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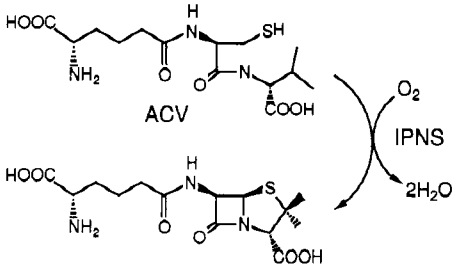
Communications

Coordination Chemistry of the Metal Binding Site of Isopenicillin N Synthase

Isopenicillin N synthase (IPNS) is a non-heme Fe(II)-dependent enzyme found in β-lactam antibiotic-producing microorganisms that catalyzes the formation of isopenicillin N from δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV).[1] Unlike the reactions



Isopenicillin N

catalyzed by the Fe(II)-containing dioxygenases which incorporate the elements of dioxygen into their substrates,[2] the two oxidative ring closures of ACV forming β-lactam and thiazolidine rings catalyzed by IPNS result in the complete four-electron reduction of 1 equiv of dioxygen to 2 equiv of water.[1] Evidence for exogenous ligand binding has been derived from EPR, Mössbauer, and electronic spectral studies of Fe-IPNS and its substrate and NO complexes, where sites for NO (as an analogue for O_2), substrate, and solvent (H_2O) are inferred.[3] In this communication, we take advantage of the different spectroscopic properties of Fe(II), Co(II), and Cu(II) to probe the metal binding site of IPNS and present insights into the IPNS metal coordination environment and the nature of its endogenous ligands.

Metal-reconstituted IPNS can be obtained by treating apo-IPNS[4] with 1 equiv of Fe(II), Cu(II), or Co(II). That all three ions bind reversibly to the same site is suggested by the inhibition of Fe-IPNS activity by the presence of 100-fold excess Cu(II) or Co(II) and the recovery of activity by prolonged incubation of Cu- and Co-IPNS with 100-fold excess Fe(II) under inert atmosphere. While the electronic spectrum of Fe-IPNS is featureless in the visible region,[3] that of Co-IPNS shows a weak absorption band at 490 nm (ϵ = 35 M^{-1} cm^{-1}) and two shoulders at 475 and 510 nm. The energy and the low absorptivity as well as the shape of the bands are very similar to those of two Co(II)-substituted metalloenzymes, Co(II)-iron alcohol dehydrogenase[5a] and Co(II)-glyoxalase I,[5b] and are consistent with ligand field transitions of six-coordinate Co(II) complexes and metalloproteins.[5,6] Similarly, the electronic spectrum of Cu-IPNS (Figure 1) shows a broad d–d band at 654 nm (77 M^{-1} cm^{-1}) consistent with a five- or six-coordinate Cu(II) site as observed in Cu(II) complexes and proteins.[6] The X-band EPR spectrum of Cu-IPNS (Figure 2A) shows a tetragonally distorted copper binding site with some site heterogeneity and can be classified as arising from a type 2 copper center.[7] Taken together, the data strongly suggest a six-coordinate metal binding site for IPNS.

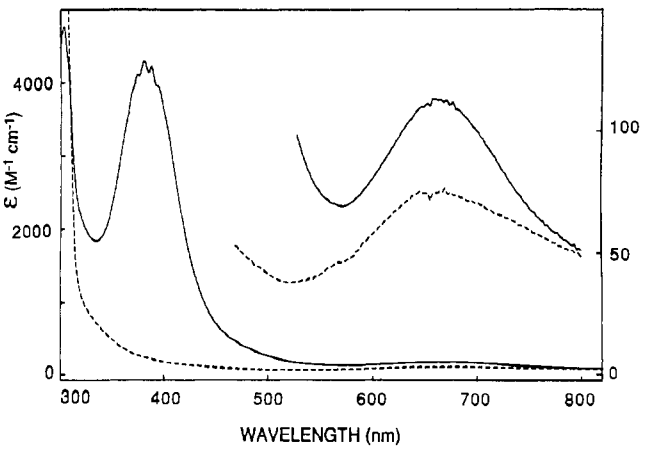


Figure 1. Electronic spectra (HP 8451A diode-array spectrophotometer) of Cu-IPNS (dashed line) and the Cu-IPNS–ACV complex (solid line) in 0.1 M MOPS at pH 7.1 and ambient temperature.

