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EPR OF Co(II) AS A STRUCTURAL AND MECHANISTIC PROBE OF METALLOPROTEIN ACTIVE SITES: CHARACTERISATION OF AN AMINOPEPTIDASE

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Co(II) can often be substituted for Zn(II) in zinc-dependent metalloenzymes to provide spectroscopically accessible forms of the enzymes. Co(II) is an excellent spectroscopic probe as it is both optically active and EPR active. Further, its fast relaxation properties make it a useful paramagnetic shift reagent in NMR. In EPR, the dependence of the spectra of high-spin Co(II) on *E/D* and the sensitivity of the resolvability of the ^{59}Co hyperfine structure to strain terms allow structural information to be inferred from the EPR spectra. In addition to its useful spectroscopic properties, Co(II) is often an extremely good functional mimic of Zn(II), and Co(II)-substituted zinc-dependent enzymes often display catalytic activities analogous to the native Zn(II)-containing enzyme forms. It is therefore somewhat surprising that there are few examples of EPR studies of Co(II)-substituted enzymes. The most detailed studies carried out to date are those on the aminopeptidase from *Aeromonas proteolytica*. Therefore, the methodology of extracting structural information from EPR of Co(II)-containing proteins is described using studies on *A. proteolytica* aminopeptidase as an example.

INTRODUCTION

The aminopeptidase ('AAP') from the marine bacterium *Aeromonas proteolytica* is a prototypical dinuclear metallohydrolase. The class of dinuclear and trinuclear metallohydrolases include the enzymes phospholipase C (Hough, Hansen, Birknes, Jynge, Hansen, Hordvik, Little, Dodson & Derewenda, 1989), alkaline phosphatase (Kim & Wyckoff, 1991), inositol monophosphatase (Bone, Frank, Springer, Pollack, Osborne, Atack, Knowles, McAllister, Ragan, Broughton, Baker & Fletcher, 1994), DNA polymerase 1 (Beese & Steitz, 1991), the ribonuclease H domain of HIV-1 reverse transcriptase (Davies, Hostomska, Hostomsky, Jordan & Mathews, 1991), P1 nuclease (Lahm, Volbeda & Suck, 1990), urease (Jabri, Carr, Hausinger & Karplus, 1995), purple acid phosphatases (Holz, Que & Ming, 1992; True, Scarrow, Randall, Holz & Que, 1993; Sträter, Klabunde, Tucker, Witzel & Krebs, 1995) and the aminopeptidases (Roderick & Matthews, 1993; Burley, David, Taylor & Lipscomb, 1990). These enzymes are key players in processes including protein degradation, cellular targeting, tissue repair, angiogenesis and carcinogenesis (Lipscomb & Sträter, 1996; Wilcox, 1996; Dismukes, 1996). Aminopeptidases themselves have been found to be the targets for some anti-cancer drugs and thus elucidation of modes of inhibition of aminopeptidases is likely to be of great value in rational drug design (Huntington, Bienvenue, Wei, Bennett, Holz & Pei, 1999; Ustynyuk, Bennett, Edwards & Holz, 1999; Bienvenue, Bennett & Holz, 2000). The aminopeptidase (AAP) from *A. proteolytica* is an easily obtained, highly stable, small (32 kDa) monomeric enzyme and has been well characterised with regard to its catalytic activity, substrate specificity and three dimensional structure (Prescott & Wilkes, 1976; Chevrier, Schalk, D'Orchymont, Rondeau, Moras & Tarnus, 1994). It has long been known that the apoenzyme can be activated by a number of metal ions including Co, Cu, Cd, Ni and the native Zn; the activities with Zn and Co are comparable (Bayliss & Prescott, 1986). An interesting phenomenon is the site-specific nature of metal binding. The two metal-binding sites (shown liganding Zn1 and Zn2 in Fig. 1) have distinct affinities and thus it is possible to label site 1 of AAP (shown as Zn1 in Fig. 1) with one metal ion and then site 2 with a different metal ion (Bennett & Holz, 1997b). Thus, heterodimetallic substituted forms of the enzyme [M1,M2(AAP)] can be generated (where M1 and M2 are distinct transition ions and, therefore, [M1,M2(AAP)] is distinct from [M2,M1(AAP)]).

