Conformational Studies of Peroxidase–Substrate Complexes. Structure of the Indolepropionic Acid–Horseradish Peroxidase Complex

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Summary N.m.r. spectroscopic methods are described for determining the conformation of substrate molecules bound to hemoproteins, and a structure is presented for the indolepropionic acid–horseradish peroxidase complex.

IN this communication we report the use of n.m.r. methods[1] to describe the orientation of a bound substrate, indolepropionic acid (IPA), to the heme of the enzyme horseradish peroxidase (HRP). The heme protein perturbs the n.m.r. spectrum of the substrate through (1) ring current shifts, (2) pseudocontact shifts, and (3) dipolar broadening. Each perturbation yields information on the substrate–heme unit.[1]

All n.m.r. spectroscopic measurements were made at 270 MHz using a Bruker spectrometer with an Oxford Instruments Co. magnet. Resolution was improved by using difference spectroscopy and pulse sequences[2] which differentiate resonances of different relaxation times. Longitudinal relaxation times (T_1) were determined using the conventional $(180°–\tau–90°–T)_n$ pulse sequence.

TABLE. Shift and relaxation data for the indolepropionic acid complexes of horseradish peroxidase.

Proton	Experimental shift ratio	Computed shift ratio	Experimental $1/T_{1p}$ ratio	Experimental distance ratio	Computed distance ratio
H-4	100 ($\Delta\delta$ = −0·72)[a]	100	100	100	100 (r = 9·3 Å)[a]
H-2	21	21	99	101	103
H-7	29	29	97	103	104
H-5[b]	*ca.* 70	88	—	—	104
H-6[b]	*ca.* 35	66	—	—	107
CH_2	79	113 63	66	108	95 112
CH_2CO_2H	74	105 54	51	112	90 108

[a] The fully bound shift given is calculated using $K_d = 6·2 \pm 2·9 \times 10^{-3}$ M and a negative shift is to low field. One absolute distance is given. [b] The data on these protons are of low accuracy through problems of resolution.

In the complexes of the high-spin Fe^{III} heme ($S = 5/2$) form of HRP resonances of the substrate suffer both pseudocontact shifts and paramagnetic dipolar broadening.[1] The shift is proportional to $(3\cos^2\theta - 1)/r^3$, where r is the distance of a given nucleus from the iron and θ is the angle between $\rightarrow_r$ and the principal magnetic axis. Here the g-tensor is very close to axial[3] and the principal axis is close to perpendicular to the heme plane. In the low-spin state of Fe^{III} heme there is no paramagnetic dipolar broadening as the electron relaxation time is very short.[4] Thus the low-spin state of the protein can be used as a relaxation blank for the high-spin form assuming that the two forms of the protein form very similar complexes. That this is the case can be shown for the binding of indolepropionic acid by studying binding constants,[5] which are the same within the error limits at $1·0 \times 10^2$ M^{-1}, and by noting that the effect of substrate binding is upon the same protein resonances. The diamagnetic Fe^{II} low-spin CO complex also binds substrate equally strongly but no shifts on the substrate resonances were observed and ring current effects can therefore be neglected. We show below that this circumstance arises as the substrate binds some distance from the heme and close to the ring-current cone angle of zero shift.

