available in the titration of Me_2SO-d_6 with H_2O.[22] As more H_2O is added, H_2O and Me_2SO-d_6 compete for NH hydrogen bond donors. These effects are under investigation in our laboratories with compounds containing stable hydrogen bonds and linear peptides that do not form stable intramolecular hydrogen bonds.

The temperature dependence of chemical shifts arises from an increase in molecular motion which disrupts peptide hydrogen bonds to solvent as the temperature is increased. Hydrogen bonding deshields protons; thus, a reduction in the time the proton is hydrogen bonded causes an upfield shift of its resonance. The magnitude of temperature coefficients increases as H_2O is added to Me_2SO-d_6 (Figure 6). This implies that hydrogen bonds from the cyclic peptide to solvent molecules in solutions of Me_2SO-d_6 plus H_2O are disrupted more easily than those in pure Me_2SO-d_6.

Several of the proposed important conformations suggested for enkephalins cannot be obtained with this constrained cyclic analogue as determined by CPK model studies.[5] The bend models described by Roques et al.[23] and Jones et al.[24] and the antiparallel Phe to Tyr hydrogen bonding pattern observed by crystallography[25] cannot be formed by this analogue. We further find that the β-bend structure proposed by Bradbury et al.[26] containing a hydrogen bond between the Phe NH and Tyr CO is not observed.

The computer simulations suggest some interesting conformational features involving side chains and backbone atoms external to the ring. A hydrogen bond between Tyr CO and Gly NH is seen in low-energy conformations although it is not substantiated by NMR solution data. The effect of solvation of the Tyr side chain and the charged amino terminus prevents the formation of this hydrogen bond in Me_2SO-d_6 and mixed H_2O/Me_2SO-d_6 solutions. Hydrogen bonding between the Tyr OH and Gly CO has also been observed in similar isomers (unpublished data). Although the biological significance of such structures cannot easily be probed experimentally, their importance may lie in defining conformations of the enkephalin at a receptor site.

Conclusions

We have investigated the dynamics and conformations of a 14-membered cyclic enkephalin analogue by proton NMR and computer simulations. Elucidation of hydrogen bonds has provided a framework for the three-dimensional shape of the ring without the ambiguity of torsions calculated from coupling constants. The approximate conformations of the compound in Me_2SO-d_6 and mixed H_2O/Me_2SO-d_6 solutions have been deduced by combining experimental NMR results and theoretical computer calculations. A double hydrogen bond structure is found in pure Me_2SO-d_6 with Leu NH bound to Gly CO and D-A_2bu side-chain NH bound to either D-A_2bu CO or Phe CO or a combination thereof. The preferred conformation in mixed H_2O/Me_2SO-d_6 solutions is similar, but the hydrogen bond involving Leu NH is disrupted. The severe constraints on this cyclic peptide make it an important compound. Novel peptide opiates are being synthesized in our laboratories which incorporate additional constraints to obtain an essentially rigid biologically active enkephalin.

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Electronic Structures of Active Site Models for Compounds I and II of Peroxidase

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Abstract: Xα multiple scattering calculations are reported for the d^4 oxo complexes $Fe(P)(py)(O)^{0,1+}$ (P = porphine, py = pyridine), models for the active sites of intermediates I and II of horseradish peroxidase. Orbital energy trends for the addition of pyridine and then oxygen to Fe(II) porphine are used to rationalize spin and oxidation states of these clusters. Particular attention is paid to comparisons with ENDOR spectral data on intermediate I. Our results suggest that both σ and π spin transfer to the ligands is important and that the spins localized at the FeO and porphyrin sites interact strongly.

