaCl_2$. The α-amylase was eluted with a gradient of NaCl (0–1 M) in the same buffer.

Table I: cDNA-Derived Amino Acid Sequence of *A. niger* Acid α-Amylase

--------10	--------20	--------30	--------40
LSAAEWRTQS	IYFLLTDRFG	RTDNSTTATC	DTGDQIYCGG
--------50	--------60	--------70	--------80
SWQGIINHLD	YIQGMGFTAI	WISPITEQLP	QDTADGEAYH
--------90	-------100	-------110	-------120
GYWQQKIYDV	NSNFGTADDL	KSLSDALHAR	GMYLMVDVVP
-------130	-------140	-------150	-------160
NHMGYAGNGN	DVDYSVFDPF	DSSSYFHPYC	LITDWDNLTM
-------170	-------180	-------190	-------200
VQDCWEGDTI	VSLPDLNTTE	TAVRTIWYDW	VADLVSNYSV
-------210	-------220	-------230	-------240
DGLRIDSVLE	VEPDFFPGYQ	EAAGVYCVGE	VDNGNPALDC
-------250	-------260	-------270	-------280
PYQKVLDGVL	NYPTYWQLLY	AFESSSGSIS	NLYNMIKSVA
-------290	-------300	-------310	-------320
SDCSDPTLLG	NFIENHDNPR	FASYTSDYSQ	AKNVLSYIFL
-------330	-------340	-------350	-------360
SDGIPIVYAG	EEQHYSGGKV	PYNREATWLS	GYDTSAELYT
-------370	-------380	-------390	-------400
WIATTNAIRK	LAISADSAYI	TYANDAFYTD	SNTIAMRKGT
-------410	-------420	-------430	-------440
SGSQVITVLS	NKGSSGSSYT	LTLSGSGYTS	GTKLIEAYTC
-------450	-------460	-------470	-------480
TSVTVDSSGD	IPVPMASGLP	RVLLPASVVD	SSSLCGGSGR
-------490			
LYVE			

A crude TAKA amylase product (Fungamyl Novo) extensively washed free from low molecular weight substances by ultrafiltration using tap water was further purified on a Q-Sepharose column equilibrated with 20 mM piperazine hydrochloride (pH 6.5) buffer. The bound proteins were eluted with NaCl (0–0.6M) in the same buffer, and the amylase was eluted with a very flat linear gradient around 0.2 M NaCl (30–33% 0.6 M NaCl). Amylase fractions giving only one peak on a TSK 3000 column were pooled, concentrated by ultrafiltration, and washed with 1 mM calcium acetate before lyophilization.

X-ray Crystallography. Crystals of *A. niger* acid α-amylase, suitable for X-ray studies, were obtained from poly(ethylene glycol) solutions (MW 8000). The pH was 3.0–4.0, close to the range of maximum enzymatic activity. The crystals were orthorhombic (space group $C222_1$, $a = 81.1$, $b = 98.3$, and $c = 138.0$ Å) with one molecule per asymmetric unit and

