Magnetic Field Dependence of Electron Transfer and the Role of Electron Spin in Heme Enzymes: Horseradish Peroxidase

Marc B. Taraban* and Tatyana V. Leshina

Institute of Chemical Kinetics and Combustion
Novosibirsk-90, 630090 Russia

Mark A. Anderson and Charles B. Grissom*

Department of Chemistry, University of Utah
Salt Lake City, Utah 84112

Received August 28, 1996
Revised Manuscript Received March 5, 1997

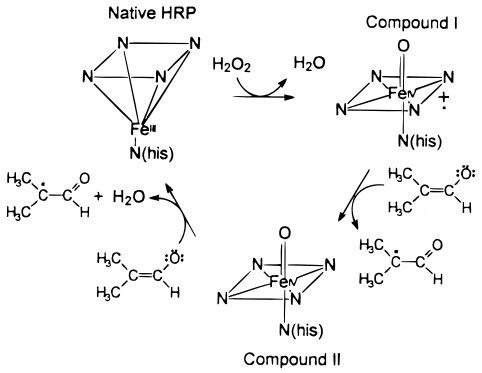
For the first time, kinetic magnetic field effects have been used to study the redox cycling of the paramagnetic intermediates in a heme-containing enzyme. In the horseradish peroxidase (HRP) reaction, the rate of conversion of compound I (HRP-I) to compound II (HRP-II) decreases by 15% at 750 G and the rate of conversion of compound II to native HRP decreases by 35% at 750 G.[1] This is consistent with the reversible formation of a triplet radical pair (RP) in the single electron transfer from diamagnetic substrate to low-spin $S = 1$ heme.

The rate of chemical reactions with radical pair intermediates can be altered by a DC magnetic field of modest magnitude (10−1000 G).[2,3] As a probe of mechanism, this technique has most often been used to change the rate of intersystem crossing (ISC) between singlet and triplet electron spin states in reactions with radical (or, more generally, paramagnetic) intermediates.[2] Recently, we have extended this technique to study paramagnetic intermediates in enzymatic reactions,[3] including the B_{12}-dependent enzyme, ethanolamine ammonia lyase.[4]

Horseradish peroxidase is one of a family of heme-containing enzymes that catalyzes the reduction of peroxides at the expense of electron donation by a variety of organic reductants.[5] Scheme 1 illustrates the catalytic cycle that begins with $2e^-$ oxidation of native ferric-HRP to ferryl-HRP and the porphyrin radical cation, termed compound I.[6] One-electron reduction converts HRP-I to spectroscopically-distinct HRP-II. Substrate binding and $1e^-$ reduction by a second organic molecule completes the catalytic cycle by converting ferryl HRP-II to native ferric HRP. Low-spin Fe(IV) in the HRP-I and HRP-II intermediates, as well as the organic substrate radicals, are paramagnetic species that have multiple electron spin angular momentum states when considered in the enzyme−substrate complex.

Pre-steady-state (stopped-flow) kinetic experiments were carried out with a rapid-scanning stopped-flow spectrophotometer outfitted with an electromagnet surrounding the mixing and observation cell.[7−9] Global fitting analysis of the data allows identification of the spectrally-distinct species HRP-I, HRP-II, and native HRP.[10] [Complete absorbance spectra of HRP, HRP-I, and HRP-II, as well as sample kinetic traces, are given in the Supporting Information.] The kinetic trace was extracted at 418 nm in order to fit the time-dependent absorbance traces to various kinetic models that feature rapid formation of HRP-I, followed by first-order conversion to HRP-II, and subsequent first-order conversion to native HRP.[11]

Scheme 1. Catalytic Cycle of Horseradish Peroxidase (adapted from ref 6)



Stopped-flow kinetic traces were collected in a random order of magnetic field strengths, but always with a corresponding zero magnetic field control trace that was collected immediately before or after the non-zero magnetic field trace. This procedure allows the precise comparison of kinetic traces as *relative* rates. Figure 1 shows the magnetic field dependence of the relative rate constants k_1 and k_2 (Scheme 1). Both k_1 and k_2 are *net* rate constants that include any possible reversal of these steps. Typical values of the effective rate constants k_1 and k_2 at zero magnetic field are about 6 and 20 s^{-1}, respectively.[12] Under the reaction conditions, the enolate is present at a steady-state concentration of about 1.3×10^{-7} M, to give values of $k_1 = 1.5 \times 10^8$ M^{-1} s^{-1} and $k_2 = 4.6 \times 10^7$ M^{-1} s^{-1}. The standard error reported for each data point reflects an average value obtained from multiple measurements at each magnetic field, but collected in a random order.

