Peroxidase-benzhydroxamic acid complexes: Spectroscopic evidence that a Fe-H2O distance of 2.6 Å can correspond to hexa-coordinate high-spin heme

ARTICLE *in* JBIC JOURNAL OF BIOLOGICAL INORGANIC CHEMISTRY · MARCH 1999

Impact Factor: 2.54 · DOI: 10.1007/s007750050287 · Source: PubMed

5 AUTHORS, INCLUDING:

Giulietta Smulevich
University of Florence
186 PUBLICATIONS **4,531** CITATIONS
SEE PROFILE

Chiara Indiani
Manhattan College
29 PUBLICATIONS **1,081** CITATIONS
SEE PROFILE

Maurizio Becucci
University of Florence
89 PUBLICATIONS **1,002** CITATIONS
SEE PROFILE

JBIC (1999) 4:39–47

ORIGINAL ARTICLE

Giulietta Smulevich · Alessandro Feis
Chiara Indiani · Maurizio Becucci
Mario P. Marzocchi

Peroxidase-benzhydroxamic acid complexes: spectroscopic evidence that a Fe-H_2O distance of 2.6 Å can correspond to hexa-coordinate high-spin heme

Received: 17 July 1998 / Accepted: 17 November 1998

Abstract Resonance Raman (RR) spectra have been obtained for single-crystal horseradish peroxidase isozyme C complexed with benzhydroxamic acid (BHA). The data are compared with those obtained in solution by both RR and electronic absorption spectroscopies at room and low (12–80 K) temperatures. Moreover, the analysis has been extended to the *Coprinus cinereus* peroxidase complexed with BHA. The results obtained for the two complexes are very similar and are consistent with the presence of an aqua six-coordinate high-spin heme. Therefore it can be concluded that despite the rather long Fe-H_2O distance of 2.6–2.7 Å found by X-ray crystallography in both complexes, the distal water molecule can still coordinate to the heme iron.

Key words Horseradish peroxidase · *Coprinus cinereus* peroxidase · Benzhydroxamic acid · Single crystal · Resonance Raman

Abbreviations *BHA* benzhydroxamic acid · *SHA* salicylhydroxamic acid · *APX* Ascorbate peroxidase · *ARP Arthromyces ramosus* peroxidase · *HRPC, HRPA1, HRPA2* horseradish peroxidase isoenzymes C, A1, and A2, respectively · *CCP* cytochrome *c* peroxidase · *CCPMI* recombinant CCP expressed in *Escherichia coli* containing Met-Ile at the N-terminus · *CIP Coprinus cinereus* peroxidase · *MetMb* metmyoglobin · *MnP* manganese peroxidase · *LIP* lignin peroxidase · *MPO* myeloperoxidase · *RR* resonance Raman · *5-c, 6-c* five- and six-coordinate · *HS* high spin · *LS* low spin · *QS* quantum admixed spin · *IS* intermediate spin · *CT1* long-wavelength (>600 nm) porphyrin-to-metal charge-transfer band

G. Smulevich (✉) · A. Feis · C. Indiani · M. P. Marzocchi
Dipartimento di Chimica, Università di Firenze,
Via G. Capponi 9, I-50121 Florence, Italy
e-mail: smulev@chim.unifi.it, Tel.: +39-055-2757596,
Fax: +39-055-2476961

M. Becucci
LENS-European Laboratory for Non Linear Spectroscopy,
Largo E. Fermi 2, I-50125 Florence, Italy

Introduction

Peroxidases are heme-containing enzymes which catalyze the reduction of hydrogen peroxide to water with the concomitant oxidation of structurally different substrates. The nature of the reducing substrates varies widely and a number of studies have been performed with the aim of characterizing their interaction with the enzymes. Moreover, peroxidases can bind aromatic substrates in the absence of hydrogen peroxide and many studies have been devoted to understand the nature of the aromatic-donor binding site [1, 2]. Although not a physiological substrate of plant peroxidases, benzhydroxamic acid (BHA) has been used extensively in binding studies as a convenient probe for the aromatic-donor site. In particular the complexes between horseradish peroxidase isoenzyme C (HRPC) and BHA have been studied by means of several spectroscopic techniques [1, 3–11] at both room and low temperatures. Furthermore, complexes of some selected site-directed mutants of HRPC with BHA have been investigated [12–14]. All the techniques supported the proposal that BHA binds to the distal side of the heme cavity and that the distal Arg38 and His42 residues are important for the binding of the aromatic donor [13, 15, 16]. However, there are conflicting interpretations as to whether BHA affects the binding of a water molecule to the Fe atom. From electronic absorption [3], resonance Raman (RR) [6, 7, 9, 13], and EPR [5] it has been concluded that the BHA complex of HRPC has a water bound to the Fe atom, whereas, recently, on the basis of altered proton hyperfine NMR shifts, the HRPC-BHA complex was assigned to a five-coordinate high-spin (5-c HS) species [11]. Other peroxidases bind BHA, namely horseradish peroxidase isozyme A2 (HRPA2) [17] and *Coprinus cinereus* peroxidase (CIP) [2]. Recently, the X-ray crystal structures of *Arthro-*