Other than the studies described herein, there have been few reports of EPR studies of high-spin Co(II)-containing enzymes. Notable earlier studies are those of Grell and Bray (1971), Rose, Dickin-

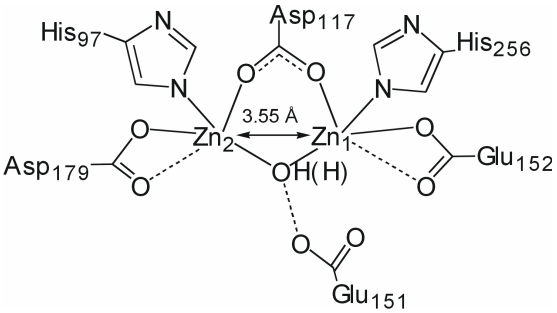

Fig. 1. The active site of the native zinc-containing aminopeptidase from *A. proteolytica*.

son and Westhead (1984), Bicknell, Hanson, Holmquist and Little (1986), Elgren, Ming and Que (1994), Kuo and Raushel (1994), Martinelli, Hanson, Thompson, Holmquist, Pilbrow, Auld and Vallee (1989), Bubacco, Magliozzo, Beltramini, Salvato and Peisach (1992), Sellin, Eriksson, Aronsson, and Mannervik (1983) and Keech, Le Brun, Wilson, Andrews, Moore and Thomson (1997). In only one notable case (Martinelli *et al.* 1989), were parameters extracted systematically by computer simulation. In many cases the spectra observed were complex due to either the presence of multiple species or distortion arising from injudicious choice of operating parameters. In such cases it is very difficult to extract reliable spin Hamiltonian parameters by mere observation and thus to meaningfully interpret the spectra. Even when reliable values for the turning points ($g_{\mathrm{eff.}(x,y,z)}$) and hyperfine interaction can be obtained by observation, interpretation of spectra in structural terms is non-trivial. There are no simple "rules of thumb" relating $g_{\mathrm{eff.}}$ and A_z to geometry or ligand environment such as those derived by Peisach and Blumberg (1974) for Cu(II). Indeed the ratio of $g_{\mathrm{eff.}}$ to A_z has been shown to be essentially constant for different coordination numbers of Co(II) (Bencini, Bertini, Canti, Gatteschi & Luchinat, 1981). The EPR-derived zero-field splitting parameter, Δ, has been used to infer coordination number (Makinen & Yim, 1981). While in principle Δ can be obtained *via* saturation measurements over a number of temperatures, in practice one can rarely obtain high-spin Co(II) EPR data over a sufficiently wide range of temperatures to be confident of identifying a clear region where the Orbach relaxation process dominates over the Raman and spin-phonon regimes, rendering the EPR-derived Δ very unreliable. Even with reliable zero-field splittings derived from MCD, Larrabee, Alessi, Asiedu, Cook, Hoerning, Klingler, Okin, Santee and Volkert (1997) showed that Δ is not a reliable predictor of geometry and therefore any geometrical prediction based on zero-field splitting parameters should be treated with a great deal of caution.

With the introduction of a straightforward method of extracting EPR parameters, Bennett and Holz (1997a, 1997b, 1998) demonstrated that useful information can, however, be extracted from the EPR spectra. Multiple species can be deconvoluted by manipulating the sample or the recording conditions so as to vary the relative amounts of the species, which can then be extracted by subtraction (Bennett & Holz, 1997b). Individual species can be verified by computer simulation. Computer simulation provides two structurally useful parameters, the rhombic distortion of the axial zero-field, *E/D*, and the amount of linewidth broadening induced by strain effects. *E/D* is an indicator of the amount of axial electronic symmetry at the Co(II) ion. While *E/D* cannot be used to predict geometry (i.e. tetrahedral *versus* octahedral), it provides an indication of distortion of the coordination geometry. *g*-, *A*-, *D*- and *E*-strains arise from microheterogeneity of the geometries of individual Co(II) sites and are difficult to deconvolute as the effects of each are somewhat similar. However, if one makes the assumption that the intrinsic linewidth is narrow (i.e. narrower than the narrowest hyperfine line) then the relative values for the strain terms provide an indication of the relative levels of microheterogeneity in different systems or differently-treated samples of the same system. It was found in practice that a combination of $g_{\mathrm{eff.}}$- and *A*-strain produces good simulations of the asymmetrically broadened hyperfine lines often observed in Co(II) spectra (Bennett & Holz, 1997a). $g_{\mathrm{eff.}}$-strain implicitly includes contributions from g_{real}-, *D*- and *E*-strains (cf. Pilbrow, 1978) and the reduction of these to a single parameter simplifies both the simulation and the interpretation. The combination of these two parameters, *E/D* and strain, can provide important structural information in that they discriminate between high-symmetry (low *E/D*) unconstrained (high *g*-strain) environments, typified by $Co(H_2O)_6^{2+}$ and low-symmetry, highly constrained, high energy structures that would be expected for the transition state of an enzyme active site. Further structural information can be obtained from the hyperfine coupling constant: smaller values of *A* correspond to electron delocalisation and structural models must take this into account.

