

The Effect on Structural and Solvent Water Molecules of Substrate Binding to Ferric Horseradish Peroxidase

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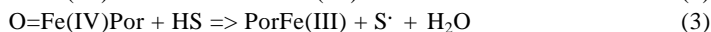
DOI: 10.1039/b000000x [DO NOT ALTER/DELETE THIS TEXT]

Ultrafast, multi-dimensional infrared spectroscopy, in the form of 2D-IR
 10 and pump-probe measurements, has been employed to investigate the effect
 of substrate binding on the structural dynamics of the horseradish
 peroxidase (HRP) enzyme. Using nitric oxide bound to the ferric haem of
 HRP as a sensitive probe of local dynamics, we report measurements of the
 frequency fluctuations (spectral diffusion) and vibrational lifetime of the
 15 NO stretching mode with benzohydroxamic acid (BHA) located in the
 substrate-binding position at the periphery of the haem pocket, in both D₂O
 and H₂O solvents. The results reveal that, with BHA bound to the enzyme,
 the local structural dynamics are insensitive to H/D exchange. These results
 are in stark contrast to those found in studies of the substrate-free enzyme,
 20 which demonstrated that the local chemical and dynamic environment of
 the haem ligand is influenced by water molecules. In light of the large
 changes in solvent accessibility caused by substrate binding, we discuss the
 potential for varying roles for the solvent in the haem pocket of HRP at
 different stages along the reaction coordinate of the enzymatic mechanism.

25 1 Introduction

The peroxidase enzymes are commonplace in biological systems, where they are
 responsible for catalysing the oxidation of substrates ranging from proteins, as in the
 case of cytochrome c peroxidase, to a variety of small organic molecules.¹ Despite
 the large number of different peroxidase enzymes, a remarkably high degree of
 30 similarity is seen across the family in terms of both the haem pocket structure and
 the mechanism by which they operate.

Horseradish peroxidase (HRP) is widely used as a typical exemplar of this category
 of enzymes, and the mechanism of HRP can be described as:



where Por represents the haem propionate moiety and S the substrate.

40 Although the physiological substrate of HRP has not been determined thus far,¹ the
 structure of HRP and the involvement of the various elements of the active site in
 this mechanism have been the subject of much study.²⁻⁹ It has been well-established

from crystallography that the haem pocket of HRP features the histidine and arginine amino acid residues, located on the distal side of the haem, that are conserved in most peroxidases (His42 and Arg38 in HRP, see Fig 1). Furthermore, molecular methods have shown these residues to be key to the formation of the oxo-ferryl intermediate species denoted Compound I (CpdI, see reaction (1)), which is one of the central aspects of the peroxidase mechanism. These residues are potentially also important in the onward reaction steps from CpdI.³

Despite this progress, the intimate molecular events that contribute to the HRP mechanism are yet to be fully established and important questions remain. One such issue relates to the mechanistic role of water in the active site of HRP. The steps leading to CpdI formation are believed to begin with the binding of a molecule of hydrogen peroxide to the 5-coordinate ferric haem of the enzyme and to progress to a precursor species similar to the Compound 0 (Cpd0) that has been observed in catalases, which takes the form Fe(III)-OOH. The formation of Cpd0 requires a proton transfer step from the bound H₂O₂ to the distal histidine side chain but the distance between the haem ligand and His42 has been shown by computational simulations to be too great to be spanned by a single H-bond interaction. Moreover, molecular dynamics simulations have suggested that proton transfer is likely to be energetically more favourable if mediated by the presence of disordered water molecules in the active site.^{10, 11} These findings are also consistent with experimental investigations suggesting a mechanistic role for water,^{1, 12, 13} though some of these conclusions have been the subject of debate.¹⁰ Finally, recent studies using ultrafast IR spectroscopy have demonstrated a clear dependence of the vibrational and structural dynamics of the haem pocket of HRP upon H/D exchange of the solvent, inferring the presence of water molecules in close contact with the haem ligand.¹⁴

Water molecules are also cited as being influential in the later stages of the peroxidase mechanism during which reduction of CpdI occurs via oxidation of larger substrates in two one-electron steps (steps (2) and (3) above). Here, the reason for the divergence of the peroxidase mechanism from that of the closely-related catalase enzyme, in which CpdI reacts via a single two-electron step involving a second molecule of H₂O₂, is widely attributed to the removal of water, or otherwise, from the vicinity of CpdI. This has led to the introduction of the concepts of a 'wet' CpdI in peroxidase and a 'dry' variant in catalase.¹⁵ In apparent support of this, it is notable that the active site of HRP is readily accessible by solvent molecules,⁹ in contrast to what is believed to be the case for catalase. More recently it has been suggested that such concepts may have a more subtle, dynamic, interpretation following spectroscopic studies indicating that both enzymes feature interactions between the haem ligand and water molecules and so are likely to undergo similar water-mediated processes leading to CpdI.¹⁴ This study did however report significant dynamic differences between the active sites of catalase and peroxidase that may contribute to the selection of different onward steps from CpdI. Specifically, HRP shows a much more flexible active site than catalase and it is plausible that this promotes the ability to bind a multitude of substrates, while the more restricted catalase pocket featuring well-defined low-frequency vibrational modes that couple to the haem ligand stretching vibration, may tailor functionality specifically toward subsequent reactions only with hydrogen peroxide.