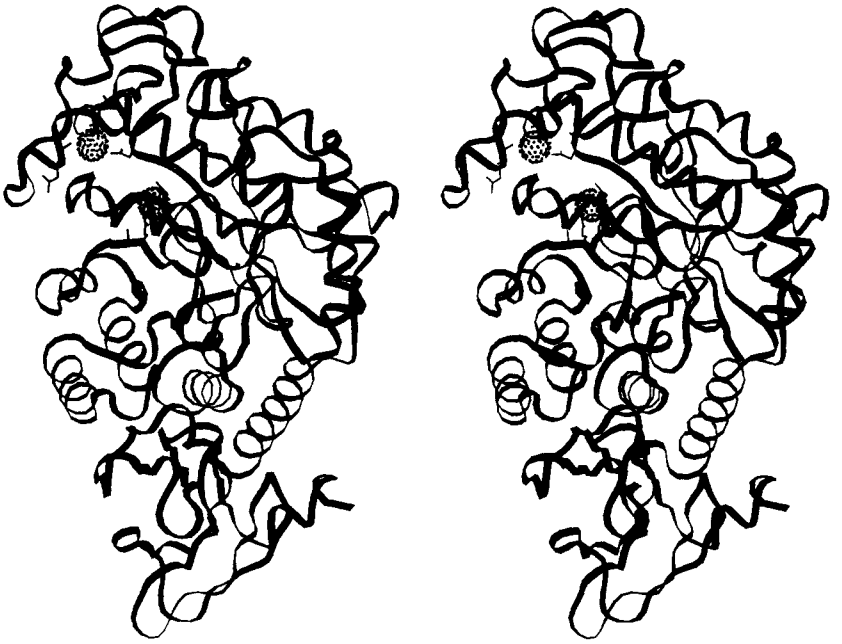

FIGURE 1: Ribbon representation of the polypeptide chain of the amylase molecule. The dotted spheres show the positions of the two calcium sites; residues involved in metal binding are shown in full.

solvent content of approximately 46%. The Siemens/Xentronics area detector system mounted on a conventional source was used to collect native and heavy metal derivative data to 3.0-Å resolution. Details of the operation of this system and of the amylase data collection have been given by Derewenda and Helliwell (1989). High-resolution (2.1-Å) data were recorded on film at the Daresbury synchrotron wiggler station (Helliwell, 1982). The structure was solved by a combination of molecular replacement (using the existing molecular model of TAKA amylase—entry 2TAA in the Protein Data Bank) and multiple isomorphous replacement methods and subsequently refined by a restrained least-squares method (Hendrickson, 1985) to an agreement factor of 16.9%.

Single crystals of TAKA α-amylase used in this study were also obtained in batch from a solution containing poly(ethylene glycol). They were orthorhombic (space group $P2_12_12_1$, a = 50.9, b = 67.2, and c = 132.7 Å); there was one molecule in the asymmetric unit. X-ray diffraction data were collected on film at the Daresbury synchrotron facility (station 9.2) and the Siemens/Xentronics area detector was used to fill in gaps and reflections overloaded on film. The structure of this form of the TAKA amylase was solved by the molecular replacement method using the refined *A. niger* structure as a model; refinement using restrained least-squares procedures as well as molecular dynamics (Brunger et al., 1989) is now almost completed and the current crystallographic R factor is 0.195. Full details of the crystallographic analyses will be published elsewhere.

Results

The atomic coordinates of the *Aspergillus* α-amylases' models are accurate, and typical errors in atomic coordinates in well-resolved regions should not exceed 0.15 Å. Neither the coordination geometry of Ca^{2+} nor the hydrogen bonds were restrained in any way during the refinement. The root mean square deviations from standard protein stereochemistry are small [for bond lenths, 0.028 Å (*A. niger*) and 0.029 Å (TAKA); for planes, 0.022 Å (*A. niger*) and 0.024 Å (TAKA)] and the temperature factors are low [average B factors 13 Å^2 (*A. niger*) and 16 Å^2 (TAKA)]. The two atomic models reveal extensive structural similarities when separate domains are considered; however, we observe a rotation of the C-terminal domain in the *A. oryzae* amylase relative to the *A. niger* enzyme. It is possible that this is a genuine difference between the two enzymes, although crystal packing forces cannot be excluded as a plausible explanation at this stage.