Peroxidases are hemoprotein enzymes which carry out a variety of biological functions calling for the use of peroxide as an oxidant.[2] Two spectrally distinct intermediates (HRP-I and HRP-II) have been observed on treatment of horseradish peroxidase with peroxide. A model for these intermediates involving a low-spin ferryl (FeO^{2+}) moiety is supported by electron nuclear double resonance (ENDOR),[3] Mossbauer,[4] and NMR[5] experiments, as well as by

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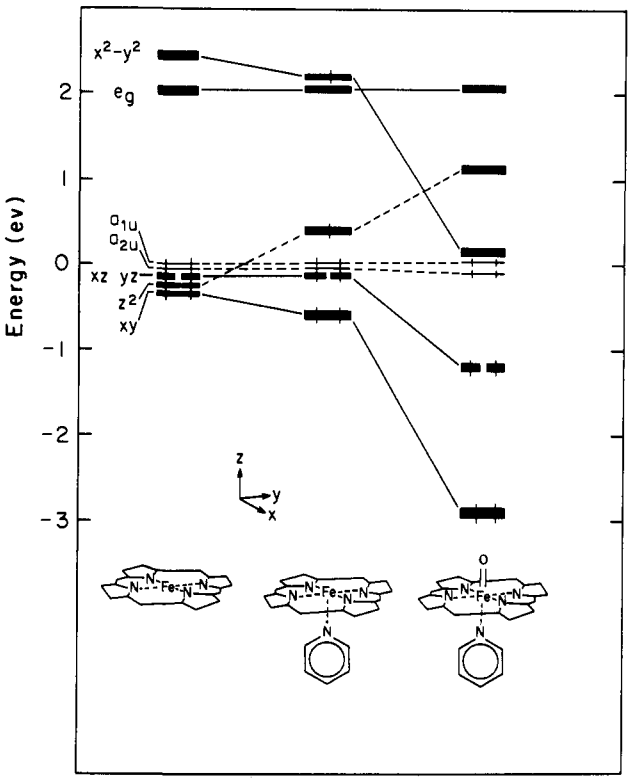

Figure 1. Orbital energy diagram for $Fe(P)^0$, $Fe(P)(py)^0$, and $Fe(P)(py)(O)^0$. Energies are in eV relative to the highest filled porphine π orbital. Only the metal d and porphine a_{1u}, a_{2u}, and e_g π orbitals are shown.

spectral and chemical similarities with synthetic analogues containing ferryl porphyrins.[6] Here we examine the description of the electronic structure of these compounds provided by the Xα multiple-scattering method.

Earlier quantum mechanical studies of these intermediates have included extended Hückel (EHT) calculations[7] and unrestricted Hartree–Fock (UHF) studies at the INDO[8] and ab initio[9] levels. Our studies on metalloporphyrins[10] have shown that the Xα method can provide results that complement other types of calculations, often providing possible alternative explanations of experimental data. This happens here for the hyperfine interactions in HRP-I.

Details of the multiple scattering procedure have been given in our earlier papers[10] and in recent reviews.[11] Our active site model Fe(P)(py)(O) (P = porphine, py = pyridine) is shown in Figure 1, along with orbital energy diagrams describing the effect of adding first pyridine and then oxygen to a four-coordinate Fe(II) porphine.[12] Compared to earlier EHT calculations, our Fe 3d orbitals are lower in energy relative to the π orbitals of the porphyrin, by about 2.5 eV for the nearly pure dxy, d_{z^2} and $d_{x^2-y^2}$ orbitals, and by about half that for the more covalent dπ (*xz*,*yz*) orbitals. Differences of this sort have been noted in earlier calculations,[10] and it is not clear which level ordering is the more correct. Our dπ orbitals mix more with occupied $e_g(\pi)$ orbitals of the porphyrin. Thus, although the Fe character of the dπ orbital is nearly the same in the Xα (37%) and EHT (42%) calculations, the remaining density is more delocalized onto the porphyrin, with 42% O and 21% porphine for the Xα calculation, compared to 50% and 8% for extended Hückel.[7a]

