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Crystal Structure of the Ternary Complex between Carboxypeptidase A, L-Phenylalanine, and the Azide Ion

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The structure of the ternary complex of carboxypeptidase A (CPA) with the amino acid L-phenylalanine and the azide anion is reported as determined by X-ray crystallography to a resolution of 2.0 Å. The structure has been refined to an R factor of 0.151 over 7234 reflections having $I > 2\sigma(I)$. The binding of the amino acid molecule to the enzyme hydrophobic pocket closely resembles that of D-phenylalanine with the carboxylate linked to Arg-145 and Asn-144 and its α-amino group linked to Glu-270. The azide anion binds to Arg-145, which is proposed as the high-affinity binding site for anions. Upon L-phe binding the Tyr-248 residue moves in the "down" position at contact distances with both the amino and the carboxylate groups of the bound amino acid. A region of electron density near the metal has been interpreted as a second azide ion directly coordinated to zinc as suggested by spectroscopic studies on cobalt-substituted CPA. The present structure confirms the proposed mechanism of CPA sensitivity toward anions.

Introduction

Bovine carboxypeptidase A (CPA) is a zinc-exopeptidase of molecular weight 34 472 which catalyzes the hydrolysis of C-terminal amino acids from polypeptide substrates showing specificity toward those substrates with aromatic side chains.[1-3]

CPA and its complexes with several classes of compounds have been the subject of extensive crystallographic studies leading to a revised proposal for the catalytic mechanism of this enzyme.[4] The active site of CPA contains several cationic groups which are relevant for binding and catalysis (Arg-71, Arg-124, Arg-127, Arg-145, Zn^{2+}). Its substrates and inhibitors possess negatively charged terminal carboxylates. For this reason much attention has been devoted to the study of binary and ternary complexes of the enzyme with zwitterionic L- or D-amino acids and small inorganic or carboxylate anions showing inhibitory properties.[5-11] Recent studies on the inhibition of peptide hydrolysis in cobalt-substituted CPA by small pseudo-halide anions such as N_3^- and NCO^-[12] have suggested the presence of two azide binding sites with different affinities. Azide binds to the first site with a K_i = 35 mM and to the second site with a K_i = 1.5 M, but only the second site binding changes the electronic absorption spectrum of Co-CPA. This and other spectroscopic studies[10] have shown that the binding to Co-CPA of products of the ester or peptide hydrolysis allows the access of such anions to metal coordination. A strong synergistic effect exists in the binding; for example, it has been found that $K_d(N_3^-)$ (the spectral K_d is only slightly different from the K_i reported above) goes from 1.4 M to 4 mM in presence of 5 mM L-phe and vice versa K_d(L-phe) goes from 1.67 mM to 0.07 mM in presence of 100 mM N_3^-.

It has been demonstrated that the metal site of CPA becomes accessible to anions at low pH[13] or after chemical modification of the residue Glu-270.[14] These findings have suggested that the hydrogen bond existing in the native enzyme between Glu-270 and the metal-bound water molecule regulates the access of small anions to the metal coordination: when this hydrogen bond is broken either by chemical modification of Glu-270 or by its involvement in a different interaction with carboxylates or amino acid inhibitors, the zinc bound water molecule becomes available for substitution by anions.

The above mechanism has been rationalized recently by the structure of the binary complexes of CPA with D-phe and D-tyr,[15] where a strong hydrogen bond between Glu-270 and the amino group of the amino acid has been observed, with consequent breaking of the hydrogen bond with the zinc-bound water molecule accompanied by a substantial movement (~1 Å) of this molecule.

An L-phe molecule bound to CPA has been observed in the structures of a hydrolyzed phosphonamidate inhibitor[16] and of an enzyme-substrate-product ternary complex.[17] Here we will describe a different binding mode of L-phe in the enzyme, leading to the formation of the ternary complex with azide. The two azide binding sites in CPA will be also proposed.

Experimental Section

Protein Crystallization and Crystal Soaking. All the reagents were of analytical grade. Bovine carboxypeptidase A prepared by the method of Cox[18] was purchased from Sigma Chemical Co. and used without further purification. CPA was crystallized in the space group $P2_1$ (a = 51.60 Å, b = 60.27 Å, c = 47.25 Å, β = 97.27°) by dialysis of an enzyme solution in 1.2 M LiCl and 0.02 M Tris-HCl at pH 7.5 against a similar solution 0.14 M in LiCl at pH 7.5. Crystals of approximate dimensions 0.3 × 0.4 × 0.6 mm were harvested after several days, slightly cross-linked in a glutaraldehyde buffer 0.02% (v/v) and soaked for 1 month at 277 K in a 0.14 M LiCL solution buffered with Hepes 0.02 M at pH 7.5 containing 0.01 M L-phe and 0.05 M NaN_3. The crystals cracked shortly after they came in contact with the soaking solution, but the cross-linking procedure prevented their complete fragmentation.