myces ramosus peroxidase (ARP) (a peroxidase essentially identical to CIP) [18] and HRPC in complex with BHA [19] have been determined. BHA binds similarly in both peroxidases and is located in the distal side of the heme pocket, with the aromatic ring nearly parallel to the heme group. The functional groups of BHA are hydrogen bonded to the distal His and Arg residues and a conserved Pro near the heme edge, but too far away to interact with the heme iron. In both HRPC and ARP complexes, only one water molecule is present in the distal heme pocket. This water forms a hydrogen bond with either the oxygen (O_9) of the hydroxyl (in ARP), or the oxygen (O_7) of the keto (in HRPC) group of BHA. In both cases the water molecule appears to be closer to the Fe atom as compared to the native structures [20, 21]. Nevertheless, the distances of 2.6–2.7 Å between the water molecule and the iron atom in these complexes are significantly greater than the 1.8–2.2 Å Fe-ligand distances seen in the X-ray crystal structures of CCP with exogenous ligands bound at the sixth coordination site of the heme [22], or in metmyoglobin (MetMb) in which a water molecule is bound to the ferric heme [23, 24].

In the present work we compare the RR spectra of HRPC-BHA in single crystal and solution, and the RR and electronic absorption spectra of CIP-BHA in solution at room and low temperatures with the aim of understanding the coordination and spin state of the heme iron. The results show unambiguously that in the complexes, for samples in both crystalline and solution states, the heme iron is six-coordinate high spin (6-c HS) and not predominantly 5-c HS, as recently suggested [25]. Therefore, the water molecule observed at 2.6–2.7 Å [20, 21] is bound to the Fe atom. The results are discussed and compared with those previously reported for the myeloperoxidase-BHA complex [26–28].

Material and methods

Recombinant CIP was obtained by expression in transformed *A. oryzae* [29] and purified as previously described [12]. HRPC (Sigma, type VI-A, $R_z = 3.1$) was used without further purification. Single crystals of recombinant HRPC with and without BHA were obtained as previously reported [19, 21] and kindly provided by Dr. A. Henriksen, University of Copenhagen, Denmark.

Solution

The absorption spectra were recorded with a Cary 5 spectrophotometer. The RR spectra in solution were obtained with excitation from the 406.7 nm line of a Kr^+ laser (Coherent) and the 496.5 nm and 514.5 nm lines of an Ar^+ laser (Coherent, Innova/5). The back-scattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Yobin-Yvon HG2S) equipped with a cooled photomultiplier (RCA C31034 A) and photon-counting electronics. Polarized spectra were obtained by inserting a polaroid analyzer between the sample and the entrance slit of the spectrometer. The depolarization ratios of the bands at 314 cm^{-1} and 460 cm^{-1} of CCl_4 were measured to check the reliability of the polarization measurements using a rotating NMR tube with a 180° back-scattered geometry. The values obtained, 0.73 and 0.0, compare favorably with the theoretical values, 0.75 and 0.0, respectively. To minimize the heating effects induced on the protein by the laser beam, the room temperature RR spectra were collected using a rotating NMR tube cooled by a gentle flow of N_2 gas passed through liquid N_2.

Low-temperature (12 K) experiments were carried out using a closed-cycle He cryostat with automatic temperature control. The samples for RR spectra were transferred with a syringe to a small groove in the copper cold finger of the cryostat at 180 K under nitrogen flow and the temperature was then slowly decreased to 12 K under vacuum. The RR spectra were measured at this temperature. The absorption spectra at low temperature (12 K) were obtained using a sealed quartz micro-cuvette (100 μl, 0.3 cm). The sealed cuvette, filled with the protein solution at room temperature, was mounted on the cold finger of the cryostat. The temperature was then slowly decreased under vacuum. The same apparatus was also used to obtain the RR spectra. Identical results were gained in the high-frequency region using the two methods. The low-frequency region showed a background due to the thick quartz windows. Electronic absorption spectra of the protein complexed with BHA were measured at 80 K using a liquid nitrogen immersion cell, since the BHA dissociated and precipitated upon a slow lowering of the temperature.