A clear need thus exists to fully characterise water involvement in the HRP active site at all stages of the enzyme mechanism. In doing so, insights into the fast

structural dynamics that accompany the presence of water and hydrogen bond interactions, which could influence individual mechanistic steps, will be of considerable value. As a result, the question we seek to address here is: what is the effect on the structural and solvation dynamics of the haem pocket of binding the model substrate molecule benzohydroxamic acid (BHA) to HRP and does this shed any light on our molecular-level understanding of the later stages of the peroxidase mechanism?

Although not a physiological substrate of HRP, BHA has been shown by co-crystallisation studies to bind strongly near the δ -edge of the haem group as a result of both hydrophobic interactions between the phenyl ring and the protein architecture and specific H-bond interactions involving the hydrophilic BHA head group and the His42 and Arg38 residues near the haem Fe (Fig 1).^{5, 16} Under conditions where the haem is not ligated by a diatomic molecule, BHA binding promotes the formation of a 6-coordinate aqua complex of the ferric haem^{17,18} but, otherwise, BHA binding seems to exclude water from the normally solvent-accessible pocket. In part, this is apparently achieved through competitive replacement of hydrogen bonding links near the haem but the binding of BHA also leads to a reorientation of the F68 residue, which serves to occlude the solvent access channel to the haem pocket.^{5, 17}

It is noted that crystallography methods are not normally sensitive to mobile water and so we employ a complementary approach, using ultrafast infrared spectroscopy to determine the spectral diffusion (2D-IR) and vibrational lifetime (pump-probe) of NO bound to the haem Fe of the HRP-BHA complex under both aqueous (H_2O) and deuterated (D_2O) solvent conditions in order to establish the effect of substrate binding on access of mobile solvent molecules to the haem centre. These measurements will further support crystal-derived data by reporting on changes in the dynamic nature of the haem site caused by substrate interactions and also on the relationship between crystal structure and the solution-phase environment.

Despite its prominent place in biological research, HRP has not been widely examined using ultrafast or dynamic spectroscopy methods. The nature of the haem pocket of HRP has been investigated using ligand rebinding studies, where NO recombination with the ferric haem following photolysis was reported to show the straightforward dynamics consistent with an active site that is readily solvent-accessible.¹⁹ The equilibrium structural dynamics of the non-physiological ferrous state of HRP have been investigated using 2D-IR methods, though the analysis predated molecular dynamics investigations into the mechanistic role of water and as such this was not considered.²⁰ Despite this, a general restriction of the dynamic nature of the haem cavity was observed on BHA binding.

Experimental

For all FTIR, IR pump-probe and 2D-IR experiments, HRP (Sigma) was concentrated to the level of ~ 7.5 mM. MAHMA NONOate was used to nitrosylate the ferric HRP as has been described previously.²¹ For experiments carried out under aqueous (H_2O) conditions, a phosphate buffer solution was employed at pH7. For deuterated samples (D_2O), repeated buffer-exchange was performed using pD7 deuterated phosphate buffer solution; care was taken to ensure deuteration of all readily exchangeable protons. BHA (Sigma) was added to the sample at concentrations of 12 mM and binding was confirmed via detection of a single,

narrow UV-Vis absorption band at 407 nm.²² For all IR experiments, the samples were held between two CaF₂ windows separated by a PTFE spacer of 100 μm thickness.

The method for obtaining IR pump-probe and 2D-IR spectra has been described previously.^{21, 23} Briefly, 2D-IR spectra were acquired using the FT-2D-IR method described in Ref²¹ using a sequence of three mid-infrared (IR) laser pulses arranged in a pseudo pump-probe beam geometry with parallel relative polarization directions (the effects of molecular rotation on the signals was found to be negligible on the timescales studied).^{24, 25} For IR pump-probe measurements, the same spectrometer was used but only two of the three pulses used for 2D-IR spectroscopy were employed. The pulses were generated by a Ti:sapphire laser pumping a white-light seeded optical parametric amplifier (OPA) equipped with difference frequency mixing of the signal and idler outputs. Mid IR pulses with a temporal duration of ~100 fs; a central frequency of 1900 cm⁻¹ with a bandwidth of ~200 cm⁻¹ were employed.

Results

FTIR Spectroscopy

Introduction of BHA and NO into solutions of HRP resulted in the observation of a single new peak in the infrared absorption spectrum near 1900 cm⁻¹ that is attributed to the NO stretching vibration (ν_{NO}) of the nitrosylated ferric haem.^{21, 23, 26} Representative spectra are shown in Fig 2A. This was observed to be the case irrespective of the order of addition of BHA and NO. When an aqueous solvent was employed, this peak was well-represented by a single Gaussian lineshape with a central frequency of 1912.1 cm⁻¹ and a full width half maximum (FWHM) value of 7.7 cm⁻¹. In deuterated solvent, this peak yielded values of 1911.5 cm⁻¹ and 8.8 cm⁻¹ for the central frequency and FWHM respectively, which do not represent a significant isotope-dependence of these parameters. The results of the data fitting are given in Table 1, where the values for the BHA-free enzyme are also quoted for reference. It is noted that the binding of BHA to HRP leads to a shift of around 8 cm⁻¹ in the frequency of the NO stretching vibration relative to the substrate-free enzyme as well as a significant narrowing of the peak by around 6 cm⁻¹.¹⁴ The absence of signals due to BHA-free HRP in the FTIR data clearly shows that the vast majority of HRP molecules in the ensemble were in the form of a BHA complex.