Inspection of preliminary difference electron density maps from data collected at 2.8 Å resolution after 4 days, 1 week, and 2 weeks of soaking showed unsatisfactory occupancy of ligand sites.

X-ray Data Collection and Solution and Refinement of the Structure. Diffraction data were collected to a maximum resolution of 2.0 Å. Data for this limit were collected in two sets on a conventional Philips PW1100 automatic four-circle diffractometer using graphite-monochromated Cu Kα radiation. The integrated intensities, estimated with an ω scan, were corrected for Lorentz and polarization effects. A linear decay correction (9% maximum) based on the intensities of three control reflections was applied to each data set. A total of 16 723 out of 19 080 possible independent reflections were collected. After scaling, the two sets were merged with an R_{merg} factor ($R_{merg} = \sum|I - \bar{I}|/\sum I$. This factor gives the

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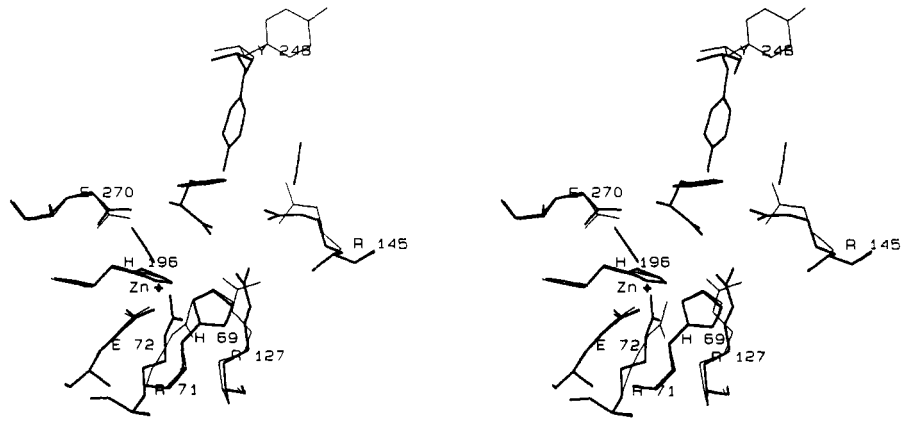

Figure 1. Binding of L-phenylalanine and azide to CPA. The coordinates from the refined model of the ternary complex (thick bonds) are superimposed on those of native CPA[22] (thin bonds).