Single crystal

The HRPC-BHA crystal was placed with a small amount of mother liquor on a slide and sealed by vacuum grease with a cover glass. A specially modified Raman microprobe apparatus was used [30]. It consists of an Olympus BHSM2 microscope equipped with a long working distance (16 mm) Leitz-Wetzlar NPL Fluotar L25 objective capable of 25× magnification with a 0.35 numerical aperture. Our estimation of the laser beam waist in the focus of the objective is about 5 μm. The same objective is used for both the sample excitation and the collection of the back-scattered radiation. The microscope holds an optical beamsplitter which sends 10% of the available laser power to the sample and transmits the 90% of the collected emission to the detection system. The spectrometer is a HR460 Jobin-Yvon monochromator with a grating of 600 grooves per mm and an ultimate resolving power of $\sim 10^4$ at 550 nm. In order to attenuate the strong Rayleigh scattering a notch filter (Kaiser Optical Systems Holographic SuperNotch) was inserted in the optical path. The detector is a liquid nitrogen-cooled Spectraview 2D CCD head of 578 × 375 pixels which allows for the contemporary recording of a 1000 cm^{-1} spectral region in our spectrometer. A 100 μm slit was used corresponding to 5 cm^{-1} spectral resolution. The grating of the spectrometer was held fixed during the experiment. The laser power on the crystal surface of the HRPC-BHA complex was 300 μW for the 514.5 nm excitation. If higher power was used or if the crystal was exposed to the beam for more than 10 min, denaturation of the protein was revealed by a fluorescence background and frequency and intensity changes of core-size marker bands in the RR spectra.

The RR spectra were calibrated with indene as standard for the high-frequency region, and with indene and CCl_4 for the low-frequency region. The frequencies were accurate to ±1 cm^{-1} for the intense isolated bands, and to about ±2 cm^{-1} for overlapped bands or shoulders.

Results

Horseradish peroxidase isoenzyme C

Figure 1 shows the electronic absorption spectra at room (panel A) and low (panel B) temperatures, respectively, of the free protein (bottom) and the com-

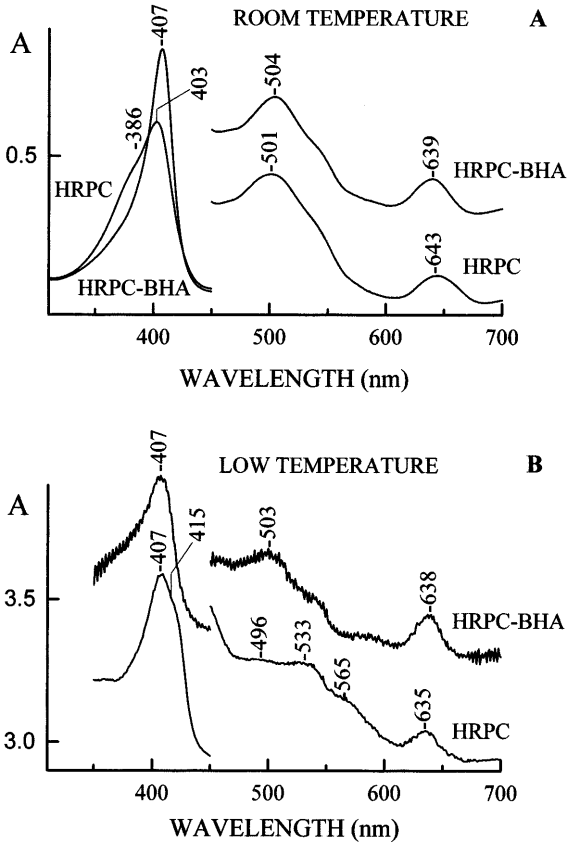

Fig. 1 Electronic absorption spectra of horseradish peroxidase isoenzyme C (HRPC) (5.1 μM) and its complex with benzhydroxamic acid (BHA) (4.8 μM HRPC plus 30.5 μM BHA) in 0.1 M phosphate buffer, pH 7, at room temperature (**A**) and of HRPC (~34 μM) at 12 K and HRPC-BHA (~14 μM) at 80 K in 0.1 M phosphate buffer, pH 7 (**B**). The region between 450 nm and 700 nm has been expanded eightfold (**A**) and threefold (**B**), respectively

plex with BHA (top) in solution. A general inspection of the electronic absorption spectrum of the free protein at room temperature indicates that it corresponds to a 5-c state [31]. Upon addition of BHA the electronic spectrum shows marked changes: the Soret band red-shifts by 4 nm and sharpens with an increase in the extinction coefficient by about 35% and in the visible region the long-wavelength (>600 nm) porphyrin-to-metal charge-transfer (CT1) band blue-shifts by about 4 nm [3]. These changes have been interpreted as due to the formation of a 6-c species, with a water molecule coordinated to the sixth position of the iron atom [9, 13]. Upon lowering the temperature, the Soret band of the free protein (Fig. 1, panel B) broadens and a shoulder at 415 nm can be clearly observed. Concomitantly a new band at about 565 nm and an intensity increase of the band at 533 nm are observed. Bands at 496 nm and 635 (CT1) nm are also observed. The spectrum in the 450–700 nm region is the same as the one previously reported for HRPC at pH 6, measured at 80 K [32]. The red shift of the Soret maximum together with the appearance of the band at 565 nm and the intensity enhancement of the band at 533 nm are indicative of a 6-c low-spin (LS) heme. This species appears to coexist with another form characterized by a Soret maximum at 407 nm, the band in the visible at 496 nm, and the CT1 at 635 nm. These bands might be assigned to either a HS heme or a quantum mechanically admixed-spin (QS) heme, being very similar to the one reported for cytochrome c' at 80 K [33]. The corresponding spectrum of the BHA complex does not change at low temperature as compared to that obtained at 300 K.

