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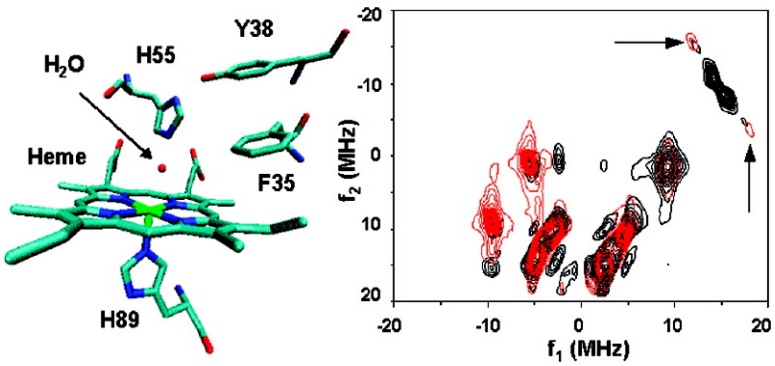

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Substrate Binding Triggers a Switch in the Iron Coordination in Dehaloperoxidase from *Amphitrite ornata*: HYSCORE Experiments

Tatyana I. Smirnova,*,† Ralph T. Weber,‡ Mike F. Davis,† and Stefan Franzen†

Department of Chemistry, North Carolina State University, 2620 Yarbrough Drive, Raleigh, North Carolina 27695, and Bruker BioSpin Corporation, EPR Division, Billerica, Massachusetts 10821

Received September 20, 2007; E-mail: tatyana_smirnova@ncsu.edu

We have explored the effect of substrate binding on the heme iron conformation in the enzyme dehaloperoxidase (DHP) that was first isolated from the terebellid polychaete *Amphitrite ornata* and is now expressed in *Escherichia coli*.[1−3] DHP is a dimeric hemoglobin[4] that also has significant peroxidase activity under physiological conditions.[5] Since hemoglobins and peroxidases require ferrous and ferric oxygen, respectively, one can hypothesize that substrate binding causes a change in protein conformation that affects the spin state of the heme iron. A recent X-ray crystal structure of metaquo DHP resting state[6] shows that water is indeed bound to the heme iron as predicted by spectroscopic measurements.[7−9] In attempting to understand the role of substrate binding, we obtained an indication from hyperfine-shifted NMR that the substrate enters the distal pocket at pH < 7.0. At pH < 7.0, the enzyme turnover is rapid and so is the inactivation of DHP.[2,3] Above pH 7.0, the turnover is significantly slower, but substantially more product is formed. Although an X-ray crystal structure[10] shows a substrate analogue, 4-iodophenol, to be bound in the distal pocket of the hemoglobin, stopped-flow experiments (not shown) indicate that DHP can function without the substrate bound to the internal binding site. The ∼23% occupancy of the substrate analogue 4-iodophenol in the X-ray structure 1EWA[10] suggests a function for the substrate interaction in the distal pocket but is inconclusive whether the distal pocket is an active site of the enzyme. We have advanced a hypothesis that substrate binding acts as a trigger event in the switch from the oxygen binding to the peroxidase function.[1−3] An experimental test correlating changes in the ligation state with the coordination of the ferric heme iron upon substrate binding is considered to be critical for verification of our hypothesis.

Herein we report on continuous wave (CW) EPR and hyperfine sublevel correlation spectroscopic (HYSCORE) analysis of the ferric form of DHP that was undertaken to characterize effects of the binding of 2,4,6-trifluorophenol (TFP) on heme iron coordination. HYSCORE experiments that correlate nuclear frequencies in the two manifolds of the electronic spin are informative for studying the hyperfine interactions of the heme iron with the surrounding nuclei.

Experimental X-band (9.5 GHz) CW EPR spectra of DHP in the absence (dotted line) and the presence (solid line) of a 10-fold excess of TFP at pH 6.0 are shown in Figure 1. The characteristic $g_{\perp}$= 6 and $g_{||}$ = 2 features of the EPR spectra show that the iron exists in a high spin (HS, $S = {}^5/_2$) state in both the absence and presence of the substrate.[14,19] In this respect, DHP resembles metmyoglobin, a known HS ferric heme protein having a six-coordinate ligation. At pH 6.0, there is no change in the spin state; however, the coordination sphere of the heme iron is clearly affected by substrate binding (Figure 1 inset).

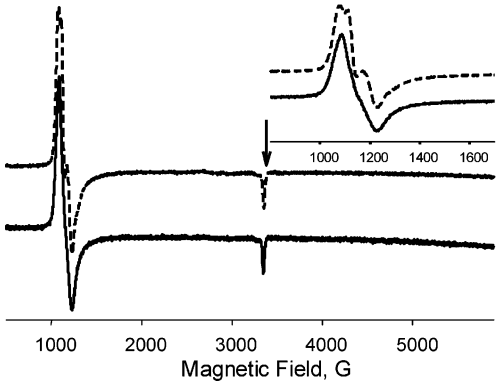

Figure 1. CW X-band (9.5 GHz) EPR spectra of DHP in the absence (dotted line) and in the presence (solid line) of 10-fold excess of TFP at pH 6.0 and T = 4 K. Arrow indicates the field of HYSCORE experiment.

Figure 2 shows HYSCORE spectra of DHP at pH = 6.0 in an H_2O buffer (A), a D_2O (99%) buffer (B), and an H_2O buffer with 10-fold excess of TFP relative to DHP (C). The spectrum of DHP in the second quadrant reveals the signals from strongly coupled nitrogen nuclei at (−9.5, 5.54) MHz that are assigned to a double quantum ($\Delta m_I = \pm 2$, dq) transition. Two less intense peaks at (−4.98, 2.95) and (−4.45, 3.15) MHz arise from single quantum ($\Delta m_I = \pm 1$, sq) transitions. These signals were assigned to four approximately equivalent nitrogen nuclei of the porphyrin ring and another nitrogen of the proximal His89.[11,12] The first quadrant revealed proton signals at 14.8 MHz that span about 6.2 MHz frequency range with a well-defined strong intensity characterized by weaker interactions of 2.5 MHz and lower.