At 10 G, a slight increase in k_1 and k_2 is observed, followed by a substantial decrease in k_1 and k_2. At higher magnetic field

(1) A preliminary report of this work was presented at the Rocky Mountain Regional ACS meeting, Denver, CO, June 10, 1996, and at the International Magnetic Spin Effects in Chemistry Conference, August 20, 1996, Novosibirsk, Russia.

(2) For reviews on magnetic field effects, see: (a) Salikhov, K. M.; Molin, Y. N.; Sagdeev, R. A.; Buchachenko, A. L. *Spin Polarization and Magnetic Effects in Radical Reactions*; Elsevier: Amsterdam, 1984. (b) Steiner, U. E.; Ulrich, T. *Chem. Rev.* **1989**, *89*, 51. (c) Steiner, U. E.; Wolff, H.-J. In *Photochemistry and Photophysics*; Rabek, J. F., Ed.; CRC Press: Boca Raton, FL, 1991; Vol. IV, Chapter 1.

(3) Grissom, C. B. *Chem. Rev.* **1995**, 95, 3.

(4) (a) Harkins, T. T.; Grissom, C. B. *Science* **1994**, *263*, 958. (b) Harkins, T. T.; Grissom, C. B. *J. Am. Chem. Soc.* **1994**, *263*, 958.

(5) For reviews on the mechanism of HRP, see: (a) Frew, J. E.; Jones, P. In *Advances in Inorganic and Bioinorganic Mechanisms*; Sykes, G., Ed.; Academic Press: New York, 1984, p 175. (b) Dunford, H. B. *Adv. Inorg. Biochem.* **1982**, *4*, 41.

(6) Dawson, J. H. *Science* **1988**, *240*, 433.

(7) The OLIS-RSM stopped-flow spectrophotometer was modified to place the thermostatted reaction cuvette (1.7 cm optical path) in the air gap of an electromagnet (see ref 4b). Absorbance spectra were recorded from 310 to 600 nm at a sampling rate of 1 kHz. Enzyme and substrate/peroxide solutions were kept in separate syringes prior to mixing.

(8) HRP was purchased from Sigma, Inc., and $R/Z > 0$ was verified for each lot purchased (typical $R/Z = 3.1$). The final concentration of HRP was 1.4 μM after mixing. The enzyme concentration was determined spectrophotometrically, using $\epsilon = 102$ mM^{-1} cm^{-1} at 403 nm. Solutions of H_2O_2 were prepared fresh daily, and the concentration was verified by iodometric titration and used at a final concentration of 1.0 μM H_2O_2. The substrate, 2-methyl-1-((trimethylsilyl)oxy)-1-propene, was solubilized in ethanol prior to mixing with buffer solution. The experiments were carried out in 100 mM KH_2PO_4 buffer titrated to pH 7.4 with KOH. All solutions were passed through a 0.45 μm filter immediately prior to use.

(9) 2-Methyl-1-((trimethylsilyl)oxy)-1-propene undergoes rapid enolization in phosphate buffer to provide a constant concentration of active enol substrate. This removes the substrate dependence from the reaction and simplifies the kinetic scheme such that rapid bimolecular combination of H_2O_2 and enzyme occurs within the mixing time of the instrument, followed by pseudo-first-order conversion of HRP-I and substrate to HRP-II, followed by pseudo-first-order conversion of HRP-II and a second molecule of substrate back to native HRP. With this concentration of H_2O_2, no higher-order oxidation products of HRP are observed.

(10) Matheson, I. B. C. *Comput. Chem.* **1990**, *14*, 49.