The RR spectra of HRPC and its BHA complex at 20 K were reported previously [9]. HRPC shows the presence of two species upon lowering the temperature, in agreement with the electronic absorption spectra. One was assigned to a 6-c LS species; the other, characterized by core-size marker bands at higher frequency than the LS form, was assigned to a 5-c QS or intermediate-spin (IS) state [9], in agreement with the EPR spectra recorded at 4 K [3]. The RR spectrum of the HRPC-BHA complex at 20 K does not differ from that observed at room temperature; the only significant difference is a sharpening of the bands [9].

Figure 2 compares the RR spectra of solution and single-crystal samples of the free and the BHA-bound proteins recorded with visible excitations. It can be seen that in both cases there are no substantial differences between the solution and crystalline states. The spectra of free HRPC have been interpreted as due to a mainly 5-c species with a small amount of the 6-c form [12, 34], in agreement with the X-ray structure which shows a water molecule at 3.2 Å from the Fe atom [21].

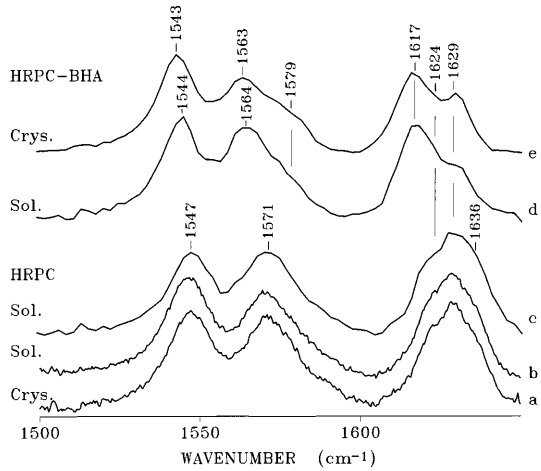

Fig. 2 Resonance Raman spectra of HRPC and HRPC-BHA in solution (0.1 M phosphate buffer, pH 7) and as a single crystal. Experimental conditions: 5 cm^{-1} spectral resolution; (*a, b*) 496.5 nm excitation: 2.5 mW laser power on the crystal surface, 1800 s accumulation time (*a*); 5 mW laser power at the sample, 600 s accumulation time (*b*); (*c, e*) 514.5 nm excitation: 25 mW laser power at the sample, 1200 s accumulation time, 50× magnification (*c*); 25 mW laser power at the sample, 1200 s accumulation time, 50× magnification (*d*); 300 μW laser power on the crystal surface, 1200 s accumulation time, 25× magnification (*e*)

Both species showed anomalously higher frequencies of the ν_3 and ν_{10} core-size marker bands than those normally observed for 5-c HS and 6-c HS hemes in model compounds and heme proteins. Therefore, they were referred as to 5-c HS# and 6-c HS#, respectively, to distinguish them from the normal hemes [12, 13]. Recently, on the basis of NMR data, the presence of a QS state, as an admixture of a low degree of the IS ($S=3/2$) state with the HS ($S=5/2$) state, has been suggested [11]. In the same work the authors studied also the resting state of HRPA1 and HRPA2. They concluded that all three proteins are in a QS state but differ according to the relative abundance of the $S=5/2$ and 3/2 states in the admixture, the latter increasing in the order HRPC, HRPA1, HRPA2. In our laboratory, HRPA2 has been characterized by RR and electronic absorption spectroscopies and the data confirm that the proteins contain a discrete amount of QS heme [35]. Addition of BHA to HRPC down-shifts the RR frequencies and the spectral changes have been interpreted as corresponding to a 6-c heme, in a pure 6-c HS state [9] or as a mixture of 6-c HS and 6-c HS# [36]. A water proton relaxation study and EPR indicated that BHA stabilizes a water ligand at the sixth coordination site of the heme iron [5] and the electronic absorption spectra, similar to that of MetMb had been interpreted accordingly [3]. However, recently, based on the NMR study, it has been concluded that the complex of BHA with HRPC is 5-c HS [11]. The present results obtained on the single crystal show that the core-size marker bands do not markedly differ from those previously observed for the solution, and that they are characteristic of a 6-c HS state [$\nu_{11}=1543\ cm^{-1}$, $\nu_{19}=1563\ cm^{-1}$, $\nu_{10}=1617\ cm^{-1}$, $\nu(C=C)=1624\ cm^{-1}$ and $1629\ cm^{-1}$]. Table 1 summarizes the frequencies observed for HRPC-BHA in solution [13] and single-crystal forms, together with those observed for CIP-BHA (see below) and a 6-c HS model compound [37].