An interesting property of Co(II) is that signals are occasionally observed from the $M_S = |\pm 3/2\rangle$ doublet. The appearance of these signals in systems hitherto exhibiting spectra from the $M_S = |\pm 1/2\rangle$ doublet, which is usually the ground

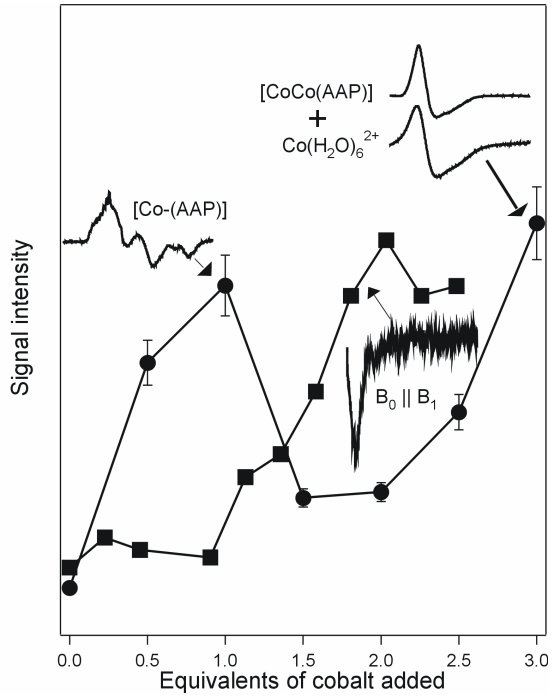

Fig. 2. EPR signals observed upon addition of Co(II) to apo-aminopeptidase. The plotted lines show the intensities of the total EPR signal (circles) and of the parallel-mode signal recorded with B0 || B1 (squares). The spectra of aminopeptidase after one equivalent (labelled "[Co-(AAP)]") and two equivalents ("[CoCo(AAP)]") of Co(II) were added are shown, as are the parallel-mode spectra of aminopeptidase plus two equivalents of Co(II) ("$B_0 \parallel B_1$") and the EPR spectrum of hexaquocobalt(II) ("$Co(H_2O)_6^{2+}$").

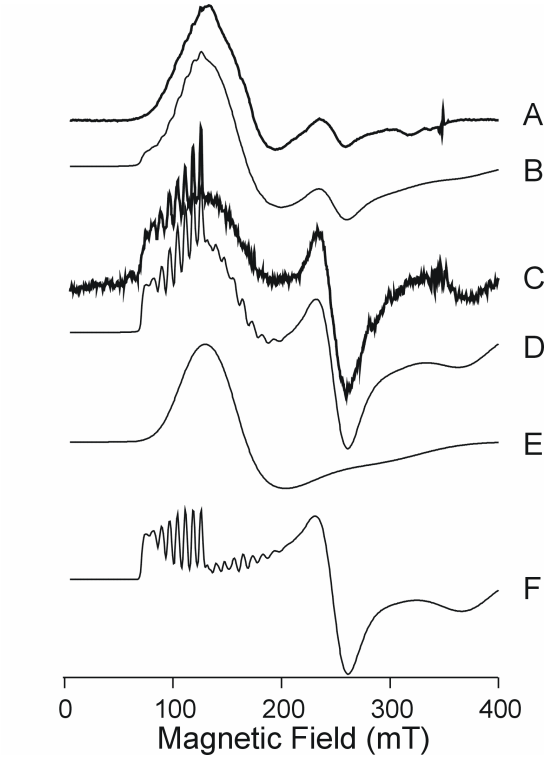

Fig. 3. EPR spectra from [CoZn]-substituted aminopeptidase. Spectra were recorded at pH 7.5 (A) and pH 10.0 (C). Each of the spectra (A) and (C) could be simulated (B and D) assuming a mixture of two pH-dependent species (E and F). The simulation B assumed 75% of the species represented by E plus 25% of species F. The simulation D assumed 45% of species E and 55% of species F. The EPR parameters used in the simulations were $g_{\mathrm{eff.}(x,y,z)} = 2.20,\ 3.92,\ 5.23$ ($M_S = |\pm 1/2\rangle$, $g_{\mathrm{real}} = 2.29$, $E/D = 0.1$) for E and $g_{\mathrm{eff.}(x,y,z)} = 1.80,\ 2.75,\ 6.88$ ($M_S = |\pm 1/2\rangle$, $g_{\mathrm{real}} = 2.54$, $E/D = 0.32$); $A_z(^{59}\mathrm{Co}) = 7.0$ mT for F.

state for the 5- or 6-coordinate sites in Co(II) enzymes, can signal either a drastic change in geometry (*e.g.* to tetrahedral) or binding of a ligand atom that severely perturbs the electronic geometry. Such signals appear, for instance, upon treatment of the Co(II)-substituted aminopeptidase from *Aeromonas proteolytica* with thiol-containing inhibitors and it has been proposed, on the basis of the lack of this effect with otherwise analogous hydroxyl-bearing inhibitors, that this is due to direct binding of the sulfur atom to Co(II) (Huntington *et al.*, 1999; Bienvenue *et al.*, 2000).