The High-Affinity Ca^{2+} Site. As concluded by Buisson et al. (1987), the essential Ca^{2+}-binding site lies between the main body of the β barrel (domain A) and the smaller domain (B), which forms an extensive loop folding over the proposed active site (Figure 1). By aligning the sequences of the PPA with TAKA, Buisson et al. (1987) proposed that in the latter Asn121, Asp163, Asp175, and His210 form the binding site. With the exception of Asp163 these assignments are correct. In the *Aspergillus* amylases the Ca^{2+} ion is coordinated by eight ligands (Figure 2): the carboxyl oxygens of Asp175 in a bidentate mode (with interatomic distances of 2.59 and 2.79 Å for *A. niger* and 2.77 and 2.80 Å for *A. oryzae*), Oδ of Asn121 (at a distance of 2.57 Å in *A. niger* and 2.52 Å in *A. oryzae*) (in a unidentate fashion), the carbonyl oxygen of Glu162 (2.63 Å in *A. niger*, 2.40 Å in *A. oryzae*), the carbonyl oxygen of Glu210 (2.42 Å in *A. niger*; in *A. oryzae* residue 210 is a His and the corresponding distance is 2.43 Å), and three water molecules (2.55, 2.54, and 2.64 Å from Ca^{2+} in *A. niger*; in *A. oryzae* the corresponding distances are 2.60, 2.69, and 2.35 Å). The discrepancies in bond lengths probably reflect the accuracy of the coordinates rather than genuine variation. Least-squares comparison of the two sites shows great similarity, with an rms difference (calculated for the calcium ions and their ligands only) of 0.24 Å and with a maximum displacement of 0.36 Å (one of the water molecules). The coordination geometry may be regarded as that of a distorted pentagonal bipyramid with the three water molecules, the oxygen of Glu162, and Oδ of Asn121 in a five-membered ring while the apices are formed by the carbonyl of Glu(His)210 and the carboxyl of Asp175 (treated here as a single ligand). There is an extensive hydrogen-bond network involving all participating water molecules, all of which are bonded directly to protein atoms; only one water molecule seems to be accessible from the surface, while the others are buried.

The Secondary Ca^{2+}-Binding Site. As already mentioned,

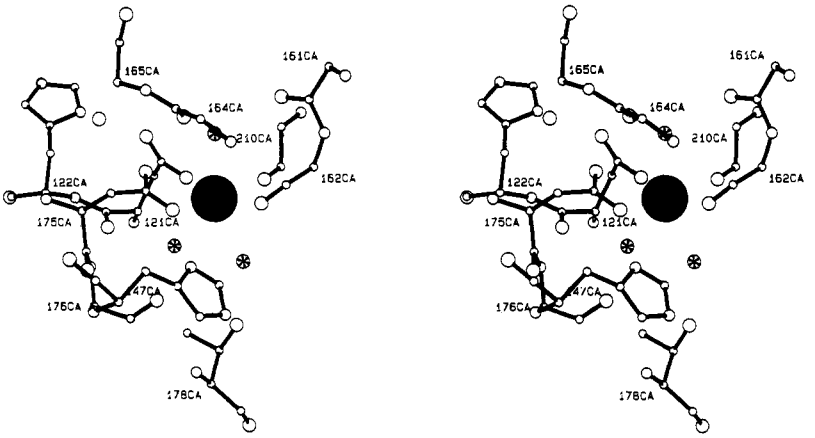

FIGURE 2: The primary calcium-binding site in *A. niger* α-amylase: a stereopair showing the atomic model. Calcium is shown as a large black sphere, and water molecules are marked for clarity. The TAKA binding site is identical within experimental error.

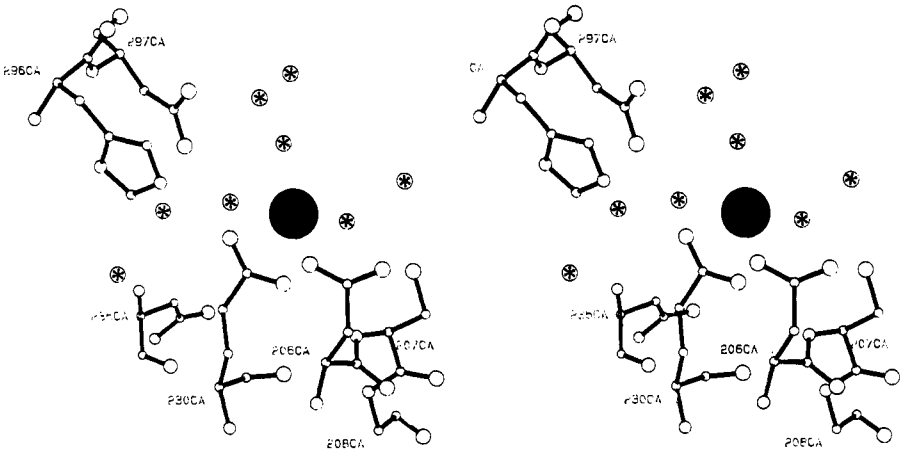

FIGURE 3: The secondary calcium-binding site in *A. niger* α-amylase: a stereoview of the atomic model in the vicinity of the bound calcium ion. Calcium and water are marked as in Figure 2. The same region in TAKA amylase is less well refined, indicating very low occupancy of the calcium ions.

