Time-resolved FTIR study of CO recombination with horseradish peroxidase

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Abstract
Vibrational changes associated with CO recombination to ferrous horseradish peroxidase were investigated by rapid-scan FTIR (Fourier-transform IR) spectroscopy in the 1200–2200 cm\(^{-1}\) range. At pH 6.0, two conformers of bound CO are present that appear as negative bands at 1905 and 1934 cm\(^{-1}\) in photolysis spectra. Their recombination rate constants are identical, confirming that they arise from two substates of bound CO that are in rapid thermal equilibrium, rather than from heterogeneous protein sites. A smaller positive band at 2134 cm\(^{-1}\) also appears on photolysis and decays with the same rate constant, indicative of an intraprotein geminate site involved in recombination or, possibly, a weak-affinity surface CO-binding site. Other signals arising from protein and haem in the 1700–1200 cm\(^{-1}\) range can also be time-resolved with similar kinetics.

Introduction
HRPC (horseradish peroxidase isoenzyme C) catalyses the oxidation of a range of organic compounds by hydrogen peroxide and is a useful model system for studies of redox catalysis involving haem ferryl states. Crystal structures have been solved with various ligands bound and in different stages of its catalytic cycle [1–3]. The protein has a haem B that is proximally ligated to His\(^{170}\). A catalytic pocket is formed at the distal face of the haem involving His\(^{42}\), Arg\(^{38}\) and Phe\(^{41}\). The distal His\(^{42}\) has a \(pK_a\) of 7.5 [4], which is shifted to 8.8 when CO is bound to the reduced enzyme [5]. IR [5–7] and Raman [8] spectra display two conformers of bound CO with \(\nu(CO)\) vibrations at 1905 and 1934 cm\(^{-1}\) at pH values in the 5–8 range that convert into a single 1934 cm\(^{-1}\) form at high pH. Mutagenesis [6] and simulation [9] studies indicate that, in the low-pH form, the bound CO has possible hydrogen-bonding partners of His\(^{42}\) and the guanidinium \(c\text{NH}\) of Arg\(^{38}\). At high pH, the \(N_c\) of His\(^{42}\) is deprotonated and cannot form a hydrogen bond with the CO, leaving only the guanidinium \(c\text{NH}\) of Arg\(^{38}\) as a hydrogen-bond donor.

CO binding to HRPC has also been studied by FTIR (Fourier-transform IR) spectroscopy using continuous illumination to generate stationary photodissociated states [7]. In this case, the signal/noise ratio is sufficient to also monitor associated vibrational changes in protein and haem. Such FTIR spectra can be time-resolved by rapid-scan [10] or step-scan [11,12] techniques. In the present study, rapid-scan FTIR spectroscopy was used to explore further the relation between bound forms of CO in HRPC and to time-resolve the associated vibrational changes of protein and haem during CO recombination.

Key words: carbon monoxide, Fourier-transform infrared spectroscopy (FTIR spectroscopy), horseradish peroxidase, rapid-scan, time-resolved spectroscopy.

Abbreviations used: FTIR, Fourier-transform IR; HRPC, horseradish peroxidase isoenzyme C.

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Time-resolved FTIR spectroscopy
Optimal samples (roughly 12 mM HRPC) had absolute absorption spectra parameters of: (i) an absorption of the 1650 cm\(^{-1}\) peak arising from amide I + water in the 1.0–1.2 range; (ii) a ratio of the maxima at 1650 and 1550 cm\(^{-1}\) between 1 and 1.5, to provide an optimal protein/water ratio; and (iii) ratios of CO/amide II peaks of at least 1/64 (1905/1550 cm\(^{-1}\)) and 1/100 (1934/1550 cm\(^{-1}\)). The pre-flash level of ferrous–CO compound was 40–50% over the time course of the measurements, with the remainder being the unligated ferrous form.

Spectra were recorded in rapid-scan mode at 275 K on a Bruker IFS66/S spectrometer fitted with a liquid nitrogen-cooled MCT-B detector. Typically, 500 interferograms were averaged to provide an initial dark baseline. Photodissociation of the HRPC ferrous haem–CO compound was achieved with a laser pulse that was synchronized with initiation of recording of 100 single interferograms with a scanner velocity of 280 kHz (time resolution of 16 ms at 4 cm\(^{-1}\) resolution). This cycle was repeated at 3 Hz, and 28000 interferograms were averaged before Fourier transformation to generate a three-dimensional data block of absorbance against frequency against time. The data from three different samples were averaged in order to improve the signal/noise ratio. Difference spectra at specific times after CO photodissociation and kinetics at specific frequencies were extracted from three-dimensional data blocks using Bruker OPUS 6.5 software.

Kinetic analyses
Because of the high protein concentrations required for viable FTIR samples, kinetic data were fitted with a generalized second-order rate equation in which various concentrations of both unligated enzyme and free CO were considered.

Figure 1 | Representative three-dimensional plot of rapid-scan FTIR spectra after laser photolysis of the CO adduct of ferrous HRPC

Samples of the CO compound of ferrous HRPC were prepared at pH 6.0 and equilibrated at 275 K. Data were acquired in rapid-scan mode after laser photolysis at zero time. The first spectrum (black) was recorded 16 ms after photolysis, and only data recorded during the first 360 ms of the reaction are shown. Each spectrum represents the average of 28,000 single interferograms recorded at 4 cm\(^{-1}\) resolution.

The averaged pre-flash ratio of (ferrous HRP–CO)/(ferrous HRPC) was estimated from the CO/amide II band ratio and photolysis yield was 40%.