For a dinuclear site, a further property of interest is the nature of any structural or electronic communication between the metal ions. EPR of cobalt-homodisubstituted centers can address this question. Two Co(II) ions can couple to give integral spin systems with $S = 0$ through $S = 3$. These are very difficult to observe under normal circumstances, but using parallel-mode EPR, *i.e.* with the microwave magnetic field, B_1, aligned parallel to the "static" (actually 100 kHz modulated) field, B_0, these signals can be observed if some part of the zero-field splitting distribution falls within the energy of the microwave (Hendrich & Debrunner, 1989). There is an inherent sensitivity enhancement of a factor of about 3 for integer spins when operating with $B_0 \parallel B_1$. More importantly, perhaps, overlapping signals due to Kramer's doublet transitions, which usually give much more intense signals than integer spin systems, are forbidden with $B_0 \parallel B_1$ and so are effectively attenuated by factors of 100-1000. The presence of integer spin signals is an important indicator of spin-spin coupling. More information can be gleaned from temperature dependence studies of $B_0 \parallel B_1$ signals: whether the coupling is ferromagnetic or antiferromagnetic, for instance, can have important structural consequences. In particular, μ(O)-bridged transition metal dinuclear sites in AAP, related enzymes and model complexes exhibit anti-ferromagnetic coupling (Holz, Bennett, Chen & Ming, 1998a; Holz, Bradshaw & Bennett,

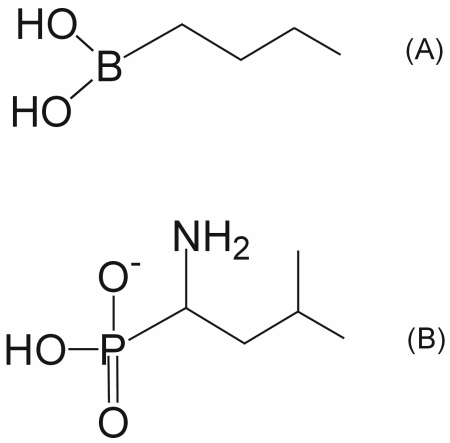

Fig. 4. Schematics of the inhibitors 1-butaneboronic acid (A) and L-leucine phosphonic acid (B).

1998b). Therefore observation of EPR signals due to ferromagnetic coupling indicates a change in the structure of the bridged moiety.

The aim of the present work is to illustrate by example how analysis of Co(II) EPR spectra can reveal structural information about a metalloenzyme by reviewing the EPR studies of Co(II)-substituted forms of AAP. The EPR studies on AAP described herein provide a particularly interesting example of the power of application of EPR spectroscopy to Co(II)-substituted forms of a zinc enzyme. The emphases will be on the $M_S = |\pm 1/2\rangle$ EPR signals of high-spin ($S = 3/2$) monocobalt(II) and the non-Kramer's signals. The reader is referred elsewhere for detailed discussion of, and further references to, kinetic studies (Bayliss & Prescott, 1986), NMR studies (Holz *et al.*, 1992, 1998a), $M_S = |\pm 3/2\rangle$ EPR studies (Huntington *et al.*, 1999; Bienvenue *et al.*, 2000), the crystal structure of the native enzyme (Chevrier *et al.*, 1994) and crystallographic studies of the zinc-containing inhibited forms of the enzyme (De Paola, Bennett, Holz, Ringe & Petsko, 1999; Stamper, Bennett, Edwards, Holz, Ringe & Petsko 2001).