IR Pump-probe Spectroscopy

IR pump-probe spectroscopy was used to recover the vibrational dynamics of the ν_{NO} mode of nitrosylated HRP-BHA (simply denoted HRP-BHA hereafter as all samples discussed are nitrosylated)); example spectra are shown in Fig 2B. In both solvents, a negative peak was detected at a frequency corresponding to the single absorption observed via FTIR spectroscopy, which is assigned to bleaching and stimulated emission processes featuring the $\nu=0-1$ transition of the ν_{NO} mode of HRP-BHA. In addition, a positive peak due to the $\nu=1-2$ transient absorption was located at 1891 cm⁻¹. The lower frequency of this transition relative to the fundamental indicates the anharmonic shift of the ν_{NO} mode of HRP-BHA.

Both the $\nu=0-1$ and $\nu=1-2$ peaks were observed to decrease in amplitude in a similar manner as a function of pump-probe delay time (Fig 2B,C). This effect is

attributable to vibrational relaxation of the ν_{NO} $v=1$ level and the data could be fitted effectively to a single exponential decay function (Fig 2C). In the case of aqueous solvents, a vibrational lifetime of 16.5 ps was extracted while in deuterated solvents a value of 16.3 ps was recovered. The results of the fitting process are given in Table 1.

Table 1: Results of fitting experimental data from FTIR, IR pump-probe and 2D-IR spectroscopy experiments to functions as described in the text.

	HRP + BHA		Substrate-free HRP ^a	
	D ₂ O	H ₂ O	D ₂ O	H ₂ O
FTIR				
<i>Frequency (cm⁻¹)</i>	1911.5	1912.1	1902.7	1903.9
<i>Linewidth (cm⁻¹)</i>	8.8	7.7	14.7	13.9
IR_{pump-probe}				
<i>$\nu=1$ lifetime (ps)</i>	16.3	16.5	18.5	14.8
2D IR				
<i>Spectral diffusion (ps)</i>	3.8	6.3	7.4	3.3
<i>Static offset</i>	0.35	0.3	0.27	0.45

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a) Data from Ref 14

2D-IR Spectroscopy

Representative 2D-IR spectra of HRP-BHA in aqueous and deuterated solvents are shown in Figs 3(A-C) and Fig 4(A-C) respectively for a range of experimental waiting times (equivalent to a pump-probe delay time). In both solvents, the spectra each show two peaks: A negative peak is located on the spectrum diagonal, with values of pump and probe frequency equal to the fundamental ν_{NO} absorption band in the FTIR spectrum. In addition, a second, positive feature is present that is shifted 26 cm⁻¹ to lower probe frequency. These peaks are assigned to the $v=0-1$ and $v=1-2$ transitions of the ν_{NO} mode respectively, consistent with the peaks observed in the pump-probe data.

Irrespective of the solvent used, the lineshapes of the 2D-IR spectral features were observed to evolve as the experimental waiting time increased; becoming more circular in profile and losing the diagonal elongation of the peaks present at short waiting times. This effect is well established for diatomic ligands bound to haem proteins and is assigned to spectral diffusion of an inhomogeneously-broadened transition.^{20, 23, 27-34}

When located in the protein haem site the ligand vibrational absorption of the ensemble of molecules in the sample is inhomogeneously broadened by fluctuations that occur in the local chemical environment of the ligand. These fluctuations can take the form of motion of the protein scaffold or of nearby solvent molecules and lead to small changes of the frequency of the NO stretching mode via electrostatic interactions with the ligand.³⁵ This is reflected as broadening of the absorption band obtained via FTIR spectroscopy but in a 2D-IR plot, when the waiting time is short

with respect to the dynamic timescale of the fluctuations, a correlation between pump and probe frequency is observed, which is manifest as diagonal elongation of the lineshape. At longer waiting times, comparable with the dynamic processes occurring, the HRP-BHA ensemble has evolved leading to a loss of the initial correlation and so a more circular lineshape. Fitting of the 2D-IR lineshapes and quantification of the temporal evolution, referred to as spectral diffusion, can be used to extract the timescales for the underlying dynamics present in the Frequency-Frequency Correlation Function (FFCF) of the NO mode, which in turn provides insight into the local chemical environment of the NO probe.^{36, 37}

For HRP-BHA in aqueous and deuterated solvents, the spectra were fit to a 2D-Gaussian lineshape featuring a cross-correlation parameter C_{2D} . It has been shown previously that the temporal dependence of C_{2D} accurately reflects the FFCF dynamics of NO in the protein environment. Figs 3D-F and 4D-F show the fitting results for the experimental data displayed in Figs 3A-C and 4A-C respectively.^{21, 23}

The variation of C_{2D} with waiting time (Fig 5) was fit to an exponential decay function featuring a static offset parameter; the results of this are also given in Table 1.