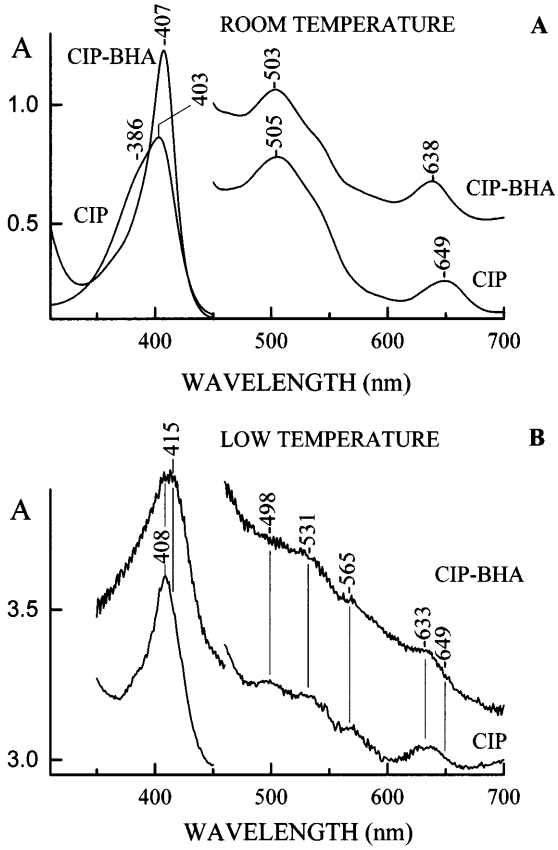

Fig. 3 Electronic absorption spectra of *Coprinus cinereus* peroxidase (CIP) (7.4 μM) and its complex with BHA (7.1 μM CIP plus 13 mM BHA) in 0.1 M phosphate buffer, pH 7, at room temperature (**A**), and of CIP (~31 μM, at 12 K) and CIP-BHA (~24 μM, at 80 K) in 0.1 M phosphate buffer, pH 7 (**B**). The region between 450 nm and 700 nm has been expanded by eightfold (**A**) and by threefold (**B**)

C. cinereus peroxidase-benzhydroxamic acid

Figure 3A shows the electronic absorption spectra of resting CIP and its complex with BHA obtained at pH 7.0 and room temperature. The spectrum of CIP, characterized by a Soret band at 403 nm with a shoulder at about 386 nm, the β band at 505 nm, and the CT1 band at 649 nm, is typical of a 5-c HS heme [12]. Upon addition of BHA the spectrum is almost identical to that of HRPC-BHA, showing the disappearance of the shoulder at 386 nm, a red shift of the Soret by about 4 nm with a concomitant 30% increase of the extinction coefficient, and a blue shift of the CT1 band to 638 nm. All these changes suggest the formation of a 6-c HS heme.

Figure 4 shows the corresponding RR spectra taken with Soret and visible excitations (left) and the polarized RR spectra (right). The totally symmetric modes (polarized) are enhanced via the A-term in the Soret excitation and the non-totally symmetric B_{1g} (depolarized) and A_{2g} (anomalous polarized) modes are enhanced via vibronic mixing in Q band excitation [37, 38]. Upon addition of BHA to CIP the spectrum converts from a typical 5-c HS heme (Fig. 4, bottom) [12] to a mainly 6-c HS (Fig. 4, middle and top) with core-size marker bands at $1485\ cm^{-1}$ (ν_3), $1520\ cm^{-1}$ (ν_{38}), $1542\ cm^{-1}$ (ν_{11}), $1559\ cm^{-1}$ (ν_{19}), $1563\ cm^{-1}$ (ν_2), $1584\ cm^{-1}$ (ν_{37}), and $1615\ cm^{-1}$ (ν_{10}). The $\nu(C=C)$ stretching mode of the vinyl groups is assigned to the band at $1624\ cm^{-1}$ which appears polarized (Fig. 4, right). These frequencies agree well with those of a 6-c HS model compound [37] (Table 1). In addition to the ligated form, a small amount of free protein is still present as judged by the bands at 1493 (ν_3), 1550 (ν_{11}), 1567 (ν_{19}), and $1631\ cm^{-1}$ (ν_{10}). This is consistent with the finding that CIP is able to form a stable 1:1 complex with BHA, albeit with a dissociation constant, K_d, which is about 1800 times higher than that measured for HRPC ($K_d=3717\pm213$ μM) [2].