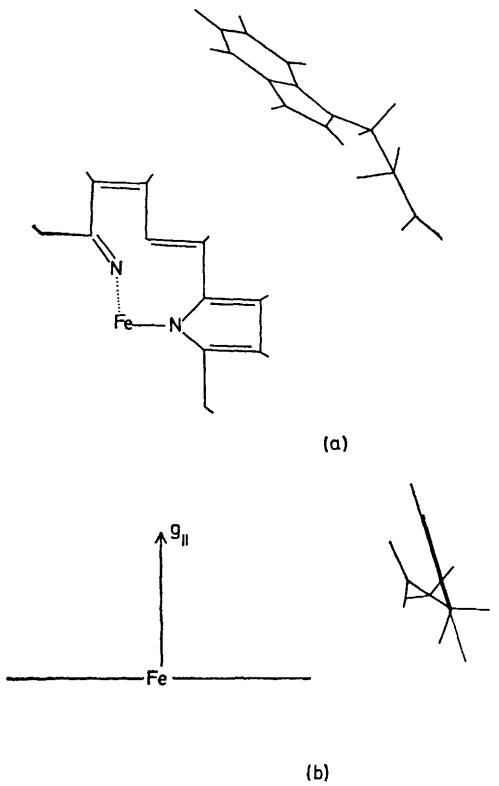

FIGURE. Structure of the indolepropionic acid–horseradish peroxidase complex: (a) projection onto the heme plane, and (b) projection on to the plane containing the symmetry axis (z) and perpendicular to the indole ring. The outline of the porphyrin ring has been included in an arbitrary orientation in the xy-plane.

Binding of the substrate to HRP was studied at pH 7·3 at 36 °C over a wide range of concentration following resonance shifts and broadenings. Under these conditions the substrate is observed to be in fast exchange. The fully bound shifts of the substrate resonances by the high-spin HRP were then estimated (Table) from the shift and binding data. Since there was no detectable ring current shift with HRP–CO these fully bound shifts were taken as the pseudocontact shifts of the IPA resonances in the HRP complex. Longitudinal relaxation times (T_1) were measured for IPA (8·5 mM) protons in the presence of HRP (0·56 and 0·65 mM,

pH 7·3) after which sufficient concentrated KCN solution was added fully to form the low-spin HRP–CN complex, the pH readjusted to 7·3, and T_1's measured again. The paramagnetic contribution by the high-spin ferric heme to the relaxation rate of the IPA protons ($1/T_{1P}$) is then the difference $1/T_{1(HRP)} - 1/T_{1(HRP-CN)}$. These values are presented in the Table. For fast exchange, which pertains in the present case, $1/T_{1P} = f/T_{1M}$, where f = fraction of substrate bound to the enzyme. From the value of $1/T_{1M}$ for any given proton its distance from the iron atom may be calculated using the Solomon–Bloembergen equation[6] provided the correlation time, τ_c, is known. The dominant correlation time is the iron unpaired electron spin relaxation time, τ_S, which is very much shorter than the rotational correlation time, τ_R, of the substrate–peroxidase complex. Assuming the value of τ_0 to be in the range 5×10^{-11} s to 10^{-10} s[4,7] the distance of H-4 from the metal is estimated to be 9·2—10·6 Å.

Computation of the conformations of enzyme-bound IPA is facilitated by the presence of the five protons of the indole ring (the NH proton is exchanged in D_2O) which constitute a rigid frame with respect to which the position of the metal ion and the orientation of the principal magnetic axis may be defined uniquely (the heme plane is assumed to be perpendicular to this axis). For the conformational search,[8] shift and relaxation data were ratioed with respect to H-4 (see Table). By varying the metal position and symmetry axis orientation over all space with the indole ring between 4 and 10·5 Å from the metal a family of solutions were found which gave very good agreement with the experimental shift and broadening ratios. The best of these is shown in the Figure with the computed shift and broadening ratios given in the Table.

Thus our n.m.r. spectroscopic methods define a structure for the IPA–horseradish peroxidase complex in which the indole ring is located 9—10 Å from the heme iron. The edge of the indole defined by C-3, C-4, and C-5 lies nearest the heme plane and the ring is inclined such that all indole protons are approximately equidistant from the metal. The propionic acid chain lies roughly in the plane of the indole ring and projects away from it.

While this work was in progress a much less detailed study[9] has been made of the geometry of the HRP complexes by relaxation methods alone. In our hands such methods give a poor definition of substrate binding sites. Again using relaxation alone the different mode of binding of different indoles and of indoles compared to phenols cannot be readily recognised. As we shall show elsewhere using the methods described here these sites can differ appreciably, and the binding relationship between heme and substrate is quite different from that in model complexes.[1]

The work was supported by the Medical Research Council. R.J.P.W. is a member of the Oxford Enzyme Group.

(*Received, 2nd July* 1975; *Com.* 758.)

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