For aqueous samples, the C_{2D} data shows a decay with a timescale of 6.3 ± 1.9 ps and a substantial static offset (~ 0.30) while, in deuterated samples, the decay timescale was found to be 3.8 ± 0.6 ps with a static offset of ~ 0.35 . In both cases, the initial value of C_{2D} is reduced from unity (as might be expected for a fully-inhomogeneously-broadened system) by a combination of fast dynamics beyond the time resolution of the spectrometer (~ 200 fs) and the homogeneous linewidth of the transition.

Discussion

Each of the three sets of experimental data presented above allow inferences to be drawn regarding the chemical or dynamic nature of the environment in which the NO ligand is located in the HRP-BHA complex.

FTIR spectroscopy

In the case of the FTIR spectra, binding of BHA to HRP leads to a significant narrowing and blue-shifting of the NO stretching vibrational mode in comparison to the substrate-free enzyme in both solvents. For ease of reference the corresponding data for the substrate-free HRP enzyme are also given in Table 1.¹⁴

It has been shown that the absorption frequency of the ν_{NO} mode in ferric haem systems is sensitive to local contacts with the protein scaffold.^{21, 23, 35} Of particular relevance here, studies on myoglobin and its mutants have indicated that the presence of a strong interaction between distal components of the haem pocket, such as the nearby histidine side chain, leads to an increase in the NO stretching vibrational frequency.^{23, 38} This effect has been attributed to the fact that, when bound to ferric haems, the electronic ground state of the Fe(III)-NO moiety is somewhat better-described by the Fe(II)-NO⁺ formalism.³⁹ This engenders interactions between the NO and the distal histidine in myoglobin that occur through a lone pair donation mechanism rather than a hydrogen bond leading to an upshift of the frequency.

In the case of substrate-free HRP, although a distal histidine residue is present in the active site, it has been shown to be more distant from the haem than would be

required for direct interaction.^{10, 11} Additionally, previous work employing ultrafast infrared spectroscopy in both aqueous and deuterated solvents has shown results that are more consistent with the primary interaction of the NO being with water molecules in the haem pocket.¹⁴ When BHA binds to HRP however, X-ray crystallography indicates that the phenyl ring is located near the δ -edge of the haem while the hydrophilic head group is located close to the Fe centre and the nearby His42 and Arg38 residues, to which the oxygen and nitrogen atoms of BHA form H-bonds (see Fig 1). In the un-liganded form of the protein, a single water molecule is observable by crystallography located in a position 2.6 Å above the haem iron that is most consistent with a weak interaction with the Fe centre.^{5, 16, 17} In the case of a haem centre with the diatomic CN ligand bound to it, the binding of BHA has been shown to be similar but there are indications of a rotated BHA head group in order to better accommodate interactions with the haem ligand.⁵

In light of this, the FTIR data are consistent with a major change in the local environment of the NO ligand occurring upon substrate coordination. Further, the spectral changes appear to be in agreement with the picture derived from the crystal structures, with the upshift of the ν_{NO} frequency supporting a close interaction between either the O or N atom of the BHA head group and the NO ligand. Alongside the frequency upshift, the marked narrowing of the ν_{NO} lineshape suggests a less inhomogeneously-broadened environment with the substrate in place, which is consistent with the replacement of water with more defined, directional interactions from BHA. Similar conclusions have been reported using IR absorption spectroscopy in concert with molecular dynamics simulations on the ferrous carbonmonoxy variant of HRP.⁴⁰ Thus, the FTIR data would seem to support removal of water from the vicinity of the haem ligand upon binding of BHA but confirmation of this requires consideration of the time-resolved experiments.

IR pump-probe spectroscopy

The vibrational lifetime of the ν_{NO} mode obtained from pump-probe spectroscopy provides a further useful indicator of the changes that are caused upon substrate binding to HRP. No solvent isotope dependence of the NO stretching mode vibrational lifetime is detected with the substrate in place; the value derived is essentially identical in both aqueous and deuterated solvents (Table 1).

This observation contrasts sharply with the situation found in the substrate-free enzyme in which the ν_{NO} mode lifetime became longer by a factor of ~25% upon deuteration of the solvent (Table 1).¹⁴ This effect was attributed to the significant increase in the solvent-related density of vibrational states in the region of the IR spectrum near the NO stretching mode frequency in aqueous versus deuterated solvents. As a result of the greater overlap between solvent modes and the ν_{NO} mode in aqueous water, more rapid energy transfer and vibrational relaxation is facilitated in relation to deuterated conditions; this effect is not limited to proteins and has been observed for various solvent-solute systems.⁴¹⁻⁴³ This isotope-dependent relaxation mechanism appears to be absent in the HRP-BHA data.

Overall, the vibrational lifetime data for HRP-BHA points to the fact that the mechanism by which the NO mode relaxes is different to that of the substrate-free enzyme in that this no longer occurs through direct involvement of the solvent. This observation is also important in that it would seem to clearly separate the impact on ν_{NO} relaxation of deuteration of the protein backbone from that of the isotopic content of the solvent. This question arises because, upon transfer into a deuterated

solvent, care is taken to ensure replacement of all readily-exchangeable protons within the enzyme structure. Thus, deuteration will necessarily alter the vibrational density of states of the protein and could, in principle, affect ν_{NO} mode relaxation dynamics. However, if this were the mechanism by which H/D exchange impacted upon the vibrational lifetime of the NO probe, this would have been observed in the substrate-bound enzyme also. That it is not confirms the original assignment of the isotope-dependent dynamics to water involvement in the haem pocket but also adds weight to the argument that this water is removed from the environment of the NO ligand upon substrate binding.

