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Magnetic Field Effects and Radical Pair Mechanisms in Enzymes: A Reappraisal of the Horseradish Peroxidase System

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Presented here is a detailed reinvestigation of a reported magnetic field effect (MFE) in the haem-dependent enzyme horseradish peroxidase (HRP),[1] using a stopped-flow device adapted to make high-precision single-wavelength measurements in the presence of a static magnetic field. We find substantial reappraisal of reported MFEs for this enzyme system is required.

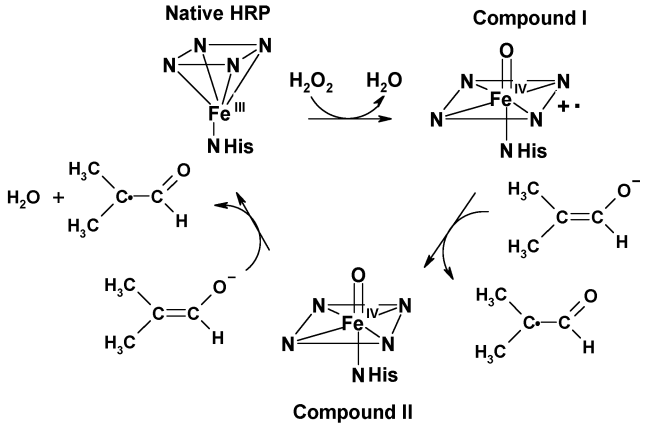
The application of external magnetic fields can perturb reaction kinetics through the constraints of the radical pair mechanism.[2a−d] It has been observed that relatively modest fields, including those of environmentally realistic magnitude, can influence the rates and yields of radical pair (RP) reactions.[3a−b] Furthermore, in recent years there have been reported effects of applied magnetic fields on enzyme-catalyzed reactions involving RPs.[1,4] Consequently, an understanding of the mechanistic origin of MFEs in biological systems is important in attempts to determine, deleterious or otherwise, the effect of environmental fields on human health. MFEs might also be of diagnostic value in providing evidence for short-lived RPs in enzyme systems.

The reaction catalyzed by HRP comprises oxidative and reductive half-reactions (Scheme 1). The oxidative half-reaction involves the conversion of the ferric form of the enzyme-bound haem to the ferryl haem (Compound I) by hydrogen peroxide. One electron reduction by substrate converts this form to Compound II and a substrate radical. Subsequent reduction by a second substrate molecule returns the enzyme to the ferric form (native) of HRP. The RP proposed in the original article comprises the notional (Compound II)• radical species (low-spin ferric,[1] oxygen bound) and the second equivalent of organic radical.

Stopped-flow kinetic experiments were performed using an Applied Photophysics SX.18MV-R reaction analyzer modified for MFE studies. The reaction cell (10-mm optical path length) housing was re-engineered from nonmagnetic material, and the external magnetic field was generated by air-Helmholtz coils supplied by a pulsed current power source (designed and built in-house). For the highest field point recorded (75 mT), the field was generated using a pair of rare-earth permanent magnets mounted to generate a homogeneous field at the sample position (see Supporting Information). Absorbance measurements were taken using a single wavelength of 418 nm and a phototmultiplier tube. Each experiment comprised 6−9 field on/field off data acquisition pairs, the order of which was randomized. Experiments were repeated several times for each magnetic field data point.

All solutions were prepared in a 100 mM KH_2PO_4 buffer, titrated to pH 7.4 with aqueous KOH, and all reagent concentrations quoted are post-mixing: HRP (Sigma type VI), 1.4 μM, determined spectrophotometrically at 403 nm using $\epsilon = 102$ mM^{-1} cm^{-1}; 2-Methyl-1-(trimethylsilyloxy)-1-propene (Aldrich), 16 mM, was solubilized in ethanol prior to mixing with buffer. The phosphate buffer mediates the rapid hydrolysis of the substrate to the free enolate;[6] the active form is represented in Scheme 1. Hydrogen peroxide, 140 μM, determined spectrophotometrically at 240 nm using $\epsilon = 39.4$ $M^{-1}cm^{-1}$, was loaded into the same drive syringe as the reducing substrate. Solutions were passed through a 0.45 μm filter on loading into the drive syringes. Reactions were carried out at 25 °C, and all conditions used remained as close to those of the original work as was possible.

Scheme 1. HRP-Catalyzed Reduction of Hydrogen Peroxide with the Enolate Hydrolysis Product of 2-Methyl-1-(trimethylsilyloxy)-1-propene as Reductant[a]



[a] The proposed RP comprises a (Compound II)• radical and the second organic radical.[1] Adapted from ref 5.

Recording each kinetic trace with magnetic field applied, with a corresponding trace with no applied field, allowed for accurate comparison of the data as relative rate coefficients after fitting to a kinetic model. Figure 1 shows relative rate coefficient versus magnetic field (mT) plots for k_1 and k_2, net rate coefficients for the first and second reductive steps, respectively. Data for both the current and original work are displayed. The range of magnetic field strengths investigated are not the same, as the purpose of this recent work was to first reproduce the observed MFE, then perform more detailed studies on the low-field region, which corresponds to magnetic fields of environmental relevance. Consequently, the field range covered by the new experimental setup is less than that attained from the large DC electromagnet employed in the original work. Typical values of k_1 and k_2 are 4 s^{-1} and 14 s^{-1}, respectively.