(11) Biphasic kinetic traces were extracted at 418 nm. The rapid decrease in absorbance is ascribed to the oxidation of native HRP to form HRP-I. This phase of the reaction is too fast to treat quantitatively under the these reaction conditions and is ignored. The phase of increasing absorbance was fitted to the kinetic expression for two sequential first-order exponential processes (see the Supporting Information for details).

(12) An applied magnetic field of 0 is defined according to the applied laboratory magnetic field and still contains the geomagnetic component of ≈0.3 G.

S0002-7863(96)03024-7 CCC: $14.00

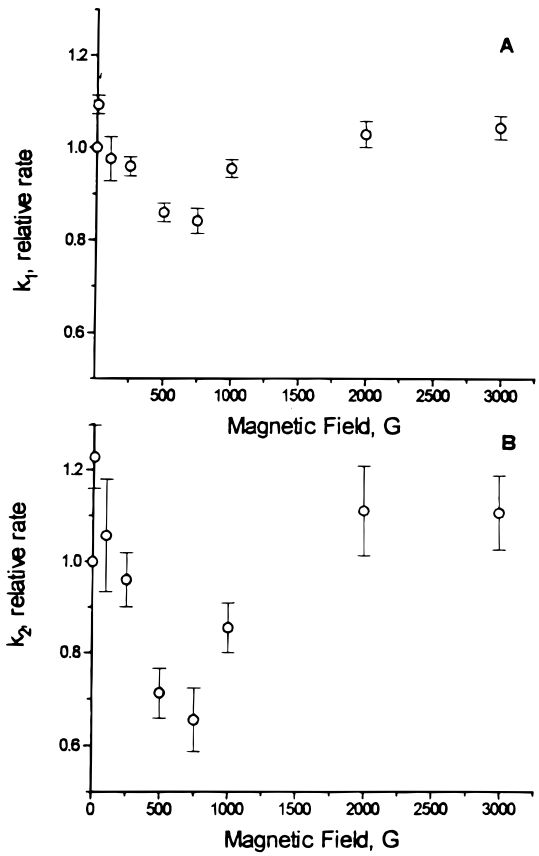


Figure 1. Magnetic field dependence of horseradish peroxidase. Kinetic traces at 418 nm are fitted to a model of sequential exponential processes, and standard error bars reflect the average of several measurements at each magnetic field strength. Reaction conditions (all concentrations specified after mixing): 100 mM KH_2PO_4 buffer, pH 7.41; 4 μM HRP (R/Z > 3.0); 1.0 M H_2O_2; 16 mM 2-methyl-1-((trimethylsilyl)oxy)-1-propene; 25.0 °C. Typical values of the effective rate constants k_1 and k_2 at zero magnetic field are about 6 and 20 s^{-1}, respectively. Under the reaction conditions, the enolate is present at a steady-state concentration of about 1.3×10^{-7} M, to give values of $k_1 = 1.5 \times 10^8$ M^{-1} s^{-1} and $k_2 = 4.6 \times 10^7$ M^{-1} s^{-1}.

strength, k_1 and k_2 return to the values observed at 0 G.[13] The same magnetic field dependence of k_1 and k_2 was observed in two replications of the data set reported herein.

Multiple paramagnetic species enter into the HRP catalytic cycle, including the porphyrin radical cation, native HRP [Fe(III)], HRP-I/HRP-II [Fe(IV)], and the two substrate radicals. A combination of any two can constitute a paramagnetic (radical) pair that can undergo spin-selective processes, wherein at least one reaction pathway is spin-forbidden. ESR Studies show HRP-I and HRP-II have g values near 5, with hyperfine coupling from the axial imidazole base of ≈7 G. HRP-I also exhibits a g value of 2.0024 that is attributed to the porphyrin radical cation. The carbon-centered substrate radical (the more stable tautomer of the enolate radical) has a g value of 2.0045 and effective hyperfine coupling of 23 G. When the parameters for the substrate radical and HRP-I or HRP-II are applied to the semiclassical model of magnetic field dependent RP recombination that allows for diffusive separation and a RP lifetime that is limited by diffusion in solution, a biphasic magnetic field dependence with the same trend as the experimental data is observed (detailed calculation methods, parameters, and results are given in the Supporting Information). A change in any of the parameters removes the qualitative agreement between the calculated and experimental results.[14]

(13) It is not known if k_1 and k_2 rise above a relative rate of 1.0 at higher magnetic field strengths.