It is interesting also to note that the vibrational relaxation of the NO stretching vibration of ferric myoglobin is similarly insensitive to solvent isotope exchange.¹⁴ As myoglobin is well-known to have no direct interaction between water and the haem ligand, rather a strong H-bond to the distal histidine residue occurs, this is also consistent with the observations for HRP-BHA.

The magnitude of the vibrational lifetime observed for HRP-BHA is not substantially different to either the aqueous or deuterated forms of the substrate-free enzyme (Table 1), indeed the values for the HRP-BHA complex fall between those of substrate-free HRP in aqueous and deuterated solvents.¹⁴ Overall, this suggests that BHA binding does not lead to the creation of a markedly more efficient route for vibrational relaxation than the water-mediated environment of HRP. A possible reason for this is that the ultimate destination of the vibrational energy imparted by the pump pulse is likely to be the same low-frequency modes of the protein architecture. The change from solvent-sensitive to solvent-independent relaxation dynamics merely seems to indicate that the initial step in this process has changed from interaction with water-related vibrational modes to that with modes of BHA. It is relevant here that BHA also possesses a range of overtone and combination band transitions near 1900 cm^{-1} arising from ring vibrations and those of the head group. These modes could potentially allow NO relaxation in place of those of the solvent for the substrate-free enzyme. Notably, these BHA-derived modes will be largely unaffected by deuteration of the solvent as BHA possesses only one or two exchangeable protons, unlike those of water. As such, large changes in the relaxation rate upon substrate binding should perhaps not be expected.

2D-IR spectroscopy

The picture emerging from the FTIR and vibrational lifetime data is further supported by the 2D-IR measurements of spectral diffusion processes affecting the ν_{NO} mode. The dynamic timescales recovered from this experiment provide information relating to the rate of fluctuation of the ν_{NO} frequency in response to local changes in the electrostatic environment of the ligand. These in turn have been shown to derive from motions within the local haem pocket.³⁵

When BHA is bound to HRP these dynamics show two main components; a picosecond-timescale decay on the order of $\sim 4\text{-}6$ ps and a static offset. Studies applying 2D-IR to a range of haem proteins have indicated that these fluctuations can have a variety of origins. Fast, few ps, dynamics typically arise from highly-specific motion of distal residues in close proximity to the ligand while slower, more global motions of the haem pocket structure contribute to the longer dynamic components.^{20, 21, 23, 27-33, 44-46} The latter tend to appear as a static offset in the data because they are too slow to fall within the temporal range of the 2D-IR experiment, which is limited by the vibrational lifetime of the probe group. More recently, it has

been shown that contributions to fast spectral diffusion processes can also occur from the presence of water near the haem ligand. These have been differentiated from protein-derived motions via a solvent isotope-dependence.¹⁴

On binding a substrate analogue to HRP the results do not appear consistent with water interacting with the haem ligand. When considering the uncertainty in the spectral diffusion timescales recovered for HRP-BHA (Fig 5), it is hard to establish a clear solvent-dependent separation between the picosecond-timescale dynamics observed in the two solvents. Perhaps the most important aspect of the data however is that the timescale recovered under aqueous conditions is longer than that in deuterated solvents, which is opposite to what one would expect for typical solvent-dependent dynamics.

As with the vibrational relaxation dynamics, it is interesting that the absolute magnitude of the spectral diffusion timescales observed in the picosecond time range for the NO stretching mode of HRP-BHA does not differ markedly from that of substrate-free HRP. The value of ~ 4-6 ps, falls between the limits of 3.3 (H₂O) and 7 ps (D₂O) observed for the substrate-free enzyme. The data from HRP experiments were explained by a model in which the water in the active site of HRP was dynamically-constrained by the nature of the haem pocket, leading to the slowing of the water motion from the 1-2 ps dynamics observed for Fe-NO units in bulk solution.⁴⁷ This was attributed to the small free volume present in the distal part of the pocket such that, rather than being free to undergo reorientational motion as in the bulk, water molecules are subject to interactions with polar, hydrophilic residues near the haem, potentially restricting motion and slowing the observed dynamics. Such an effect has been observed in reverse micelles.⁴⁸ This assignment was supported by quantitative analysis of the free-volume in the haem pocket of the substrate-free enzyme obtained from X-ray crystallography, which showed sufficient volume in the cavity for only a very small pool of <10 water molecules. While it has been shown that such a pool can support fast dynamic processes (< 2ps),⁴⁸ these simulations also suggested that the majority of the non-structural water was displaced from the centre of the haem in HRP, such that the haem-bonded NO would only interact with the extremities of the pool, where the interactions with protein residues are likely to become more dominant in restricting water motion.