overall agreement between intensities measured on different data sets.) of 0.074 based on the intensities of 1148 common unique reflections of an overlapping θ shell of 1.5° of the two data sets. All the crystals available for data collection were severely cracked. This resulted in a data set of 10663 reflections with $I > 1.0\sigma(I)$ and only 7234 reflections with $I > 2\sigma(I)$. Fourier difference maps obtained with the low cutoff data were affected by a high noise level. For this reason data at the 2σ level were used in the structure determination. Fourier difference maps were obtained with the set of programs PROTEIN.[19] Model building was performed on an Evans & Sutherland PS390 picture system with the graphic software FRODO[20] running on a host VaxStation3200 computer. The molecular model was refined by the stereochemically restrained reciprocal least-squares method of the TNT set of programs.[21] Initial phases and structure factors were calculated from the atomic coordinates of native CPA refined at 1.54 Å.[22] At this stage the crystallographic agreement factor R_{cryst} ($R_{cryst} = \sum||F_o| - |F_c||/\sum|F_o|$.) was 0.302 for 10.0–2.0-Å data. Inspection of a difference Fourier map calculated with $|F$(complex-obs)$| - |F$(nat-calcd)$|$ coefficients and phases from the refined native structure clearly indicated a region of positive electron density in the S1′ subsite of the enzyme specific for binding of C-terminal aromatic substrates. At this stage a refinement was undertaken of a model of the protein where the water molecules of the active site cavity and the residues most probably affected by the ligand binding (Arg-71, Arg-127, Arg-145, Tyr-248, Glu-270, Glu-72, His-69, His-196, Zn^{2+}) were omitted. After several cycles of refinement it became clear that part of the strand containing the Tyr-248 residue had to be rebuilt together with the side chains of the above mentioned residues, because of the movement of the Tyr-248 residue in the "down" position. While the electron density corresponding to the side chains of those residues had improved during the refinement, that of Arg-71 was still diffuse, indicating a probable disorder. Consequently a model was built in which this side chain was disordered over two positions with 50% occupancy. Of the two positions one is close to that of the native enzyme and the other has moved toward the azide ion coordinated to zinc. The relative occupancies refined to 40 and 60%, respectively. The refinement converged smoothly to an R_{cryst} of 0.176. At this point a ΔF map clearly showed density corresponding to the L-phe molecule and two other cigar-shaped regions near Arg-145 and near the metal site in which two azide molecules could be modeled. The final molecular model required the repositioning of 16 water molecules in the active-site region. Further refinement of the complete structure converged to a final R_{cryst} of 0.151 for data between 10.0- and 2.0-Å resolution. The highest peaks in the final difference electron density maps calculated with coefficients $|F$(complex-obs)$| - |F$(complex-calc)$|$ and phases from the refined model were below 2.0σ. Root-mean-square deviations from ideal values of stereochemical parameters of the refined structure of the protein complex are 0.03 Å for bond lengths, 3.27° for bond angles, 0.023 Å for planarity of trigonal atoms, and 0.043 Å for other planes. Torsion angles were not restrained during the refinement. The model includes a total of 188 water molecules. The root-mean-square deviation on the final atomic positions estimated from a Luzzati plot[23] is between 0.15 and 0.20 Å.

Table I. Zinc Coordination Distances and Angles in the L-phe–Azide CPA Complex and in Native CPA[a]

	CPA–L-phe–azide	native CPA
	Distances (Å)	
Zn–Nδ 196	2.0	2.1
Zn–Nδ 69	2.0	2.1
Zn–Oε1 72	2.1	2.2
Zn–Oε2 72	2.2	2.3
Zn–Nα′[b]	2.7	
Zn–O Wat 571		2.1
	Angles (deg)	
Nδ 69–Zn–Nδ 196	96.7	98.8
Nδ 69–Zn–D 72[c]	107.6	107.2
Nδ 196–Zn–D 72	127.0	129.2
Nδ 69–Zn–(Nα′–O 571)	109.0	121.6
Nδ 196–Zn–(Nα′–O 571)	134.2	95.6
D 72–Zn–(Nα′–O 571)	80.3	106.0

[a] Root-mean-square deviation on atomic positions ≃ 0.2 Å. [b] Nα′ indicates the azide nitrogen interacting with zinc. [c] D stands for a dummy atom positioned midway between the two carboxylate oxygens of the symmetric bidentate ligand Glu-72.

Table II. Distances (Å) between CPA, L-phe, and Azide Anions (Root-Mean-Square Deviation in Atomic Positions ≃ 0.2 Å)[a]

CPA	L-phe	azide	distance
Oε1 Glu-270	NH_3^+		2.7*
Oε2 Glu-270	NH_3^+		4.1
Oη Tyr-248	NH_3^+		3.8
	NH_3^+	Nγ′	3.3
Nδ2 Asn-144	O1		2.9*
Nη1 Arg-145	O1		3.4*
Nη Arg-145	O1		4.4
Nη1 Arg-127	O2		3.5
Nη Arg-145	O2		3.1*
Nη1 Arg-145	O2		3.4*
OH Tyr-248	O2		3.6
Oε2 Glu-270		Nγ′	3.1
Oε2 Glu-270		Nβ′	3.5
O Ser-197		Nγ′	3.2
Nη Arg-145		Nα	2.3
Nη1 Arg-145		Nα	3.6
Nε Arg-145		Nα	2.4
O Gly-155		Nβ	2.6
O Gln-249		Nγ	2.6

[a] Possible hydrogen bonds as judged from both distance and geometric criteria are indicated by an asterisk. The prime indicates the azide ion bound to zinc.