CHARACTERIZATION OF THE DINUCLEAR CENTER OF AAP

The crystal structure reported by Chevrier *et al.* (1994) indicates an active site structure consistent with the schematic of Fig. 1. The two Zn ions are separated by 3.55 Å and are bridged by the carboxylate of Asp117 and an oxygen atom. However, protein crystallography at supratomic resolution (1.8 Å) gives no information regarding hydrogen atoms and only an average position for the O atom. Thus μ-oxo, μ-OH and μ-OH_2 cannot be

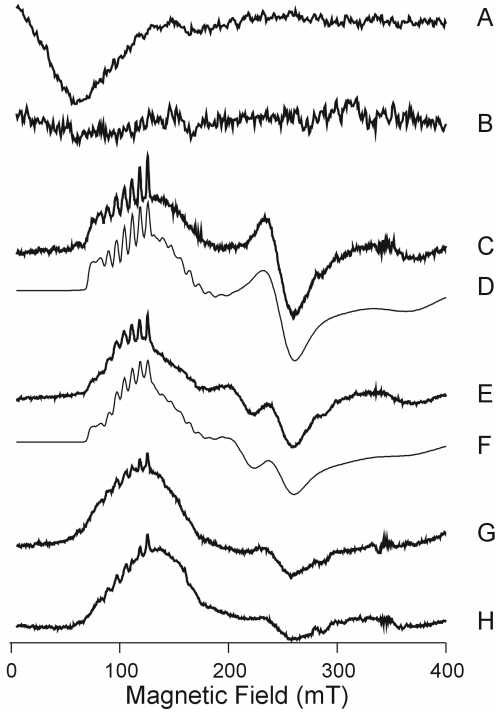

Fig. 5. The effect of 1-butaneboronic acid (BuBA) on the EPR spectra of cobalt-substituted aminopeptidase. (A) the parallel-mode ($B_0 \parallel B_1$) EPR spectrum of cobalt-disubstituted aminopeptidase [CoCo(AAP)]; (B) the parallel-mode ($B_0 \parallel B_1$) EPR spectrum of [CoCo(AAP)]+BuBA; (C) the EPR spectrum of [CoZn(AAP)] at pH 10.0; (D) simulation of C assuming two pH-dependent species with the parameters $g_{eff.(x,y,z)} = 2.20, 3.92, 5.23$ ($M_S = |\pm 1/2\rangle$, $g_{real} = 2.29$, $E/D = 0.1$) and $g_{eff.(x,y,z)} = 1.80, 2.75, 6.88$ ($M_S = |\pm 1/2\rangle$, $g_{real} = 2.54$, $E/D = 0.32$); $A_z(^{59}Co) = 7.0$ mT; (E) the EPR spectrum of [CoZn(AAP)]+BuBA at pH 10.0; (F) simulation of E assuming the two species of D plus a third, BuBA-dependent species with $g_{eff.(x,y,z)} = 2.08, 3.15, 6.15$ ($M_S = |\pm 1/2\rangle$, $g_{real} = 2.41$, $E/D = 0.22$); $A_z(^{59}Co) = 4.0$ mT; (G) the EPR spectrum of [ZnCo(AAP)]; H: the EPR spectrum of [ZnCo(AAP)] + BuBA.

distinguished, especially if a mixture of these species is present. EPR data from a sample of AAP that was titrated with Co(II) are shown in Fig. 2. Between zero and one equivalents of Co(II), a complex signal was seen that increased in intensity up to one equivalent without change in form (signal [Co-(AAP)] of Fig. 1). However, upon addition of further Co(II), the signal labelled [Co-(AAP)] was replaced by a broad, axial signal, labelled [CoCo(AAP)] in Fig. 1. Further, the integrated EPR intensity decreased steadily between one and two equivalents of added Co(II). Beyond two equivalents of Co(II), the signal intensity increased due to $Co(H_2O)_6^{2+}$. Clearly, the two Co(II) ions in the dinuclear active sites of the ma-

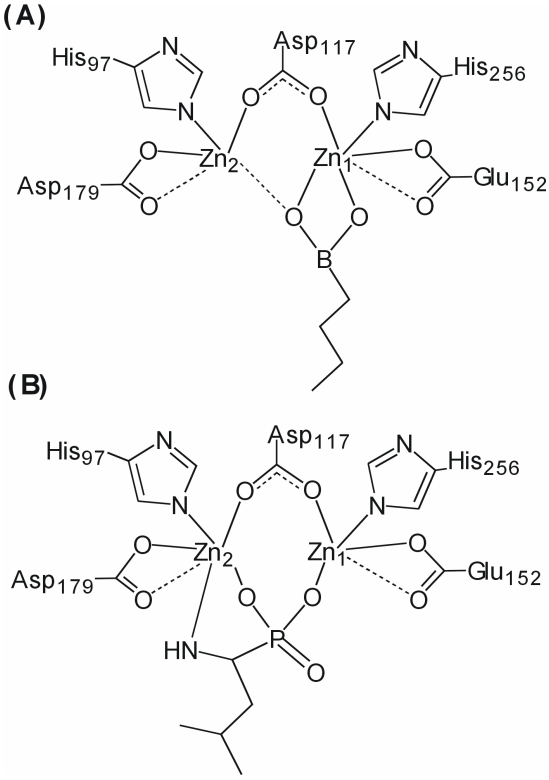

Fig. 6. Binding schemes for the aminopeptidase inhibitors 1-butaneboronic acid (A) and L-leucine phosphonic acid (B).

