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Ligand Photodissociation and Recombination Dynamics of Ferrous Cytochrome *c* Peroxidase at Alkaline pH

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The dynamics associated with ligand photodissociation and ligand binding provide an avenue through which information concerning conformational interactions involving the active site of heme proteins may be obtained. To date, most time-resolved spectroscopic investigations have involved exogenous π-acceptor ligands such as CO, O_2, and NO.[1-3] In fact, until very recently hexacoordinate low-spin hemes with strong-field σ-donor ligands were considered to be largely nonphotolabile. Magda and coworkers[4] have, however, demonstrated that both cytochrome *c* and cytochrome b_5 exhibit photodissociation of nitrogenous ligands on a very fast (<100 ps) time scale. Photodissociation of a σ ligand has also been implicated in the photodynamics of the cytochrome a_3 distal heme pocket in cytochrome *c* oxidase.[5] This study characterizes the dynamics of the low-spin alkaline form of ferrous cytochrome *c* peroxidase[6] (CCP) subsequent to heme photodissociation.

The heme pocket of CCP is specifically designed to catalyze the reduction of H_2O_2 utilizing electrons derived from cytochrome *c*. In addition, the local heme environment is conformationally sensitive to pH,[7-9] temperature,[10] and mutagenesis.[7a,11] Recently

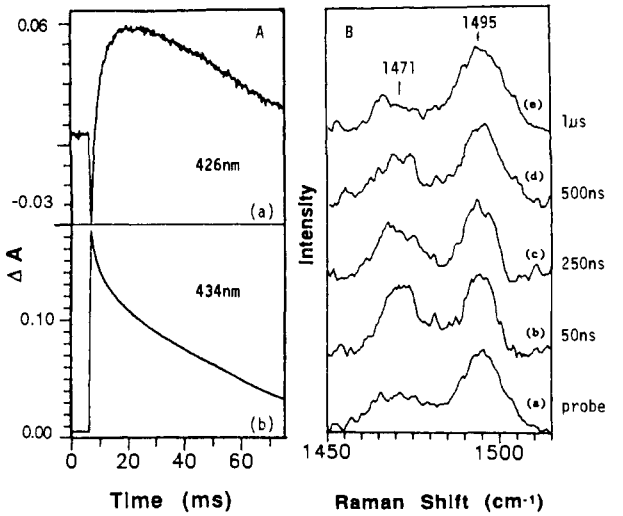

Figure 1. Transient absorption (A) and time-resolved resonance Raman (B) spectra of ferrous CCP in 100-200 mM Tris-HCl, pH = 8.50. (A) Pump laser: output of a Lambda Physik FL 3002 dye laser (at 480 nm) pumped by a Lambda Physik LPX210i excimer laser. Probe light: xenon arc lamp (~75 W) at 426 nm (a) and 434 nm (b). Transient spectra obtained in Soret region are the average of 50 shots. (B) the ν_3 (spin-state-sensitive band) region. Pump laser: Nd:YAG (Quanta-Ray DCR-II) with $\sim 4 \times 10^8$ W/cm^2 at samples (560 nm). Probe laser: output ($\sim 3 \times 10^7$ W/cm^2) of Molectron DL-24 dye laser excited by Molectron UV-24 N_2 laser, excited at 436 nm, which is close to the isosbestic point of 5c/hs and 6c/ls Soret bands of CCP(II). The spectra are the average of several scans with a rate of 10 cm^{-1} min^{-1}. Detailed descriptions of the instrumentation and sample preparation have been published elsewhere.[8,12] in detail; (a) probe only; (b) 50-ns time delay; (c) 250 ns; (d) 500 ns; (e) 1 μs.

it has been demonstrated that the equilibrium hexacoordinate low-spin ferrous form of CCP is photolabile in the pH range 7.8-9.0.[3,7,8] The time-resolved resonance Raman results presented in Figure 1B demonstrate that the photodissociation of a ligand occurs rapidly (<10 ns) and with a high yield.

The photodissociation and ligand recombination are easily followed by monitoring the time evolution of the spin-state-sensitive ν_3 band (Figure 1B). The pentacoordinate high-spin band (appearing at 1471 cm^{-1}) is generated within the 10-ns pump pulse and persists on a time scale of 500 ns. Geminate ligand recombination then occurs, and the transient species reverts back to the hexacoordinate low-spin species (observed by the increase in signal at 1495 cm^{-1}). It is likely that under alkaline conditions the distal His-52 binds to the heme Fe,[7,8] and it is this ligand which is photodissociated by pump laser pulses. The time-resolved resonance Raman data indicate that the recombination of the σ ligand in alkaline CCP(II) is quite slow ($t_{1/2} \sim 500$ ns), relative to the <50-ns half-life observed for geminate recombination in the photodissociation of HbCO or HbO_2[2] and <100 ps for ligand recombination of cytochrome *c*.[4] An endogenous ligand would not be expected to be removed from close proximity to the heme by photolysis. Thus the slow recombination kinetics most likely result from activation energetics rather than ligand mobility away from the heme. We speculate that structural dynamics within the distal heme pocket may play a significant role in these recombination energetics.