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(5) Electronic data: Co-iron alcohol dehydrogenase,[5a] ϵ_{510} = 31.5 M^{-1} cm^{-1} (shoulders at 469 and 526 nm); Co-glyoxalase I,[5b] ϵ_{493} = 35 M^{-1} cm^{-1} (shoulders at 465 and 515 nm); Co-conalbumin,[5c] ϵ_{556} = 22 M^{-1} cm^{-1}; Co-enolase,[5d] ϵ_{545} = 34 M^{-1} cm^{-1}; Co(ethyleneimine)$_6^{2+}$,[5e] ϵ_{505} = 23 M^{-1} cm^{-1}; Co(py)$_2$(CHCl$_2$COO)$_2$,[5f] ϵ_{518} ~20 M^{-1} cm^{-1}. (a) Bakshi, E. N.; Tse, P.; Murray, K. S.; Hanson, G. R.; Scopes, R. K.; Wedd, A. G. *J. Am. Chem. Soc.* **1989**, *111*, 8707–8713. (b) Sellin, S.; Eriksson, L. E. G.; Aronsson, A.-C.; Mannervik, B. *J. Biol. Chem.* **1983**, *258*, 2091–2093. (c) Bertini, I.; Luchinat, C. *Adv. Inorg. Biochem.* **1984**, *6*, 71–111. (d) Rose, S. L.; Dickinson, L. C.; Westhead, E. W. *J. Biol. Chem.* **1984**, *259*, 4405–4413. (e) Kiser, R. W.; Lapp, T. W. *Inorg. Chem.* **1962**, *1*, 401–404. (f) Lever, A. B. P.; Ogden, D. *J. Chem. Soc. A* **1967**, 2041–2048.

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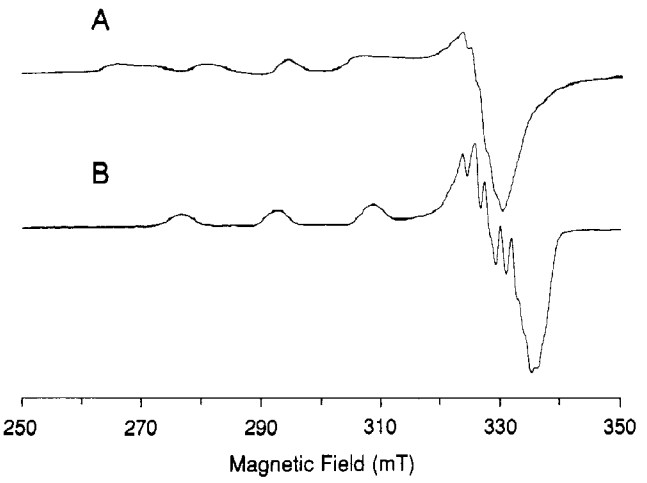


Figure 2. X-Band EPR spectra (Bruker ESP 300) of (A) Cu-IPNS ($g_{\parallel a}$ = 2.35, $g_{\parallel b}$ = 2.34, $g_{\perp}$ = 2.07, $A_{\parallel a} \sim 132$ G, $A_{\parallel b} \sim 110$ G) and (B) the Cu-IPNS–ACV complex ($g_{\parallel}$ = 2.24, $g_{\perp}$ = 2.05, $A_{\parallel}$ = 160 G) in 0.1 M MOPS at pH 7.1 and 100 K. EPR conditions: microwave frequency, 9.42 GHz; power, 1 mW; modulation amplitude, 1 G. Note that the site heterogeneity in Cu-IPNS is removed and the signals are sharpened by substrate binding.