In the case of the substrate-bound enzyme, the evidence suggests that, rather than there being an interaction between NO and the water pool, BHA displaces the water from near the haem, disrupting the pool and making H-bonding interactions with the NO ligand, consistent with the structures revealed in the crystalline phase.^{5, 16} This would explain the removal of the isotope dependence of the data. Given this scenario, it may be considered that interactions between the larger head-group of BHA and the NO ligand might be expected to yield slower spectral diffusion timescales than those found when smaller, more mobile water molecules are responsible for the dynamics. The fact that the timescales are not significantly different however suggests that the fluctuations of the BHA head group, being H-bonded to the protein architecture as they are, will be influenced to a certain extent by the same motions of the active site as water molecules were in the substrate-free enzyme, leading to similar observed dynamic timescales, albeit without the same sensitivity to solvent isotope which are superimposed on these motions in the presence of water.

In the case of the static offset parameter, this too does not vary noticeably with solvent isotopic composition in the substrate-bound system, in contrast to the data

for substrate-free HRP, which showed a noticeable increase in amplitude on progressing from D₂O to H₂O solvents.¹⁴ This also points to a reduction in the influence of water on the observed dynamics with the substrate present.

The persistence of a significant static component to the data in HRP-BHA, similar to that of the substrate-free enzyme, suggests that the overall flexibility of the haem pocket observed for HRP does not change greatly on binding BHA. It is interesting to note that previous studies of substrate binding to enzymes have determined that a significant shortening of spectral diffusion timescales are observed upon binding the correct combination of substrate and transition state analogue inhibitor molecules.⁴⁹ This was exemplified by a significant reduction in the amplitude of the pseudo static component of the spectral diffusion dynamics observed.^{17, 49} In the case of HRP-BHA however, such a shortening is not observed but it is noted that NO is not a precise structural analogue of CpDI, furthermore, BHA is not a natural substrate of HRP, merely one of a range of molecules that can bind to the substrate binding position.¹ As such, although it is instructive to employ BHA to shed light on water behaviour in substrate-bound HRP, this result suggests that it does not lead to the kind of large-scale dynamic modification of the pocket that has been indicated for some systems that are perhaps closer to the physiological situation.

Related to this, the first report of the use of 2D-IR spectroscopy to interrogate the structural dynamics of HRP, including changes to these dynamics upon BHA binding, noted that an increase in the rate of the picosecond fluctuations was observed upon substrate binding.²⁰ In that case, the ferrous form of HRP was studied with CO used as the spectroscopic probe. In addition, a general slowing of the longer timescale dynamics was attributed to a restriction of the structure following substrate binding. The change in the fast dynamics is replicated in this study of the ferric system also, though only for deuterated systems. In contrast, the reverse situation occurs for aqueous solvents with the fast timescale slowing on addition of BHA.

Finally, even upon substrate binding, the type of heavily restricted dynamics and coherent vibrational motions observed in catalase are not replicated, maintaining the view that these enzymes have dynamically very different active sites and that it is this that determines the differences in reactions catalysed.^{14, 21}

Mechanistic implications:

It is interesting to consider these results in light of the mechanism of action of HRP in which substrate molecules interact with the reactive intermediate, CpDI, undergoing oxidation. These results suggest that, although water molecules are clearly important in the formation of CpDI in HRP, as they are in catalase,¹⁴ there is a likelihood that binding of substrate molecules leads to exclusion of water from the vicinity of the haem, such that they are unlikely to play any role in later steps. Such a scenario has also been proposed following simulations.^{10, 11} This is perhaps consistent with the scenario in which water molecules are needed to bridge distances between the haem ligand and the nearby residue side chains prior to CpDI formation but the insertion of a substrate into the haem pocket, which will establish its own H-bonds to these residues, removes this need allowing subsequent steps to proceed directly.

This conclusion would seem to be at odds with the view of a 'wet' CpDI, as is widely believed to occur in HRP. However, ultrafast dynamic measurements now suggest that the concept of a wet intermediate needs to be viewed as a subtle

construct related to the fact that the dynamics near the HRP haem ligand during CpdI formation are solvent-mediated but rather more flexible, and thus slightly closer to bulk-like water, than those of catalase. These dynamics appear to specifically relate to CpdI attainment rather than the onward steps and so these results are not inconsistent with the established view.

It is noteworthy that no difference in the ability of HRP to bind NO was noted upon prior binding of BHA. This suggests that, although restricted by the motion of F68, it is still possible to gain access from solvent to haem with BHA in place. Rather, it is the close matching of H-bonding components of BHA to distal residues in the pocket that leads to exclusion of water from near the haem. Interestingly, this poses the possibility that H₂O₂ could also gain access to the haem with a substrate in place though it is not clear whether CpdI can be achieved under these conditions.