We also undertook a low-temperature study to compare the results with those previously reported for

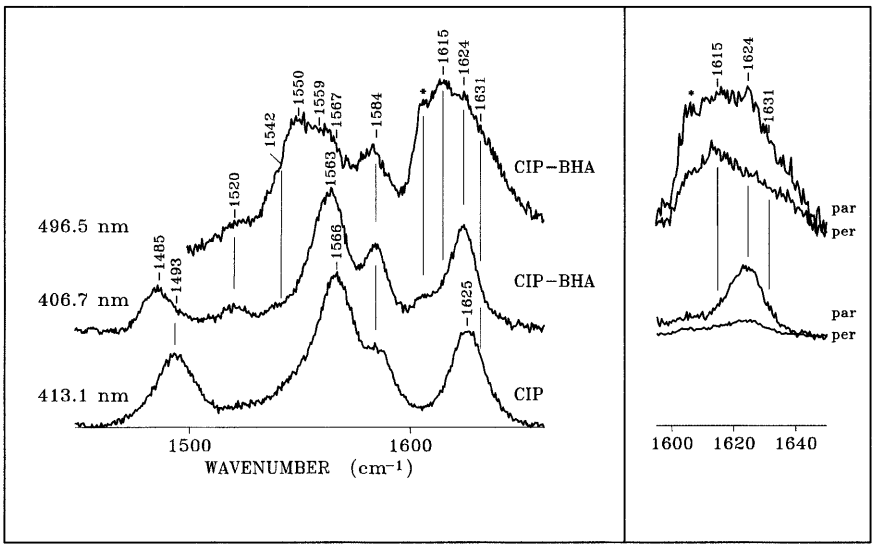

Fig. 4 Resonance Raman spectra of CIP and CIP-BHA in 0.1 M phosphate buffer, pH 7. Experimental conditions: 5 cm^{-1} spectral resolution; CIP, 413.1 nm excitation, 10 mW laser power at the sample, 15 s/0.5 cm^{-1} accumulation time; CIP-BHA, 406.7 nm excitation, 10 mW laser power at the sample, 6 s/0.5 cm^{-1} (nonpolarized, np), 8 s/0.5 cm^{-1} (par), 24 s/0.5 cm^{-1} (per) accumulation time; CIP-BHA, 496.5 nm excitation, 80 mW laser power at the sample, 81 s/0.5 cm^{-1} (np), 29 s/0.5 cm^{-1} (par), 40 s/0.5 cm^{-1} (per) accumulation time. The asterisk indicates the band due to BHA

HRPC at 20 K [9] and to take advantage of the narrowing of the RR bands. Upon lowering the temperature, the electronic spectra of both the resting form of CIP and its complex with BHA change dramatically (Fig. 3B). Native CIP is characterized by a Soret band at 408 nm, bands at 498, 531, and 565 nm, and the CT1 band at about 633 nm, with a shoulder at 649 nm. The red shift of the Soret band by about 5 nm with respect to the spectrum taken at room temperature and the wavelengths of the bands in the visible region at 531 nm and 565 nm are consistent with the presence of a 6-c LS heme. On the other hand, the bands at 498, 633, and 649 nm indicate the presence of 5-c and 6-c HS hemes. The spectrum of CIP-BHA taken at 80 K, very similar to the corresponding spectrum of the native protein, indicates the presence of a mixture of 5-c and 6-c HS and 6-c LS hemes.

The corresponding RR spectra taken at 12 K with Soret excitation (Fig. 5, bottom) and visible excitation (Fig. 5, top) confirm the presence of a temperature-induced transition in both samples as observed in the electronic absorption spectra. From the combined analysis of the RR spectra of CIP obtained with both excitations (Fig. 5, left), it can be concluded that the protein undergoes a transition from an almost pure 5-c HS