there seemed to be uncertainty regarding the exact number of Ca^{2+} ions in amylase. Our electron density map of *A. niger* amylase originally revealed only one large peak, easily interpretable as Ca^{2+} and coinciding with the main site. However, an analysis of the water molecules identified in the structure showed that one of them was in contact with six other ligands [the carboxyl of Glu230 in a bidentate mode, Asp206 in a unidentate mode, and three water molecules (Figure 3)]. This incomplete distorted octahedral stereochemistry was much more indicative of a metal ion; in addition, residual density was also found in the place of a possible seventh ligand. This site is also the major substitution site for Pb^{2+} and Sm^{2+}.

We find that a 20 mM excess of Ca^{2+} reduces the *A. niger* amylase activity in a reaction mixture containing 6.11×10^{-3} mg/mL enzyme to only 1%. Since Glu230, Asp206, and Asp297 (all close to the peak identified as a possible secondary binding site for Ca^{2+}) have all been implicated in the hydrolysis of the substrate, the reduction of catalytic activity is consistent with the binding of Ca^{2+} at the low-affinity site. To elucidate the action of Ca^{2+} further, its influence on K_M and k_{cat} has been determined by using *p*-nitrophenyl α-D-maltoheptaoside as substrate. Table II shows the results; K_M is influenced to a much higher degree than k_{cat}. Although this firmly supports the conclusion that the secondary Ca^{2+} binding site is a part of or is identical with the active site, the effect of Ca^{2+} on k_{cat} suggests that inhibition by Ca^{2+} is not of a purely competitive type.

In the original structure determination the secondary Ca^{2+}-binding site was clearly partly occupied, since a water molecule with a temperature (*B*) factor of 10 Å^2 was sufficient to account for the density. We have therefore soaked a single crystal of *A. niger* amylase in a 20 mM solution of $CaCl_2$ and collected X-ray data to 2.5-Å resolution. The subsequent difference Fourier calculated with coefficients ($F_{Ca} - F_{native}$) and phased on the refined native structure revealed pronounced features associated with Glu230 and its ligand (Figure 4). We take this result as conclusive evidence for the location of the secondary Ca^{2+} site in amylase.

In the case of the TAKA amylase, there is also a well-ordered water molecule bound to Glu230. Its temperature factor, however, is slightly higher (15 Å^2), and the coordination sphere is less well defined. Our conclusion was that this site has even less affinity than in the *A. niger* enzyme. Kinetic studies (Table III) supported this result. At a 20 mM concentration of calcium the TAKA amylase retains almost 50% of its activity. Nonetheless, the inhibitory effect of surplus calcium is clearly detectable.

Table II: Effect of Ca^{2+} on the K_M and k_{cat} of *A. niger* Acid α-Amylase[a]

$[Ca^{2+}]$ (mM)	K_M (mM)	k_{cat} (s^{-1})
0	1.58	8.69
5	6.81	5.63
10	11.20	5.30

[a] The measurements were done by using a modification of the Boehringer method, catalog no. 568651. Substrate, *p*-nitrophenyl α-D-maltoheptaoside; buffer, 0.1 M acetate, pH 6.0; enzyme concentration, 8.08×10^{-3} mg/mL of reaction mixture; temperature, 25 °C.