The magnetic properties of HRP-I are usually interpreted in terms of a coupling between two spin systems, one at the iron center which has $S = 1$ and a zero-field splitting of $D = 25$ cm^{-1} and the other an a_{2u} porphyrin radical, with $S = {}^1/_2$.[4a] Since nonrelativistic molecular orbital calculations do not contain the spin–orbit effects that lead to the zero-field splitting, it is not a simple matter to make direct comparisons with observed hyperfine interactions. Nevertheless, the spin distributions calculated for a simple $S = {}^3/_2$ state with three unpaired electrons (two in dπ and one in a_{2u}) should give some insight into the origins of the observed interactions. There are several interesting features of our spin distributions:

(i) The partially occupied dπ orbitals contain 0.1% s character on the porphyrin nitrogens. By itself this s population would lead to a Fermi contact term of 4 MHz (for a spin Hamiltonian with $S = {}^1/_2$), compared to the observed value of 7.2 MHz.[3] Additional nitrogen hyperfine interactions will come from polarization effects arising from the spin in its pπ orbital; our value of 7.1% for this population is slightly higher than the 4.3–6.0% empirical estimate based on the ENDOR observations.[3]

(ii) Our a_{2u} orbital has 0.1% s character on the axial pyridine nitrogen, along with about 0.3% pσ character. This amount of s character would result in a Fermi term of 3 MHz, consistent with line-broadening effects seen in ESR studies of proteins and model complexes.[7c] The EHT results show a larger total spin population (about 5.7%), but no separation into s and p character was reported.[7c]

(iii) We find significant π spin population (5.6%) at the Cβ positions, arising in about equal amounts from the a_{2u} and dπ orbitals. This is in contrast to both the EHT and the UHF calculations, which predict very small (<0.3%) spin populations at Cβ, but is consistent with an average spin population of about 3.6% estimated from ENDOR measurements. Again, spin transfer from the dπ orbitals on the FeO unit into the porphyrin appears to be important in explaining the observed hyperfine tensors. Our π spin population for the meso carbons (14.3%) is also consistent with the assignment of the proton ENDOR signals seen at 9.26 and 11.90 MHz to meso protons.[13]

(iv) ^{17}O ENDOR signals have also been detected for HRP-I, suggesting that about 25% of the spin at the FeO center is located at the oxygen site.[3] Our oxygen pπ spin population (~42%) is larger than this empirical estimate, but it is smaller than the EHT value of 50%. In spite of the fact that the two dπ orbitals are split by only 0.03 eV, we see a large difference between the oxygen p_x and p_y densities (39% and 46%, respectively). This is at odds with the EHT results and with the observed axial character of the ^{17}O hyperfine tensor.

In summary, the results presented here offer a qualitatively satisfactory model for the hyperfine interactions observed for HRP-I. We invoke spin populations arising from an a_{2u} porphyrin radical and an antibonding FeO dπ/pπ orbital, where the former may be delocalized onto the axial pyridine (or imidazole) ligand and the latter into the porphyrin moiety. We note that this

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(13) This estimate assumes a value of −63 MHz for the proportionality factor relating a carbon π spin population to the hyperfine interaction at a neighboring proton; see ref 3.

provides a reasonable explanation for the observed Cβ spin densities, and that it is important to distinguish s and p populations when estimating hyperfine interactions for the nitrogens. Because of the strong interaction between the two spin subsystems, it may be an oversimplification to label HRP-I as an "a_{2u} cation radical".[14] The differences in π spin populations between the present calculations and earlier ones may not be significant, but the possible consequences of significant mixing between the FeO and porphyrin spin systems need to be considered when empirical assignments are made. Further studies on these systems, incorporating spin-polarization and spin–orbit effects, are in progress.[15]

Registry No. $Fe(P)^0$, 32647-22-6; $Fe(P)(py)^0$, 60817-63-2; $Fe(P)(py)(O)^0$, 96453-16-6; peroxidase, 9003-99-0.