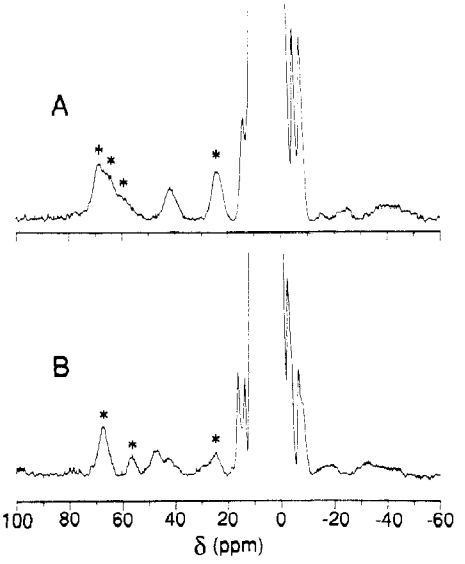
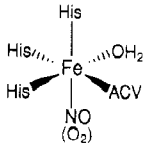


Figure 3. Isotropically shifted ^{1}H NMR spectra (IBM NR/300 at 300 MHz, 10 °C) of ~2 mM (A) Fe-IPNS and (B) Fe-IPNS–ACV complex under argon in 0.1 M MOPS at pH 7.1. The signals marked by asterisks are solvent exchangeable.

The nature of the active site ligands can be probed by magnetic resonance techniques.[7,8] The ^{1}H NMR spectrum of Fe-IPNS under argon shows signals centered near 65 (~3 H), 42 (~2 H), and 24 (~2 H) ppm, of which only the 42 ppm feature remains in D_2O solution (Figure 3A). This spectrum remains unchanged for hours even when Fe-IPNS is exposed to air. The solvent exchangeable 65 ppm resonances may be assigned to the imidazole NH protons of ligated histidines on the basis of the chemical shifts of Fe(II)–imidazole complexes and proteins.[9] A similar solvent-exchangeable signal is observed at 78 ppm for Co-IPNS in the range expected for Co(II)–imidazole interactions.[8] The ^{1}H NMR spectra of Fe-IPNS and Co-IPNS are both perturbed by the presence of 5 mM ACV (Incell Co., Milwaukee, WI) under inert atmosphere, indicating that ACV may bind to the enzyme prior to O_2 (or NO) binding. The histidyl imidazole N–H signals of Fe-IPNS are split at 66 and 56 ppm with a 2:1 intensity ratio (Figure 3B). Similarly, the histidyl imidazole N–H signals in Co-IPNS are split into three features at 94, 81, and 79 ppm when substrate binds. None of these features is observed in the solution of Fe(II) or Co(II) with ACV in the absence of apo-IPNS. These observations suggest that three histidines are involved in the metal binding site of IPNS and that substrate significantly perturbs the metal site. The assignment of the other isotropically shifted ^{1}H NMR signals of Fe-IPNS and Co-IPNS is not straightforward and is under further investigation.

Dramatic changes also occur in the electronic and EPR spectra of Cu-IPNS in the presence of 5 mM ACV (Figures 1 and 2). The d–d transition shifts to 664 nm and increases in intensity (115 M^{-1} cm^{-1}). In addition, a new intense absorption appears at 385 nm (4050 M^{-1} cm^{-1}), which may be assigned to an RS $\rightarrow$ Cu charge-transfer band by analogy to features found in Cu(II)–SR complexes,[10] such as Cu(tet b)(o-SC_6H_4COO),[10a] and the HS^- complex of plasma amine oxidase.[11a] The EPR spectrum of the Cu-IPNS–ACV complex shows a shift of $g_{\parallel}$ from 2.35 to 2.24 and an increase of $A_{\parallel}$ to 160 G (Figure 2B). Both changes are consistent with thiolate binding to the Cu(II) in Cu-IPNS and analogous to the effect of RS^- or diethyldithiocarbamate binding to several type 2 copper proteins.[11] The observed perturbations also indicate that ACV may interact with the Cu(II) at an equatorial site, since both $g_{\parallel}$ and $A_{\parallel}$ are relatively insensitive to axial perturbations.[12] The spectral changes induced by ACV binding to Cu-IPNS along with the 0.2 mm/s decrease in the Mössbauer isomer shift of Fe-IPNS upon ACV binding[3] strongly implicate the coordination of thiolate to the metal center.

When taken together with the observations on Fe-IPNS and its complexes with ACV and NO,[3] the present data indicate a six-coordinate metal center with three endogenous histidine ligands and sites for the thiolate of ACV, NO (or O_2 by analogy), and solvent. The following model is proposed as a working hypothesis for the active site of IPNS:



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