Results and Discussion

Figure 1 shows the relevant residues of the refined model of the CPA–L-phe–azide ternary complex superimposed with the

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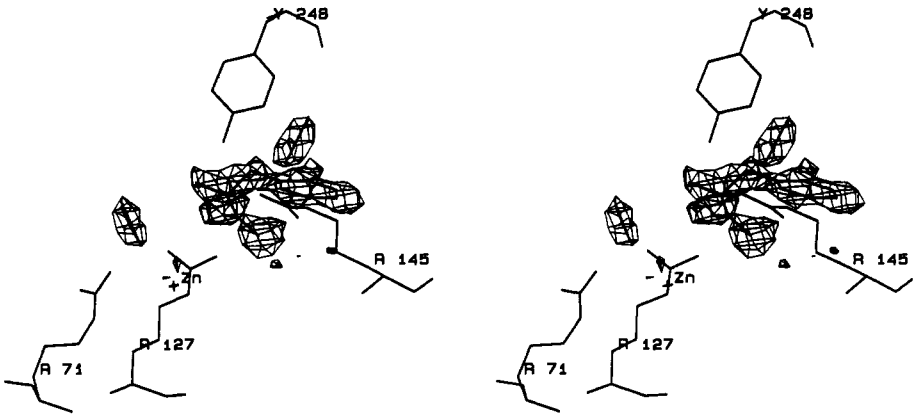


Figure 2. Difference electron density map of the CPA–L-phenylalanine–azide complex calculated with coefficients $|F(\text{complex-obs})| - |F(\text{complex-calcd})|$ and phases from the refined model of CPA in this structure at $R_{cryst} = 0.176$ before modeling of L-phenylalanine and the azide ions. The positive electron density is contoured at the level of 3.0σ. The coordinates of the final model are superimposed.

same residues of the native CPA at 1.54 Å.[22] Figure 2 shows the difference electron density of the complex obtained with coefficients and phases from the structure refined at $R_{cryst} = 0.176$ together with the model of the complexing molecules. Table I reports the geometry of zinc coordination in this structure compared with that of native CPA. In Table II the interactions of the enzyme with the inhibitor molecules are reported.

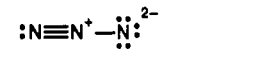
The structure of this CPA ternary complex shows one molecule of L-phenylalanine bound to the enzyme (Figure 2). The aromatic ring of the bound L-phe molecule lies in the hydrophobic pocket S1′, and the carboxylate group of L-phe is involved in a salt link with the guanidinium group of Arg-145 and in a further hydrogen bond donated by the amide of Asn-144, as always observed in CPA complexes with pseudosubstrates or inhibitors.[4,15-17] The charged amino group of the L-phe zwitterion is involved in a short contact with a carboxylate oxygen of the residue Glu-270 (see Table II).

Interactions between CPA and isolated L-phe were previously observed in two cases. In a hydrolyzed phosphonamidate inhibitor[16] the L-phe molecule does not make any hydrogen-bond interaction with the enzyme, the amino acid carboxylate group interacting with a phosphonate oxygen and the α-amino group pointing toward solution. In an enzyme–substrate–product ternary complex (CPA–BZF–L-phe)[17] the amino acid interacts with the enzyme by a salt link between its carboxylate group and Arg-145 while the amino group is engaged in an "incipient" formation of a peptide bond with an intact molecule of the benzoyl-L-phenylalanine substrate and is 3.3 Å away from the Glu-270 carboxylate oxygen. The contacts found between L-phe and the enzyme in this structure (see Table II) are somewhat between those found in the BZF–L-phe and in the D-phe CPA complexes. The distance of 2.7 (2) Å found for the interaction of NH_3^+ with Glu-270 is not significantly different from that found in the D-phe complex (2.3 (2) Å). However, it must be pointed out that the geometry of this interaction is not ideal for hydrogen bonding as in the D-phe complex, the angle (L-phe)Cα–NH_3^+–Oϵ1(Glu-270) being acute (≈78°). This observation is quite accurate because the well-defined shape of Glu-270 and L-phe electron density allows a precise positioning of the two residues. Perhaps in this case we are observing a pure electrostatic interaction which can explain the different affinity of L- and D-phe for CPA (estimated K_i values are 18 and 2 mM, respectively[24-26]) in the sense that the latter is able to establish a hydrogen bond because of the different orientation of the charged amino group.

Upon binding the L-phe molecule, the Tyr-248 phenolic side chain moves in the characteristic "down" position and remains at contact distance with the carboxylate of L-phe (tyr-248 OH–L-phe O distance = 3.7 Å), making hydrogen bonds with tightly bound water molecules of the cavity.