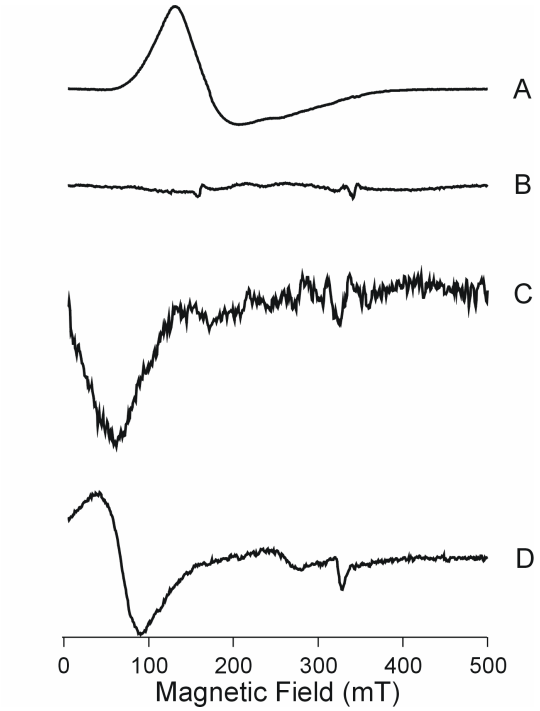

Fig. 7. The effect of L-leucine phosphonic acid (LPA) on the EPR spectra of cobalt-disubstituted aminopeptidase. The EPR spectra of [CoCo(AAP)] are shown before (A) and after (B) the addition of LPA. The parallel mode ($B_0 \parallel B_1$) EPR spectra are also shown for [CoCo(AAP)] before (C) and after (D) the addition of LPA.

jority of enzyme molecules exhibit either ferromagnetic or antiferromagnetic spin-spin coupling.

The nature of the bridging moiety was addressed by parallel mode EPR. With $B_0 \parallel B_1$, a signal (Fig. 2) was observed that appeared upon the addition of > 1 equivalent Co(II) and increased linearly until two equivalents of Co(II) had been added. In addition, the signal was highly pH dependent and decreased in intensity upon raising the pH above 7.5 and could not be observed at all at pH 10 (Bennett & Holz, 1997a). The signal due to the heterodimetallic [CoZn(AAP)] also displayed marked pH dependence (Fig. 3). The signals recorded at pH 7.5 (Fig. 3A) and pH 10.0 (Fig. 3C) are complex and distinct. However, both signals could be simulated (Fig. 3B and 3D; simulation parameters are given in the figure legend) assuming mixtures of the same two species, shown as Fig 3E and 3F. At pH 7.5, the predominant species is a broad, axial species (Fig. 3E) with low *E/D* (0.1) and sufficient strain to render unresolvable any ^{59}Co hyperfine splitting. In contrast, the predominant signal at pH 10.0 is a rhombic (*E/D* = 0.32) species with very well resolved ^{59}Co hyperfine lines (Fig. 3F). These data suggest a pH-dependent change in geometry with a pK_a around 9. Similarly, the pH dependence of the intensity of the $B_0 \parallel B_1$ signal due to [CoCo(AAP)] suggests a pH-dependent change in the inter-cobalt bridging moiety. These data are consistent with a change from water to hydroxyl ligation upon increasing pH. At low pH, water forms a μ-aquo bridge. Water is a weak ligand and is thus sufficiently flexible as to allow the Co(II) ion in [CoZn(AAP)] to adopt a coordination with a fairly high degree of axial symmetry (low *E/D*). However, as a weak ligand, its coordination geometry is poorly defined and this heterogeneity results in high strains and loss of resolution of the ^{59}Co hyperfine splitting in the dominant species. In [CoCo(AAP)] at low pH, the bridging water ligand is weak enough to allow at least some of the Co-μ(H_2O)-Co moieties to adopt a conformation with a Co-O-Co angle sufficiently acute as to result in ferromagnetic coupling between the two Co ions, which then exhibit a parallel-mode, ground state (Bennett & Holz, 1997a) integer spin EPR signal. At high pH, however, the much stronger hydroxyl ligand imposes constraints upon the geometry of the single Co of [CoZn(AAP)], resulting in a highly constrained (low strain, resolved hyperfine), low symmetry (*E/D* = 0.32) environment. In [CoCo(AAP)], the μ-OH bridge effectively mediates strong anti-