From Figure 1 it is clear that the current investigation was unable to reproduce the MFE previously observed for this system. To ensure that the apparatus was capable of correctly monitoring field-induced changes, a model chemical system was employed to confirm that this was the case. The photoexcitation of pyrene (10^{-4} M) in the presence of dicyanobenzene (10^{-2} M) proceeds via the

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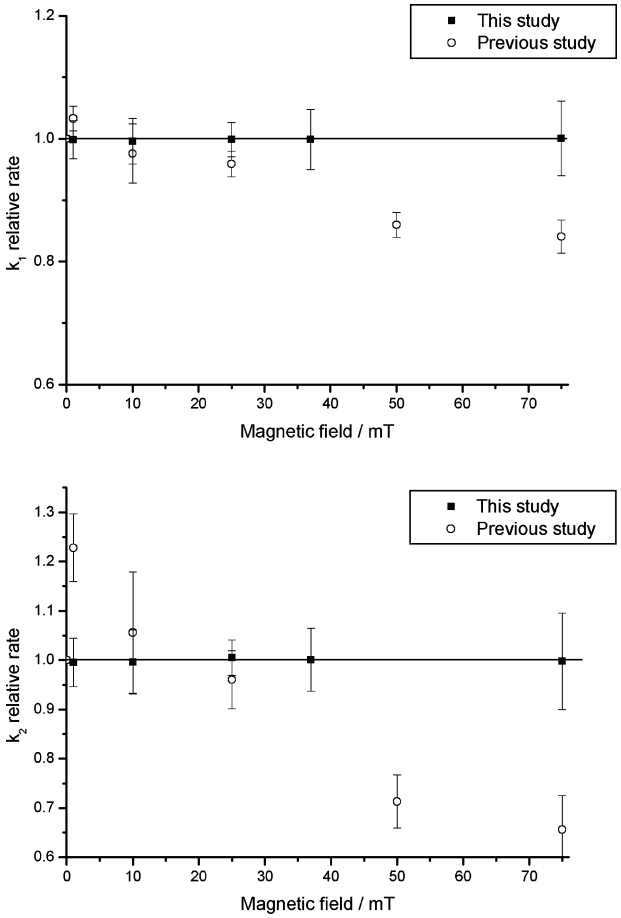

Figure 1. Plots of relative rate coefficient vs magnetic field for k_1 (top graph) and k_2 (bottom graph). k_1 and k_2 are net rate coefficients for the two stages in the reductive half-reaction of the HRP (1.4 μM)-catalyzed reduction of hydrogen peroxide (140 μM), with 2-methyl-1-(trimethylsilyloxy)-1-propene (16 mM) as reductant. All reagent solutions were prepared in 100 mM KH_2PO_4 buffer of pH 7.4. Reaction temperature: 25 °C. Relevant data for both the current and previous study are shown.

formation of a singlet RP, and the singlet product is an exciplex that fluoresces in the blue part of the visible spectrum. This system has previously been extensively studied in both time-resolved and field modulation experiments.[7] An experiment, described in detail in the Supporting Information accompanying this communication, demonstrated a clear MFE within the same experimental apparatus. The observed field effect saturation was in good agreement with previously published work.

Our inability to recreate the effect in the enzymatic system prompted us to re-evaluate the way in which the original analysis was performed. Several potential problems were uncovered as a result of this reanalysis, which, again, are detailed in the Supporting Information. Using values of k_1 and k_2 and the relative values under magnetic field conditions quoted in the original publication, fitting curves were reconstructed using the function supplied. Even at the field point where the magnitude of the MFE was greatest (75 mT), the field on/field off curves are virtually indistinguishable. This arises because the effects on the shape of the decay curve for changes to k_1 and k_2 are dependent upon the extinction coefficients of Compound II and Native HRP (the former being approximately twice the latter). Increasing the value of k_1 yields an opposite change in the shape of the decay curve to an increase in k_2. The observed MFE on the values of k_1 and k_2 correspond to a counterbalancing effect on the shape of the decay curve for all field points recorded.

Another significant problem with the original analysis was the omission of a term for Compound I in the fitting function. The basis of such an omission was that the relative extinction coefficient of this species at 418 nm is around a sixth the value of Compound II and a third that of the native enzyme. This is not an insignificant absorbance at this wavelength, and consequently, a modified fitting equation was written to include a term for Compound I. Using this new function to fit the current data improved the accuracy of the fit significantly, and the rate coefficients in Figure 1 were obtained with this new function.

Our study demonstrates the absence of a MFE in the reductive half-reaction of this HRP-catalyzed cycle and identifies problems with the analysis of kinetic data in the original study. The RP model proposed in the original work only predicts changes to the value of k_2 and not k_1, but the observed changes were to both k_1 and k_2. Additionally, there are a number of reasons why the system might not be field-sensitive, even if the originally proposed model is correct. These include the possibility of rapid electron spin relaxation in the RP[8] (although the proposed low-spin ferric haem radical is perhaps less likely to induce rapid spin relaxation than the Compound II triplet itself[9]) or a large electron-exchange interaction caused by the close proximity of RP members. The RP described in the original work was proposed solely to explain the observed MFE based on its likely magnetic parameters. There is no experimental evidence for the formation of a (Compound II)$^\bullet$ radical and clearly no measured magnetic or distance parameters for the RP itself. The original work also gives no suggestion about how the (Compound II)$^\bullet$ radical (low-spin ferric[1], oxygen bound) might convert back to Native HRP (quantum mixed mid/high spin ferric,[10] no oxygen bound). Field effects would also be unobservable were the rate of this conversion rapid relative to the rate of singlet−triplet interconversion in the RP.

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Note Added after ASAP Publication. After this paper was published ASAP on June 10, 2006, a sign error was corrected in eqs 4 and 5 in the Supporting Information. The corrected PDF was posted June 13, 2006.

Supporting Information Available: Full experimental and analytical details. This material is available free of charge via the Internet at http://pubs.acs.org.

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