In this structure the zinc coordination is essentially the same as that found in the native CPA (Figure 2) with Glu-72, His-69, and His-196 bound to zinc with analogous geometry. The differences in bond lengths and angles are within experimental error (see Table I). The main difference lies in the fourth position of the distorted zinc coordination tetrahedron. In place of the water molecule bound at 2.1 Å from zinc, the present structure shows an elongated region of electron density (between 3σ and 3.5σ) that we have interpreted as the metal site (low-affinity site) for the azide ion in CPA. The azide modeled in this site has the terminal nitrogen at 2.7 (2) Å from Zn^{2+} and 2.8 (2) Å from the disordered Arg-71. These distances indicate that the interaction of the anion with the metal is mostly electrostatic and that the positioning of azide is influenced by the positively charged guanidinium group of Arg-71. The prevalent electrostatic character of the Zn^{2+}–azide interaction is also supported by the value of 135 (3)° found for the Zn–N_α(azide)–N_β(azide) angle, which is larger than the usual metal–azide angle found in model complexes.[28]

The description of the two azide binding sites found in the present structure deserves a detailed discussion. Even from the first Fourier maps it was evident that a cigarlike density was present at the end of the Arg-145 side chain extending toward the region of Gln-249. After refinement the size and the shape of this density was ideal to accommodate an azide anion interacting electrostatically with the positively charged side chain of Arg-145 in one side and with the backbone carbonyls of Gln-249 and Gly-155 at the other end. This interaction, resulting in short contacts (see Table II), can be explained by considering the polar nature of the azide molecule especially in the



resonant form. The azide dipole orients itself with respect to the other polar groups constituted by the positive guanidinium group of Arg-145 and the carbonyl groups of Gln-249 and Gly-155. Although the experimental electronic density might be interpretable as disordered solvent, its height (3.5–4.0σ) and its linear shape strengthen the assignment of this electron density to the azide anion. We propose that this site is the spectroscopically silent high-affinity binding site found in kinetic studies[12,27] in which azide binding is partially competitive toward peptide hydrolysis. Of course, the possibility of binding sites corresponding to other positively charged groups in the active-site region of the enzyme cannot be excluded. For example a region of weak electron density extending from Arg-127 is found in this structure, but its shape is not as well defined as that one on Arg-145 and may also be interpreted as hydrogen-bonded water molecules. The partially competitive inhibitory behavior of azide can be understood if we consider the conformational change experienced by Arg-145 upon

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azide binding (Figure 1). In the present structure the side chain of Arg-145 has extended toward the interior of the active site cavity by 0.7 Å and the plane of the guanidinium group is rotated in a position almost perpendicular with respect to its orientation in the native enzyme. The L-phe binding to the enzyme is not prevented by this new conformation, but it is actually favored. In fact the new position of the Arg-145 side chain makes possible a closer approach of the L-phe amino nitrogen to the carboxylate group of Glu-270. On the contrary, the binding of a polypeptide substrate can be hindered because once the terminal carboxylate is bound to the rotated Arg-145, the peptide link is not pointing any longer toward the S1 catalytic site, but it is pushed toward the cavity wall.

The second azide binding site can be tentatively assigned to the zinc ion. As already noted, this density is lower than that on Arg-145 (3.0–3.5σ), but its shape is very similar (Figure 2). It must be stressed again that electron density corresponding to disordered solvent will always be indistinguishable from that of the azide ion. This observation, together with the fact that the structure was determined with a limited amount of observed reflections, suggests great caution in the above interpretation. Our interpretation is consistent with the proposed model of activation of the zinc-bound water molecule toward anion substitution.[4,12] The low-affinity-site model is also consistent with the spectroscopic observations of a charge transfer between a cobalt-bound azide ion and the metal in the corresponding ternary complex with CoCPA.[12]

The synergism in the azide and the L-phe binding to CPA is explained by the presence of the azide molecule on the high-affinity site near Arg-145 which forces its guanidinium group to adopt the extended conformation described above (Figure 1). This new conformation allows the simultaneous near-optimal interactions of L-phe both with Arg-145 and with Glu-270 which would be otherways mutually exclusive as observed in the other L-phe-containing complexes[16,17] (vide supra). It is interesting to point out that due to the different chirality of the α carbon, the D-phe amino acid can also perform such simultaneous hydrogen bonds.[15] On the other hand, the interaction of L-phe with Glu-270 weakens the hydrogen bond between Glu-270 and the metal-bound water molecule, favoring its replacement by the second azide ion. Further stabilization of the ternary complex can be achieved by the network of hydrogen bonds and electrostatic interactions which link the L-phe molecule to the metal-bound azide anion through the bridging Glu-270 carboxylate and the stabilization of low-affinity azide by contacts with Ser-197 and water molecules bound in the cavity.