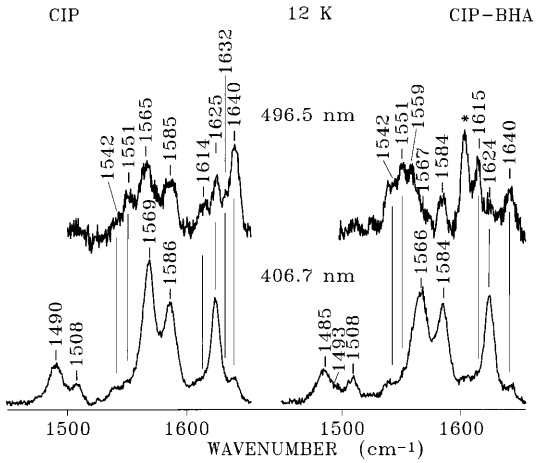

Fig. 5 Resonance Raman spectra of CIP (*left*) and CIP-BHA (*right*) in 0.1 M phosphate buffer, pH 7, at 12 K, taken with Soret (*bottom*) and visible (*top*) excitation. Experimental conditions: 5 cm^{-1} spectral resolution; *bottom*: CIP, 413.1 nm excitation, 20 mW laser power at the sample, 5 s/0.5 cm^{-1} accumulation time; CIP-BHA, 406.7 nm excitation, 27 mW laser power at the sample, 4 s/0.5 cm^{-1} accumulation time; *top*: 496.5 nm excitation, CIP, 50 mW laser power at the sample, 17 s/0.5 cm^{-1} accumulation time; CIP-BHA, 100 mW laser power at the sample, 4 s/0.5 cm^{-1} accumulation time. The asterisk indicates the band due to BHA

Table 1 Resonance Raman frequencies (cm^{-1}) observed for horseradish peroxidase isoenzyme C-benzhydroxamic acid (HRPC-BHA) and *Coprinus cinereus* peroxidase-benzhydroxamic acid (CIP-BHA) complexes, as compared to metmyoglobin (MetMb) and a six-coordinate high-spin (6-c HS) model compound

Mode	HRPC-BHA (crystal)	HRPC-BHA[a] (solution)	CIP-BHA (solution)	MetMb[b] (solution)	Model compound[c] 6-c HS
ν_3		1486–1491	1485	1483	1480
ν_{38}		1522	1520	1511–1521	1518
ν_{11}	1543	1544	1542	1544	1545
ν_2		1564	1563	1563	1559
ν_{19}	1563	1564	1559	1562[d]	1560
ν_{37}	1579	1579	1584	1583	1580
$\nu_{(C=C)}$	1624–1629	1624–1630	1624	1621	1621
ν_{10}	1617	1618	1615	1611[d]	1610

[a] From [9]
[b] From [41]
[c] From [37]
[d] From [74]

heme at room temperature to a mixture of three species at 12 K. The bands at 1508 cm^{-1} (ν_3), 1565 cm^{-1} (ν_{11}), 1586 cm^{-1} (ν_2), 1585 cm^{-1} (ν_{19}), and 1640 cm^{-1} (ν_{10}) are assigned to a 6-c LS heme. The bands at 1490 cm^{-1} and 1569 cm^{-1} observed upon 406.7 nm excitation are due to the ν_3 and ν_2 modes of a HS species. The presence of two distinct ν_{11} at 1542 cm^{-1} (6-cHS) and 1551 cm^{-1} (5-c HS), as well as two ν_{10} at 1614 cm^{-1} (6-c HS) and 1632 cm^{-1} (5-c HS), in the spectrum obtained with the 496.5 nm excitation, suggests that the features observed with Soret excitation are due to the overlapping contributions of a 5-c HS and a 6-c HS species. As previously reported, the frequency of the core-size marker bands of the 6-c LS heme are all upshifted at 12 K, indicating that core contraction occurs. This effect was ascribed to the increase of the protein packing forces with decreasing temperature [39].

By comparing the RR spectrum of the CIP-BHA complex obtained at 12 K (Fig. 5, right) with that obtained at room temperature (Fig. 4), it appears that the frequencies of the bands due to the 6-c HS of the BHA-bound form are not affected by the decreased temperature and the new bands are due to the 5-c HS and LS species of the unligated form. Therefore, the CIP-BHA complex is characterized by the presence of three species, namely 5-c HS, 6-c HS, and 6-c LS species, as the native protein, but the 6-c HS is the predominant form.

Discussion

The crystal structures of the ARP-BHA and HRPC-BHA complexes have been determined at 1.6 Å and 2.0 Å resolution, respectively [18, 19]. They show that the BHA is located in the distal heme pocket, nearly parallel to the heme, but too far away to directly interact with the iron atom. The functional groups of BHA are held by hydrogen bonds to the N_ε of the distal His, the N_ε of the distal Arg, the O of a Pro residue (Pro154 in ARP and Pro139 in HRPC), and the only water molecule present in the distal cavity is located at 2.6–2.7 Å from the iron atom. Therefore, the binding of BHA to the distal cavity of the heme has pushed the distal water molecule down toward the heme iron by more than 0.6 Å as compared to the native proteins [20, 21] and has replaced the hydrogen bond of distal water to the N_ε of the distal His. Nevertheless, the distance between the water molecule and the iron atom is still significantly greater than the 1.8–2.2 Å Fe-ligand distances observed in the X-ray crystal structure of cytochrome *c* peroxidase (CCP) with exogenous ligands bound at the sixth coordination site of the heme [22]. It is also longer than the Fe-O distance in MetMb (2.0–2.1 Å) [23, 24] and cytochrome P450 (2.1 Å), in which a water molecule is bound to the ferric heme [40].