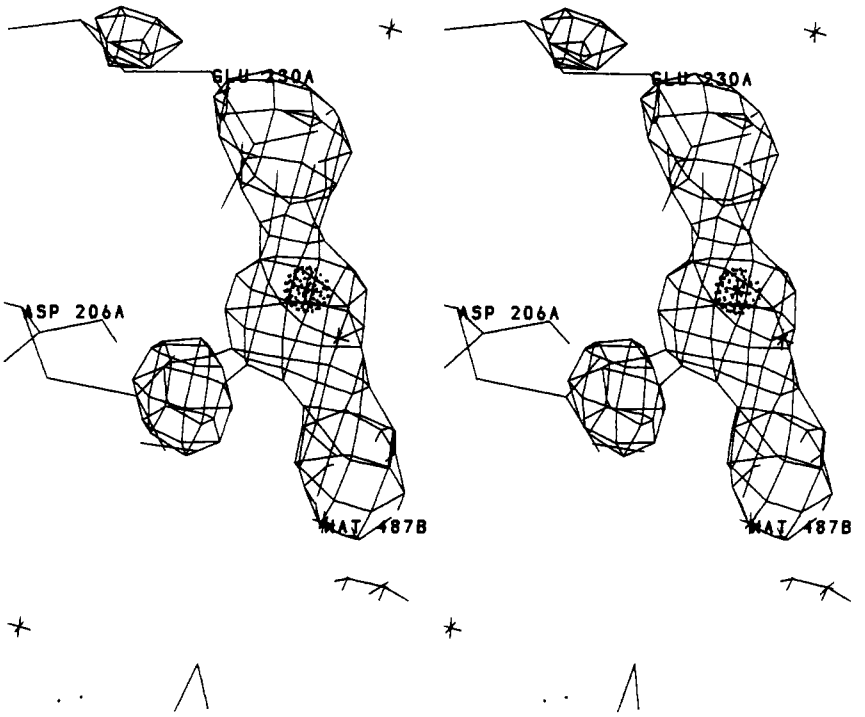

FIGURE 4: Stereoview of the difference electron density ($F_{Ca} - F_{native}$) associated with the secondary calcium-binding site. The calcium position is denoted by the dotted sphere and a skeletal representation of the two acidic side chains involved in metal binding is added. For clarity only one positive contour is shown. The positive features flanking the ligands indicate higher occupancy (in case of water molecules) or the reduction in mobility (lower *B* factors) for the side chains; very small shifts of the side chains have also taken place as judged by the presence of several negative features (not shown here).

Table III: Decrease in Enzymatic Activity of *A. oryzae* (TAKA) α-Amylase in the Presence of Ca^{2+}

$[Ca^{2+}]$ (mM)	TAKA α-amylase act.[a] (%)	$[Ca^{2+}]$ (mM)	TAKA α-amylase act.[a] (%)
0	100.0	30	17.9
10	93.7	40	9.2
20	45.9	50	2.4

[a] The measurements were done according to the Phadebas analytical method (Pharmacia Diagnostics). Buffer, 0.1 M acetate, pH 4.5; enzyme concentration, 1.01×10^{-4} mg/mL of reaction mixture; temperature, 30 °C.

Discussion

The high-affinity binding site in *Aspergillus* amylases is unique in two ways. First, it is made up of a constellation of residues belonging to two distinct domains: the β barrel and the smaller domain B. It has been postulated by Buisson (1987) that, by linking these two fragments, Ca^{2+} preserves the structural integrity of the active site, which is presumed to be a big cleft between the β barrel and the B domain. By contrast, the EF hand site is formed by a single 12-residue-long stretch of a polypeptide chain.

The second unusual feature is the stereochemistry of ligand coordination. In organic complexes, Ca^{2+} has been observed to bind up to nine ligands, with a marked preference for seven or eight (Einspahr & Bugg, 1980, 1981). In proteins, however, the packing of residues around the cation imposes restrictions upon the spatial arrangement of ligands. In practically all cases the Ca^{2+} coordination may be viewed as that of an octahedron with varying degrees of distortion. Table IV shows a diagrammatic representation of a number of Ca^{2+}-binding sites, including those found in amylase. The alignment is based