Conclusions

Ultrafast, multi-dimensional infrared spectroscopy methods have been employed to investigate the effect of substrate binding on the structural dynamics of the Horseradish peroxidase (HRP) enzyme. Measurements of the frequency fluctuations (spectral diffusion) and vibrational lifetime of the stretching vibrational mode of NO bound to ferric HRP with benzohydroxamic acid (BHA) located in the substrate-binding position at the periphery of the haem pocket, in both D₂O and H₂O solvents reveal no sensitivity of these parameters to H/D exchange. Comparison of these results with those for the substrate-free enzyme suggests that large changes in solvent residence in the haem pocket are caused by substrate binding. Overall, these observations imply that, although water molecules are vital for the formation of CpdI, they are likely to play a less important role in onward reaction processes of peroxidase and that it is the overall dynamics and relative flexibility of the respective pockets that determines the differences in behaviour between peroxidases and catalases. In turn, we suggest that the concept of the 'wet' and 'dry' CpdI intermediates proposed for these enzymes^{9, 15} can be modified to a more nuanced picture upon consideration of ultrafast dynamics.

Acknowledgements

NTH, KA, GH and NS would like to acknowledge funding for this work from the EPSRC and support from STFC for access to the Central Laser Facility. Useful discussions with Dr A Gumiero are also gratefully acknowledged.

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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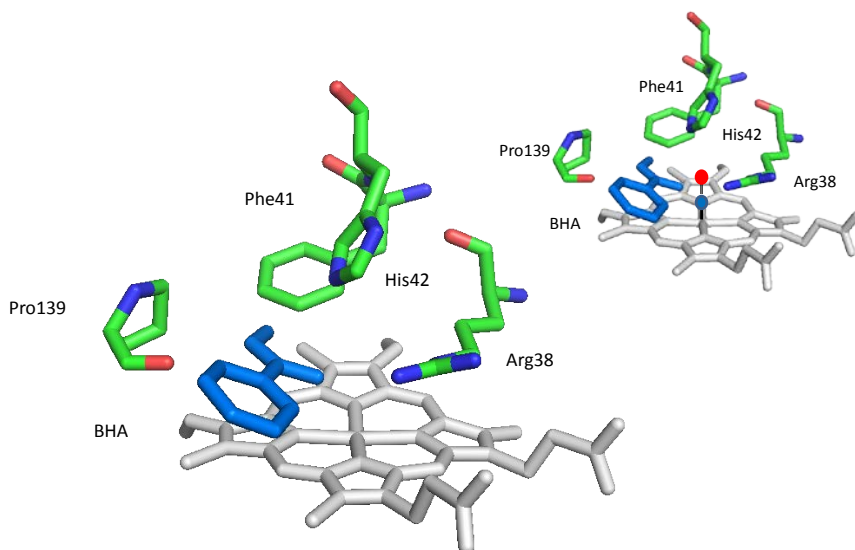


Figure 1: Structure of the haem pocket of HRP-BHA showing residues with side chains located in close proximity to the haem (PDB: 2ATJ)⁵ The resolution of the structure was 2 Å. The inset shows a schematic representation of the same structure with the NO binding position indicated; N is shown as a blue sphere; O, red.

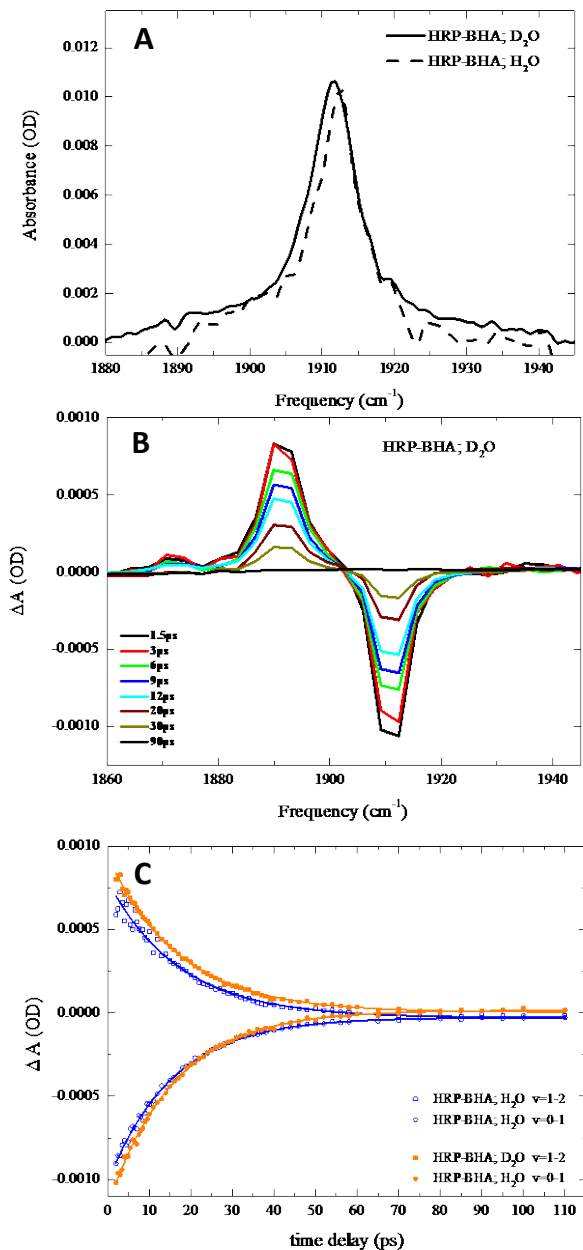


Figure 2: A) FTIR spectra of HRP-BHA in deuterated (solid line) and aqueous (dashed line) solvents. B) Ultrafast IR pump-probe spectra of HRP-BHA in deuterated solvent at a range of pump-probe delay times. C) Temporal decay profiles of the peaks observed in pump-probe spectra for HRP-BHA in aqueous (blue) and deuterated (orange) solvents. Solid lines indicate the results of fitting to exponential functions as described in the text.