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Substrate Analogue Binding to the Coupled Binuclear Copper Active Site in Tyrosinase

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Abstract: Chemical and spectroscopic studies are presented for the binding of a series of carboxylate competitive inhibitors to the oxy ($[2Cu^{II}–O_2^{2-}]$), met ($[2Cu^{II}]$), and half-met ($[Cu^{II}Cu^{I}]$) derivatives of the binuclear copper active site in tyrosinase. These inhibitors are found to divide into two groups: (1) poor inhibitors, which show an equilibrium constant for binding to the enzyme similar to that for binding to aqueous Cu(II) complexes, and (2) good inhibitors, which are substrate analogues in that the carboxylate is conjugated into an aromatic ring, producing a planar structure and which bind with an equilibrium constant higher by an order of magnitude relative to aqueous copper. Associated with this increased stability are unusual Cu(II) spectral features which relate to a difference in the geometry of substrate binding to the copper site. The poor inhibitors produce normal EPR, absorption, and CD spectral features typical of tetragonal Cu(II) in a square-pyramidal structure with the Cu displaced by ~0.3 Å toward the axial ligand. Alternatively, the unusual spectral features associated with good competitive inhibitor binding to the copper site (large rhombic splitting of the $g_\perp$ region, large hyperfine splitting of the lowest g value, and a low energy (<10 000 cm^{-1}) transition in the CD spectrum) result from a significant distortion of the Cu(II) site toward a trigonal bipyramidal geometry along the C_s distortion coordinate for associative ligand substitution reactions of square-planar and tetragonal complexes. A ligand field analysis of this coordinate is presented and used to estimate the geometry of this substrate-bound active site complex. From this analysis, the protein pocket appears to contribute to the stabilization of substrate analogue binding in a geometry which is midway along this reaction coordinate. The contribution of this substrate–protein interaction to the ortho-hydroxylation reaction of oxytyrosinase is discussed, and possible electronic pathways for this reaction are considered, based on the geometric and electronic structure of the spectroscopically effective model for the ternary complex formed by substrate, dioxygen, and the binuclear copper active site.

Tyrosinase contains a coupled binuclear copper site which catalyzes the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the two-electron oxidation of *o*-diphenols to *o*-quinones (catecholase activity). Isotopic studies have demonstrated that the oxygen atom incorporated into the monophenol during oxidation comes from molecular oxygen.[1] Chemical and spectroscopic studies of tyrosinase have shown that its coupled binuclear copper active site is very similar to that found in the hemocyanins.[2] This binuclear copper site can be prepared in a variety of forms, and the systematic study[3] of these derivatives has greatly added to our understanding of the geometric and electronic structure of this active site.

In the oxygenated form, the spectroscopically effective model of the active site contains two tetragonal Cu(II) ions with nitrogen and oxygen ligation. These coppers are bridged by an endogenous group (phenolate, hydroxide, or alkoxide) which provides antiferromagnetic coupling between them and results in the lack of an EPR signal.[4] In addition, the exogenous oxygen molecule is bound as peroxide,[2a] also bridging the coppers in a μ-1,2 geometry.[2b] Peroxide bound in this mode produces a distinct $O_2^{2-} \rightarrow$ Cu(II) charge-transfer spectrum which can be correlated to the optical features of the oxy enzyme and includes an extremely intense absorption band centered at 350 nm (ϵ = 26 000 M^{-1} cm^{-1}).[3]

Mettyrosinase, like the oxy form, contains two tetragonal copper(II) ions antiferromagnetically coupled through an endogenous bridge, but exogenous ligands other than peroxide are bound to the copper site.[2b,3] This derivative can be converted by addition of peroxide to oxytyrosinase, which in turn decays back to mettyrosinase upon loss of peroxide. The resting form of tyrosinase is found to be a mixture of ≥85% met and ≤15% oxy.[4] In addition, a half met derivative can be prepared which contains the two coppers in a mixed valence oxidation state $[Cu^{I}–Cu^{II}]$ and which is EPR detectable. The EPR and optical spectral features of this form reflect electron delocalization between the coppers

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