Table IV: Stereochemistry of Selected Calcium-Binding Sites in Proteins[a]

protein	ligands									
	$-z$	$+z$	$+x$		$-y$		$-x$		$+y$	
proteinase K (1)	>Asp	O=	O=	OW		OW		OW		OW
phospholipase A_2	>Asp	O=	O=		OW		OW		O=	
trypsin (*S. griseus*)	>Asp	OGlu	O=		OW		OW		O=	
bovine trypsin	OGlu	O=	O=		OW		OGlu		OW	
galactose-binding protein	>Glu	OAsp	O=		OGlu		OAsp		OAsp	
concanavalin A	>Asp	OAsp	O=		OAsp		OW		OAsp	
troponin C (3, 4)	>Glu	OAsp	O=		OW		OAsn		OAsp	
calmodulin (1–4)	>Glu	OAsx	O=		OW		OAsp		OAsx	
parvalbumin (1)	>Glu	OAsp	O=		OW		OAsp		OAsp	
calcium-binding protein (1)	>Glu	O=	O=		OW		O=		O=	
calcium-binding protein (2)	>Glu	OAsp	O=		OW		OAsn		OAsp	
amylase (1)	>Asp	O=	O=	OW		OAsp		OW		OW
acid mold protease	OW	OW	O=	OW		OW		OW		OW
subtilisin NOVO (1)	>Asp	OGln	O=		O=		OAsn		O=	
thermolysin (3)	>Asp	OW	O=		OW		OW		OAsp	

[a] Only those single sites where the complete coordination sphere is observed at high resolution are shown in this table; if there is more than one site, conventional numbering of the site shown is used; the coordinates are taken from the Protein Data Bank or (in the case of concanavalin A and amylase) are as yet unpublished; the stereochemistry of the galactose binding protein site is based on figures published by Vyas et al. (1987). > denotes a bidentate ligand, O= denotes a carbonyl oxygen, OW denotes a water molecule, and OAsp, OGlu, etc. denote side-chain oxygen atoms.

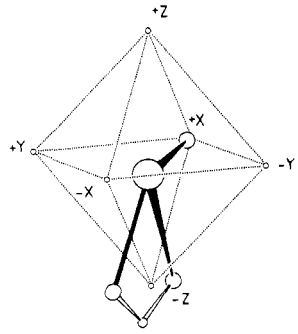

FIGURE 5: Diagrammatic representation of the stereochemistry of the calcium-binding sites surveyed in Table IV. The sites are aligned for comparison so that the invariant carbonyl is in position $+x$, while the virtually invariant bidentate carboxyl is in position $-z$. Other details are given in the text.

on the positions of an invariant carbonyl group and a bidentate carboxyl group (see Figure 5).

In practically all cases, a carboxyl group (Glu in the case of the EF hand proteins and Asp in other Ca^{2+}-binding sites) bound in a bidentate mode serves as a "primary" ligand in the $-z$ position (it is absent only in acid proteinase, which has an unusual Ca^{2+} coordination and which will be later discussed). The $+z$ position is occupied either by Asp in a unidentate fashion (all EF sites) or by a carbonyl (proteinase K, phospholipase A_2, and amylase). Another carbonyl occupies the $+x$ position. The remaining ligands all lie in (or very close to) the xy plane. If there are no other protein ligands, then four water molecules complete a pentagonal ring (proteinase K), thus bringing the coordination number to eight. An identical ring can be seen in mold acid proteinase, where it is made up entirely of water molecules, as the only protein ligand is the invariant $+x$ carbonyl. However, if there is at least one more protein atom ligand in the xy plane, then its exact location determines the total coordination number. A location close to any of the three apices $(-x, +y, -y)$ seems to impose a four-membered ring (phospholipase A_2, *Streptomyces griseus* trypsin, concanavalin A, all EF hand proteins). A position close to an apex of a pentagon allows three more water molecules to be coordinated in a fashion similar to that of the water ring in proteinase K. Amylase is the only protein known so far to exhibit such stereochemistry.

The pattern of coordination that emerges from Table IV provides a useful guide for modifying and designing Ca^{2+}-binding sites in a protein engineering experiments.

The location of the secondary calcium-binding sites in the two proteins explains the inhibitory effect of an excess of these ions. Considering the relatively high Ca^{2+} concentrations at which the effect occurs, its physiological effect is difficult to evaluate.

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