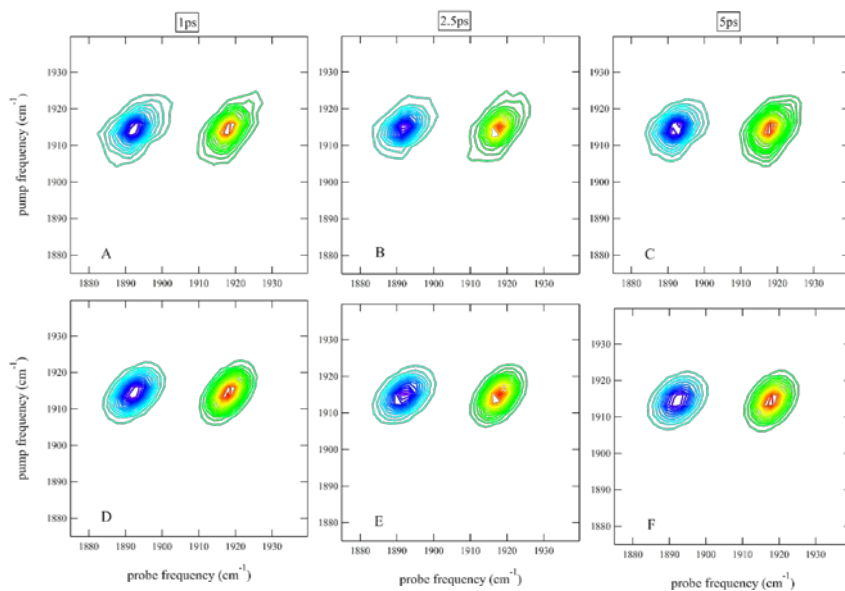


Figure 3: A-C) 2D-IR spectra of HRP-BHA in aqueous solvents at a range of experimental waiting times. D-F) Fits of the data in A-C) to a two-dimensional Gaussian function as discussed in the text.

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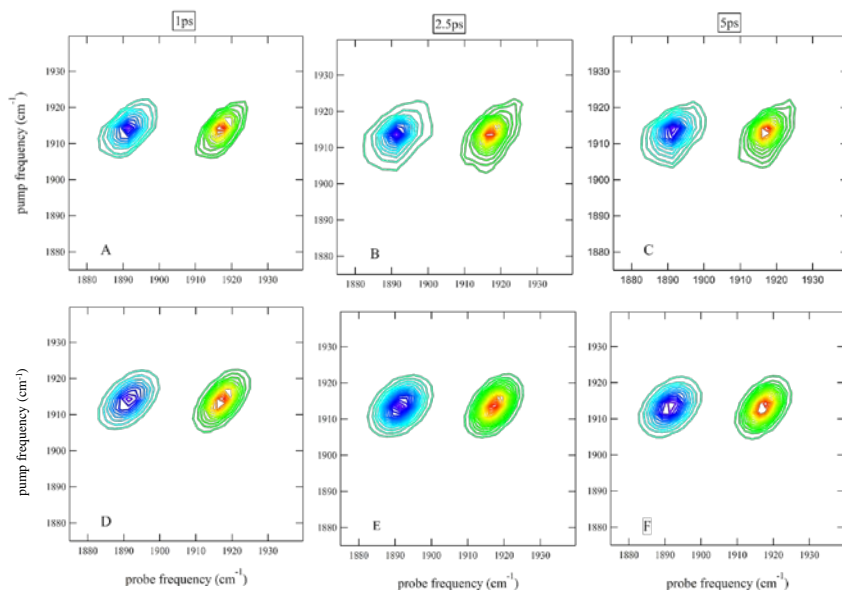


Figure 4: A-C) 2D-IR spectra of HRP-BHA in deuterated solvents at a range of experimental waiting times. D-F) Fits of the data in A-C) to a two-dimensional Gaussian function as discussed in the text.

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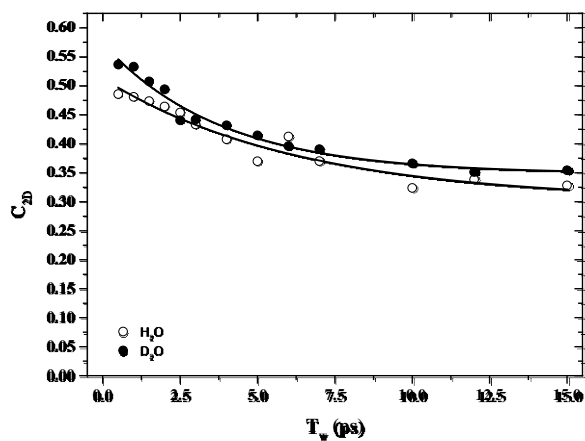


Figure 5: Spectral diffusion dynamics for HRP-BHA in both aqueous (open circles) and deuterated (filled circles) solvents. Solid lines indicate fits to a single exponential decay as described in the text.

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