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Synthesis, Structure, and Properties of $[Mn(salpn)(EtOH)_2](ClO_4)$ and Its Aerobic Oxidation Product $[Mn(salpn)O]_2$[1]

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Preparation and isolation of the mononuclear Mn^{III} complex $[Mn(salpn)(EtOH)_2](ClO_4)$ (**1a**) (H_2salpn = *N,N'*-bis(salicylidene)-1,3-diaminopropane) were accomplished by air oxidation of a solution containing H_2salpn, NaOH, and $Mn(ClO_4)_2 \cdot 6H_2O$ in MeOH/EtOH (1:1), followed by addition of Et_2O. Compound **1a**·EtOH·H_2O crystallizes in the triclinic space group $P\bar{1}$ with a = 11.649 (3) Å, b = 13.902 (6) Å, c = 19.849 (7) Å, α = 70.18 (2)°, β = 70.52 (2)°, γ = 60.33 (2)°, V = 2726 (2) Å^3, and Z = 4. For **1a**·EtOH·H_2O there are two mononuclear Mn^{III} complexes in the crystallographic asymmetric unit. The Mn–O and Mn–N distances for **1** are in good agreement with those found for other Mn^{III} Schiff base complexes. The ^{1}H NMR spectrum of **1** in CD_3CN has well-resolved, paramagnetically shifted resonances at 19.8, 11.6, 7.4, −2.8, −24.8, −26.2, and −94.2 ppm. Compound **1** has a $Mn^{III/II}$ reduction potential of −0.154 V vs SSCE as measured by cyclic voltammetry in DMSO. Compound **1** was converted into $[Mn_2O_2(salpn)_2]$ (**2**) by air oxidation in basic MeOH followed by recrystallization from DMF. Alternatively, **2** can be prepared by air oxidation of $Mn(salpn)(H_2O)$ in pyridine, followed by crystallization from CH_2Cl_2/cyclohexane. Compound **2**·2DMF crystallizes in the monoclinic space group $P2_1/c$ with a = 10.722 (2) Å, b = 10.163 (3) Å, c = 17.564 (4) Å, β = 100.67 (2)°, V = 1890 (1) Å^3, and Z = 2. Using an isotropic spin-exchange Hamiltonian $\mathcal{H} = -2J\hat{S}_1 \cdot \hat{S}_2$, variable-temperature magnetic data for **2** were fit well with a J value of −82 cm^{-1}. The magnitude of J is significantly less than that for several other species containing the $\{Mn_2O_2\}^{4+}$ core yet comparable to the value obtained for $[Mn_2O_2(pic)_2]$ (pic = 2-pyridinecarboxylate) (*Inorg. Chem.* **1989**, *28*, 4037). The reduction potential for **2** in DMSO (−0.39 V vs SSCE) for the IV,IV/IV,III couple is the lowest determined for a bis(μ-oxo)dimanganese complex.

Manganese plays an important role in several biological redox-active systems. In three of these systems—the oxygen-evolving complex in photosystem II of green plants,[2] Mn catalases,[3] and Mn ribonucleotide reductase[4]—the active sites are thought to exist as binuclear species or manganese aggregates of higher nuclearity. Because these metalloenzymes function as multielectron-redox catalysts, it is reasonable to assume that the manganese sites serve as reservoirs of reducing or oxidizing equivalents. The accessibility of at least two oxidation states for most manganese coordination complexes makes it a suitable metal for this function. The most extensively studied of the biological systems has been the photosystem II oxygen-evolving complex (PSII OEC). Although it is known that there are four manganese atoms per OEC,[5] the precise arrangement of these four atoms has not been determined unambiguously.[6] EXAFS analysis of the OEC indicates the

(1) Abbreviations used: salpn = *N,N'*-bis(salicylidene)-1,3-diaminopropane; EtOH = ethanol; EXAFS = extended X-ray absorption fine structure; PSII OEC = photosystem II oxygen-evolving complex; phen = phenanthroline; bispicen = *N,N'*-bis(2-pyridylmethyl)-1,2-ethanediamine; pic = 2-pyridinecarboxylate; DMF = *N,N*-dimethylformamide.

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