To better understand the origin of the proton signals, a spectrum of DHP prepared in pH 6.0 D_2O buffer was obtained (Figure 2B). For this sample preparation, all of the spectral features in both quadrants remained the same, except the signals corresponding to strongly coupled (6 MHz) proton(s) that disappeared. This indicates that the signal from the weakly coupled proton(s) with interaction of about 2.5 MHz originates from nonexchangeable protein protons. Hyperfine interactions of similar magnitude have been observed for hydrogen atoms in the heme and proximal histidine.[13,14] The disappearance of the strongly coupled 6 MHz proton signal in D_2O buffer (Figure 2B) is attributed to exchangeable hydrogen atom-(s). Previous ENDOR studies of metmyoglobin reported a 6.1 ± 0.1 MHz hyperfine coupling for the protons of a water molecule coordinated to the iron as the sixth ligand,[15,16] which is essentially the same as the 6 MHz signal observed for DHP. Following buffer exchange, we have observed an additional intensity in the first quadrant at (1.83, 2.63) MHz that is consistent with the deuteron signals (ν_D = 2.3 MHz). Unfortunately, for DHP, in both H_2O and D_2O buffers, this quadrant contains a strong spectral feature, extending from 1.5 to 3.8 MHz that prohibits unambiguous

† North Carolina State University.
‡ Bruker BioSpin Corporation.

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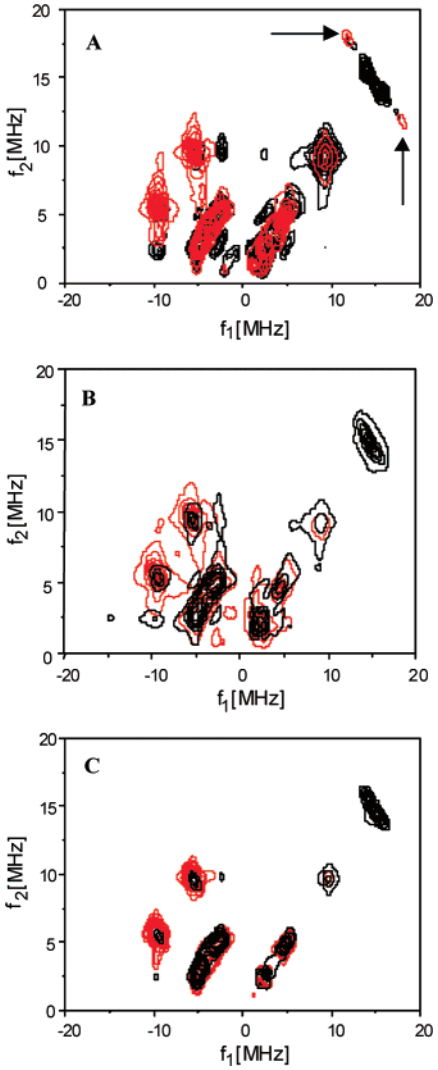

Figure 2. HYSCORE spectra of DHP in pH = 6.0 buffers prepared from H_2O (A) and D_2O (B) and DHP in H_2O buffer with 10-fold excess of TFP (C). The spectra were recorded at magnetic field of 346 mT with τ =128 ns (red trace) and 100 ns (black trace) at T = 4.5 K.

determination of the deuteron nuclei coupling constant. The assignment of the 6 MHz protons to heme-bound water agrees with the X-ray crystal structure[6] and other spectroscopic data.[7−9]

The major change in the pH 6.0 DHP HYSCORE spectra upon TFP substrate binding is the disappearance of the signal from the strongly coupled protons (Figure 2C). We have also observed changes in the positions of nitrogen resonance lines corresponding to a small but measurable increase in the hyperfine coupling constant from approximately 7.5 to 7.7 MHz. On the other hand, although the line shape of the signals from the weakly coupled nonexchangeable protons alters slightly in the presence of TFP, the maximum hyperfine splitting remained similar in all three spectra. The changes in strongly coupled protons demonstrate that the binding of TFP at pH 6.0 results in a displacement of the water molecule and transition from five- to six-coordinated iron. Increase in the hyperfine coupling of nitrogen nuclei of the heme is consistent with the change in the iron coordination.[13] Since no spectral features from ^{19}F were observed, the TFP substrate does not appear to ligate to the heme iron in this process.

The reported HYSCORE data provide information on the molecular mechanism by which substrate binding can alter the function of DHP. The data indicate that the heme-bound water molecule in the resting state of the ferric form is displaced when the substrate binds, at least under the conditions of the experiment. Such behavior can be compared to the effect observed in cytochrome P450cam, where the transition from the low-spin $S = {}^1/_2$ to the high-spin state, observed upon substrate binding, is associated with displacement of a water molecule from the axial ligand position.[17−19] However, in contrast to cytochrome P450cam, displacement of a water molecule from the heme iron of DHP at pH 6.0 does not result in a change of the spin state since the iron is initially in the HS state. Thus, in DHP, the EPR data show a change in rhombicity that is consistent with a change in the coordination without affecting the spin state. The power of HYSCORE is that it shows changes in the coupling of protons on the heme-bound water that further substantiate the hypothesis that substrate binding displaces the water from the heme iron.

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Supporting Information Available: Experimental procedures and HYSCORE spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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