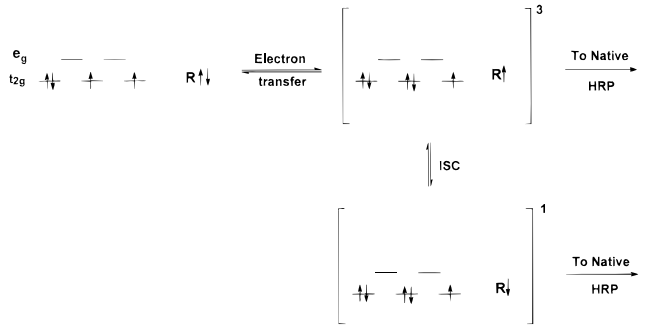


Figure 2. Electron transfer from diamagnetic substrate to low-spin Fe(IV) in HRP-II will occur to produce the triplet RP and conserve the total electron angular momentum of the system. Only the triplet RP can undergo reverse electron transfer to regenerate HRP-II. Intersystem crossing will lead to the singlet spin state that is unreactive toward recombination. In this scheme, both the singlet and triplet electron spin states can undergo forward reaction in the catalytic cycle, but only the triplet can undergo reverse electron transfer.

Identification of a unique magnetosensitive step is not yet possible, but the most likely candidate occurs in the electron transfer complex formed between HRP-II and substrate.[15] The transfer of one electron from substrate to HRP-II regenerates native HRP and, according to the rules of total electron spin retention, should produce the *triplet* RP (Figure 2; low-spin HRP-II, Fe(IV), $S = 1$) that partitions between formation of native HRP and recombination to regenerate HRP-II. In this unusual case, the singlet RP is *unreactive* toward RP recombination because it would lead to loss of conservation of total angular momentum. The sign of the calculated magnetic field dependence of RP recombination agrees with a scheme in which the triplet RP is formed initially, and only the triplet RP can undergo recombination.

This observation has profound implications for the role of electron spin in all reactions that involve paramagnetic metal ions. When a diamagnetic substrate donates an electron to an $S = 1$ paramagnetic acceptor, total electron spin will be conserved and reverse electron transfer will be limited to the triplet spin state. Intersystem crossing to the singlet spin state creates a species that is unreactive toward reverse electron transfer. Forward electron transfer to a low-spin metal center can be made less reversible (and kinetically more favorable) by the incorporation of nearby heavy atoms with the ability to induce intersystem crossing through spin−orbit coupling (e.g., S or Se).

Acknowledgment. This work is supported by grants from the NIEHS (ES05728 and ES08896), the CAST program of the U.S. National Academy of Sciences, and the Russian Federation for Scientific Research. We wish to thank Dr. Alexander Kruppa for assistance with MatLab and Dr. Walther Ellis for helpful discussions.

Supporting Information Available: Absorbance spectra for native HRP, HRP-I, and HRP-II extracted from kinetic traces by global fitting analysis of absorbance *vs* wavelength *vs* time, methods and results for calculated magnetic field dependence of RP recombination probability in the HRP reaction, tabulated values of k_1 and k_2 *vs* magnetic field, and Monte-Carlo simulation of HRP magnetic field dependence employing the semiclassical approximation of RP recombination (12 pages). See any current masthead page for ordering and Internet access instructions.

JA9630248

(14) If the model of magnetic field dependent recombination involving electron transfer is correct, the extreme magnetic anisotropy of the heme center must not dominate electron spin relaxation to the extent that electron spin correlation is lost. Otherwise, no magnetic field effect on reverse electron transfer would be observed.

(15) Magnetic field dependent reverse electron transfer involving k_2 does not adequately explain the slight magnetic field dependence of k_1. Additional experiments are underway to examine these microscopic rate constants at a finer level of dissection.