Kinetics of CO rebinding to ferrous HRPC

Figure 1 shows a three-dimensional data block of rapid-scan difference spectra of CO recombination with ferrous HRPC after laser photolysis at 275 K and pH 6.0. The bands correspond closely to those reported previously in photostationary photolysis spectra [7]. Most prominent are the 1934 and 1905 cm\(^{-1}\) troughs that have been assigned to \(v(CO)\) bands of two conformations of haem-bound CO, stabilized by different strengths of hydrogen-bonding interaction(s), either to Arg\(^{38}\) alone (1934 cm\(^{-1}\) conformer) or to both Arg\(^{38}\) and His\(^{62}\) (1905 cm\(^{-1}\) conformer) [6,9]. The decay kinetics of both bands could be fitted with the same second-order rate constant (k) of 1.7 ± 0.1 mM\(^{-1}\) · s\(^{-1}\) (Table 1). This confirms that the conformers do not arise from two separate forms of HRPC, but instead are two states that are in rapid thermal equilibrium within the whole HRPC population. Good fits of second-order simulations were achieved by taking a value of 40% photolysis of HRPC–CO (determined from band intensities in dark absolute spectra) to yield 8.8 mM free HRPC and 2.2 mM free CO. Kertesz et al. [13] and Coletta et al. [14] measured the second-order rate constant for CO binding to HRPC at 3.4 and 3.0 mM\(^{-1}\) · s\(^{-1}\) respectively at pH 7.0 and 293 K. Given that the kinetics are unchanged between pH 5.0 and 7.0 [6,14] and slowed by a factor of 3 upon temperature change from 293 to 275 K [15], the IR-derived rate constant is roughly in agreement with literature values.

Table 1 | Rate constants of relaxation of selected frequencies associated with CO rebinding to ferrous HRPC after laser flash photolysis

<table>
<thead>
<tr>
<th>Frequencies ((mM^{-1} · s^{-1}))</th>
<th>Assignments</th>
</tr>
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<tbody>
<tr>
<td>2134 cm(^{-1})</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>1934 cm(^{-1})</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>1905 cm(^{-1})</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>1651−1641 cm(^{-1})</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>1548−1539 cm(^{-1})</td>
<td>1.7 ± 0.2</td>
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</table>

A positive band of lower intensity also appears after photolysis at 2134 cm\(^{-1}\) (Figures 1 and 2). This band, which is evident in earlier work [7], must arise from an additional bound state of CO that is formed by the photolyzed CO. It could be an intraprotein geminate state as is seen, for example, in myoglobin [16,17] and, possibly, other proteins such as NO synthase [18] and cytochrome \(b_5\) [19] or it could also arise from a weak-affinity surface site that reacts to the increased solution CO concentration after photolysis [20]. The time-resolved spectra of Figures 1 and 2 show that this peak decays with the same rate constant as the bands of haem-bound CO (Table 1). Hence, it can be concluded that the 2134 cm\(^{-1}\) species is already in equilibrium with solution CO in the first (16 ms) trace and must represent a transient site that binds CO before it forms the stable recombination product with ferrous haem or CO binding to a weak-affinity surface site. If a geminate site, a higher occupancy immediately after photolysis might be expected, and this warrants further investigation at smaller time scales by step-scan methods.

Vibrational modes associated with protein and haem

Photolysis/recombination of CO is accompanied by complex vibrational changes in protein and haem that appear below 1800 cm\(^{-1}\) in the ‘fingerprint’ region. Tentative assignments of major bands have been made on the basis of effects of hydrogen/deuterium exchange and comparisons with model materials [7]. Within the available signal/noise levels, all of these bands were found to relax with the same kinetics as the bands of bound CO. For example, the peak/trough at 1548/1539 cm\(^{-1}\), which was assigned to a haem band rather than amide II from its insensitivity to hydrogen/deuterium exchange, had a decay rate constant of 1.7 ± 0.2 mM\(^{-1}\) · s\(^{-1}\) and that at 1651−1641 cm\(^{-1}\), which is probably an amide I bandshift, one of 1.6 ± 0.2 mM\(^{-1}\) · s\(^{-1}\) (Table 1). Hence, the haem and surrounding protein rearrangements that accompany CO rebinding to haem must occur in synchrony, with no slower post-recombination phases of reorganization.
Figure 2 | Transient FTIR difference spectra after laser photolysis of the CO adduct of ferrous HRPC

(A) Spectra extracted and averaged from three separate three-dimensional datasets obtained as in Figure 1 (i.e. each spectrum is an average of 84,000 individual interferograms) at 16 (black), 73 (red), 130 (blue), 244 (dark green), 357 (purple) and 1098 (light green) ms after photolysis. Spectra represent absorbance differences from the dark state before photolysis. (B) Plots of absorbance against time extracted from three-dimensional datasets at 1905 (H17033), 1934 (H17039) and 2134 (H17009.) cm$^{-1}$ with second-order fits overlaid.

Conclusions
This rapid-scan time-resolved FTIR spectroscopy study of CO recombination with ferrous HRPC confirms that the two low-pH conformers of CO are thermally interconvertable substates within the whole protein population. It also reveals a synchronous 2134 cm$^{-1}$ transient that must arise from CO binding to a transient geminate site during rebinding or to a weak-affinity surface site. This work also establishes that the signal/noise ratio is sufficient to extend the kinetic studies into the region below 1800 cm$^{-1}$ where vibrational changes in the protein and haem cofactor can also be observed to relax in concert with CO recombination. These studies are being extended to smaller time scales with step-scan methods [11] in order to ascertain geminate states and whether recombination and structural events can be temporally resolved. Progression to application to the functionally more complex cytochrome c oxidase is also underway.

References
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