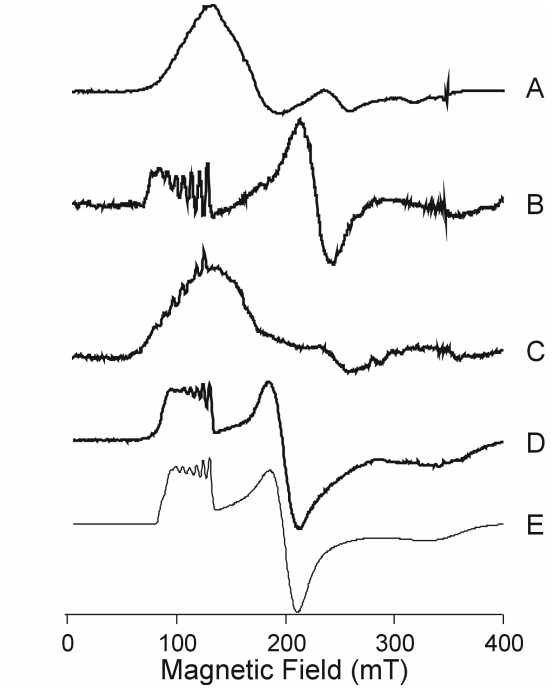

Fig. 8. The effect of L-leucine phosphonic acid (LPA) on the EPR spectra of cobalt-substituted heterodimetallic forms of aminopeptidase. The EPR spectra of [CoZn(AAP)] are shown before (A) and after (B) the addition of LPA. The EPR spectra of the distinct [ZnCo(AAP)] are also shown before (C) and after (D) the addition of LPA. The simulation E assumes the parameters $g_{\text{eff.(x,y,z)}}$ = 1.95, 3.40, 6.23 (M_S = |±1/2⟩, $g_{\text{real(x,y,z)}}$ = 2.20, 2.20, 2.48, E/D = 0.20); $A_z(^{59}\text{Co})$ = 4.95 mT.

ferromagnetic interaction between the two Co ions and the $B_0 \parallel B_1$ signal is lost.

CHARACTERIZATION OF SUBSTRATE BINDING: STUDIES ON THE INHIBITOR 1-BUTANEBORONIC ACID

The inhibitor 1-butaneboronic acid (BuBA; Fig 4A) is a potent inhibitor of native [ZnZn(AAP)]. The mode of binding of BuBA was investigated by EPR spectroscopy of [CoCo(AAP)] and of the heterodimetallic substituted forms [CoZn(AAP)] and [ZnCo(AAP)]. Upon addition of BuBA to [CoCo(AAP)], signal intensity corresponding to two equivalents of Co(II) was restored in the $B_0 \perp B_1$ EPR mode (Bennett & Holz, 1997b) and the $B_0 \parallel B_1$signal was abolished (Fig. 5A and 5B). Thus it appears that the μ-$OH_{(1, 2)}$ bridge is broken upon binding of BuBA. To investigate whether BuBA interacts with a single distinct Co, with either of the Co ions or with both, the EPR spectra of [CoZn(AAP)] and [ZnCo(AAP)] were recorded before and after addition of BuBA. Upon addition of BuBA to [CoZn(AAP)] the resting spectrum (Fig. 5C) changed markedly (Fig. 5E). Particularly obvious was a new derivative feature at $g \sim 3.15$ (~ 200 mT). Subtraction of Fig. 5C from 5F revealed a novel, BuBA-dependent rhombic species that was best simulated assuming $g_{\text{eff.(x,y,z)}}$ = 2.08, 3.15, 6.15 (M_S = |±1/2⟩, g_{real} = 2.41, E/D = 0.22) and $A_z(^{59}\text{Co})$ = 4.0 mT (Bennett & Holz, 1997b). Incorporation of this species results in an excellent simulation for the spectrum of BuBA-treated [CoZn(AAP)] (Fig. 5F). In contrast, the spectrum of [ZnCo(AAP)] (Fig. 5G) was unaffected by the addition of BuBA. Therefore it appears clear from the EPR data that BuBA binds specifically to the metal ion labelled "Zn1" in Fig. 1 and, in doing so, breaks the μ-$OH_{(1, 2)}$ bridge. Thus, the BuBA-inhibited species appeared to mimic the substrate-bound species. However, the small $A_z(^{59}\text{Co})$ of 4.0 mT (cf. 7.2 mT in the resting enzyme) suggests that BuBA is not merely binding *via* a single oxygen but that an electrophilic moiety such as a ring structure is formed, as shown in Fig 6A. Consistent with this asymmetric structure was the rhombicity (E/D = 0.22) of the BuBA-dependent EPR species. Thus the BuBA-bound form may represent a species beyond the substrate-bound form on the reaction coordinate. This proposed mode of binding (Bennett & Holz, 1997b) was subsequently confirmed in the native [ZnZn(AAP)] by X-ray crystallographic analysis (De Paola *et al*., 1999).