Subsequent transient absorption measurements of the alkaline form of CCP(II) indicate that ligand recombination initiates relatively slow and complex conformational dynamics in the protein (Figure 1A). Heme ligand photodissociation is indicated by the

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rapid initial deflection at 426 nm (Soret maximum for alkaline-reduced low-spin CCP), which is followed by an *increase* in absorbance with $t_{1/2} < 1.5$ ms, indicating the presence of a transient species with a higher extinction at this wavelength than the equilibrium low-spin species. This transient then decays with $t_{1/2} < 40$ ms to the equilibrium low-spin species. It can be concluded, however, that this transient consists of a nonequilibrium low-spin heme since the resonance Raman data mentioned earlier demonstrate that ligand recombination is complete within <1 μs. In addition, the multiphasic decay of the 434-nm transient (isosbestic between equilibrium ferrous high- and low-spin CCP) indicates multiple conformational interactions of the protein in response to the rebinding of the σ ligand. These processes are distinct from the ligand recombination itself and may represent more global protein dynamics conformationally linked to the heme active site.

In summary, we have observed that the photodissociation of an endogenous σ ligand from ferrous CCP in the high-pH form induces ligand recombination dynamics that are unique from those of other protein systems. The multiphasic behavior and long time scale involved indicate that these processes are intimately associated with global protein dynamics. This study demonstrates that the conformational flexibility of CCP may be directly linked to the heme active site and hence peroxidase activity.

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A New Approach toward the Inhibition of Ribonucleases: A Water-Stable Ribonucleoside–Technetium Chelate

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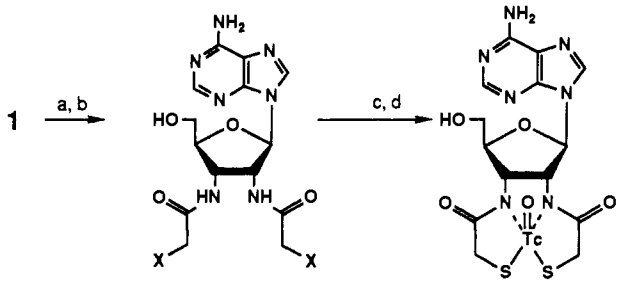
The ribonuclease-mediated hydrolysis of RNA is believed to proceed via a cyclic 2′,3′-monophosphate transition state in which the attacking 2′-hydroxyl and the departing 5′-nucleoside occupy the axial positions of a pentacoordinate trigonal-bipyramidal phosphorane.[1] Since phosphorus does not form a stable pentacoordinate complex, other elements, namely, vanadium,[2a-d] have been used to model the postulated transition state for RNA hydrolysis.[2e] Biophysical studies, including X-ray crystallography, have been performed on the complex formed between vanadate, a ribonucleoside, and RNase A.[3] All the evidence points toward the formation of a trigonal-bipyramidal vanadate ribonucleoside complex bound in the active site of the enzyme. Unfortunately, the complexes exist only when bound to RNAse A and disproportionate in aqueous solution into a complex mixture.[2,4] Recently, a cyclic vanadium(V) chloro alkoxide has been synthesized and the crystal structure of the compound determined.[4] It is unlikely that this complex is stable in aqueous media. Thus, no water-soluble metal-based pentacoordinate analogues that are potent inhibitors of ribonucleases exist. In this communication, we report the synthesis of the first pentacoordinate metallochelate of a ribonucleoside **4** and its potency as an inhibitor of ribonuclease U2.

Scheme I.[a] Synthesis of Ribonuclease Inhibitor **4**



[a] Key: (a) 2 equiv of $(ClCH_2CO)_2O$, CH_3CN, DMF, 0 °C → 25 °C, 12 h; (b) $C_6H_5COS^-Na^+$, EtOH, 78 °C, 3 h, 20% from **1**; (c) EtOH, 5 N NaOH, 78 °C, 5 min; (d) 0.95 equiv of Tc(VII), $Na_2S_2O_4$, 82 °C, 30 min, 30% from **3**.

Table I. Substrate and Inhibition Constants of Ribonuclease U2[a]

substrate	K_m (mM)	V_{max} (pmol/min)	K_i (μM)
ApA	3.1	47.4	134
ApU	18.7	95.8	220

[a] Assays were performed as described.[11]

The inhibitor **4** is a derivative of a class of technetium-99 complexes that are used as imaging agents in nuclear medicine.[5,6] These metallocycles are stable in aqueous solution and exist as pentacoordinate species free in solution.[5-7] Diffractable crystals of one ethylenediamine complex have been obtained and its X-ray structure, independent of protein, solved showing that the parent complex adopts a square-pyramidal geometry.[7] Molecular modeling suggested that if the ethylenediamine is incorporated as part of a ribonucleoside, the resulting constrained complex adopts a geometry that approaches a trigonal bipyramid.[8] The technetium chelate was synthesized from 2′,3′-diamino-2′,3′-dideoxyadenosine, **1**,[9] in three steps (Scheme I), and purified by reverse-phase chromatography on a Sep-Pak C-18 cartridge (Millipore). Analysis of the complex by HPLC (RP C-18) showed the product to be a 2.4:1 mixture of diastereomers.[10]

Addition of the diastereomeric mixture **4** to assays of ribonuclease U2 with diribonucleotide substrates (ApA or ApU) yielded a concentration-dependent inhibition of U2 activity.[11]

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(10) Compound **4** exhibited satisfactory spectral and analytical analyses.

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