CHARACTERIZATION OF A TRANSITION STATE INHIBITOR, L-LEUCINE PHOSPHONIC ACID

L-leucine phosphonic acid (LPA; Fig 4B) is a well-characterized transition-state inhibitor of the AAP-related bovine lens leucine aminopeptidase (BLLAP) and binds to form a complex in which one of the phosphate oxygen atoms forms a bridge between the two metal ions, a second oxygen interacts with the metal ion analogous to Zn1 of Fig. 1 and the N-terminal amine interacts with Zn2 (Sträter and Lipscomb (1995). For an analogous structure in [CoCo(AAP)], one would expect a strongly antiferromagnetically-coupled system and, therefore, no EPR signals. Upon adding LPA to [CoCo(AAP)], the $B_0 \perp B_1$ signal (Fig. 7A) was indeed lost (Fig. 7B). However, the weak $B_0 \parallel B_1$ signal from [CoCo(AAP)] at $g \sim 12$ (Fig. 7C) was replaced by an intense ground-state signal with $g \sim 10$ (Fig. 10D). These data suggest strong ferromagnetic coupling in the LPA complex of AAP

and a different bridging structure to that in BLLAP. In particular, there cannot be a single phosphonyl-derived oxygen atom bridging the metal ions. Studies of the heterodisubstituted [CoZn(AAP)] and [ZnCo(AAP)] provided more information. At pH 7.5, the spectrum of [CoZn(AAP)] (Fig. 8A) is dominated by a broad axial species and contains a minor rhombic species. Upon addition of LPA, however, a single species was observed (Fig 8B) with $g_{\mathrm{eff.(x,y,z)}} = 1.90,\ 3.05,\ 6.66$ ($M_S = |\pm 1/2\rangle$, $g_{\mathrm{real}} = 2.53$, $E/D = 0.26$) and $A_z(^{59}\mathrm{Co}) = 7.0$ mT (Bennett & Holz, 1998; Stamper *et al.*, 2001). Similarly, the complex spectrum of [ZnCo(AAP)] (Fig. 8C) was replaced by a single rhombic species (Fig. 8D) that was readily simulated (Fig. 8E). The data show that LPA interacts with both metal ions and that a bridge is formed between the metal ions which mediates ferromagnetic rather than antiferromagnetic coupling. This is highly unlikely to be a single atom bridge. In contrast to BLLAP, the EPR data suggests a μ-phosphonate bridge in the LPA complex of AAP, as shown in Fig. 6B. Further structural conclusions can be derived from the EPR data: while the spectrum of [CoZn(AAP)]-LPA is similar to the rhombic species of naked [CoZn(AAP)], the simulation of [ZnCo(AAP)] indicates a hyperfine interaction of only 4.95 mT compared to 7.2 mT in naked [ZnCo(AAP)] (Bennett & Holz, 1997b). Thus, as with BuBA, an electrophilic structure or ligand must be bound that allows significant delocalisation of the paramagnetic electron density. By analogy with BLLAP, it was proposed (Bennett & Holz, 1998) that the aminogroup of LPA was also bound to the metal ion corresponding to Zn1 of Fig. 1, thus forming a five membered ring. Subsequent studies, including X-ray crystallographic analysis, have since shown this structure to indeed be correct (Stamper *et al.*, 2001).

DISCUSSION

The studies reviewed herein serve to highlight the potential of extracting structural information from EPR of cobalt-substituted metalloproteins. EPR of inorganic Co(II) high-spin complexes in solution often yields little information due to the high symmetry coordination. However, enzyme active sites often contain metals in high energy, low symmetry environments. The consequent reduction in strain-induced line-broadening and the separation of $g_{\mathrm{eff.}}$ values can yield spectra rich in information. The use of parallel-mode EPR is invaluable in studying polynuclear sites. The structural information derived from EPR and since confirmed by X-ray crystallography has been useful in two respects. Using inhibitors such as BuBA and LPA, which form complexes that mimic transient species in the catalytic pathway, a detailed mechanism for the enzyme was proposed and has been refined (Bennett & Holz 1997a, 1997b, 1998; De Paola *et al.* 1999; Stamper *et al.* 2001). Second, an understanding of how inhibitors interact with the active sites of this class of enzymes has led to a systematic search for new inhibitors and is now leading to rational design of potent inhibitors with potential chemotherapeutic application (Ustynyuk *et al.*, 1999; Huntington *et al*., 1999; Bienvenue *et al.*, 2000).

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