The present results show unambiguously that the heme of the HRPC-BHA single crystal is 6-c HS. The RR frequencies, very similar to those previously observed for MetMb [41] and cytochrome P450 [42], suggest that the most likely candidate for the sixth ligand is a water molecule. The CIP-BHA complex in solution behaves in a very similar manner. Accordingly, the electronic absorption spectra of the complexes of both proteins with BHA are consistent with the presence of a 6-c HS heme, being characterized by the disappearance of the shoulder at about 386 nm, a red-shifted Soret band, and an extinction coefficient increased by about 30% with respect to the native enzyme. The wavelength of the CT1 at 639 nm, about 7 nm red-shifted with respect to that observed for MetMb [43], is consistent with a hydrogen bond between the iron-coordinated water molecule and the positively charged guanidinium group of the distal Arg present in both CIP-BHA [18] and HRPC-BHA [19], as previously suggested for the HRPC-BHA complex in solution [13]. In fact, it has been recently shown that the energy of the d_π orbitals of the heme iron is correlated to the nature of the axial ligand in terms of *p* electron interactions with the iron orbitals [44]. In particular, hydrogen bonding affects the ligand *p* donor capability. When the ligand acts as hydrogen-bond acceptor, then the stronger the hydrogen bond the longer the wavelength of the CT1, as for fluoride bound to the Fe atom [45].

Upon lowering the temperature the RR spectra of the CIP-BHA complex show the existence of three species: a 5-c HS and a 6-c LS heme are present in addition to the 6-c HS heme observed at room temperature, whose band frequencies are not affected by the temperature change. Since the protein does not fully bind the aromatic donor, a small amount of 5-c HS heme, due to the free protein, is still present upon BHA addition at room temperature. Therefore, for the native protein, we carried out a thorough analysis of the spectral changes induced by lowering the temperature in order to obtain a more complete understanding of the spectral features of the complex. The appearance at low temperature of a 6-c heme in CIP appears to be in common with other peroxidases belonging to class I and II of the "plant peroxidase superfamily" [46], as a similar behavior has been previously observed in CCP and some site-directed mutants [47–49], APX [50], and LIP [51]. In all these cases the RR frequencies of the 5-c and 6-c HS species do not change at low temperature. On the contrary, the frequencies of the 6-c LS proteins at low temperature appear to be 3–4 cm^{-1} higher than those observed for LS proteins or models at room temperature [37]. This behavior has been explained to be a consequence of heme contraction, induced by the increased packing forces at low temperature [39, 49]. The X-ray structures of all peroxidases solved so far have shown the presence of a distal water molecule at a distance from the Fe atom in the range of 2.4–5.7 Å [34]. Therefore, the temperature-induced transition can be explained by considering that upon cooling the increase in protein packing forces reduces the distance between the Fe atom and the distal water molecule, enabling the water molecule to bind. The appearance of a 6c-HS

and/or 6c-LS species depends on the polar character of the water molecule. In CCP the aqua ligand, involved in three strong hydrogen bonds [52], has an anionic character and, therefore, is expected to behave as a strong ligand. This hypothesis seems to be confirmed by the RR study of single-crystal CCP at various temperatures, which showed that as the temperature was lowered the spectrum converted from 5-c HS to one characteristic of a LS heme. The conversion, readily reversible, was quite gradual and at no temperature was there evidence for a 6-c HS species [53].

The RR experiments on single crystals so far reported are all consistent with the conclusion drawn from the X-ray structure [53–56] and, to our knowledge, this is the first time that a Fe-H_2O distance of about 2.6–2.7 Å has been demonstrated to correspond to an aqua-ligated species. In fact, the binding of BHA to the Fe atom can be excluded as the shortest distance between the heme iron and the BHA molecule is 4.4 Å, and between the porphyrin ring and BHA are 3.7 Å (ARP-BHA) and 3.9 Å (HRPC-BHA). Therefore, our results disagree with the conclusion drawn from NMR measurements of the HRPC-BHA complex, assigned to a 5-c HS species on the basis of altered hyperfine shifts that reflect an increase of the zero-field splitting [11].

The hexacoordination, observed in solution and in the single crystal of the peroxidase-BHA complexes, seems to derive from the presence of a water molecule in the close vicinity of the heme iron. A comparison of the present results with the complexes formed between human myeloperoxidase (MPO) and hydroxamic acids, such as BHA ($K_d = 5000\ \mu M$) and salicylhydroxamic acid (SHA) ($K_d = 2\ \mu M$) [26] (which differs from BHA only by the presence of an OH group on the C_6 of the phenyl ring) appears to be useful to clarify this point. The resting enzyme MPO in solution contains a ferric heme which has HS pentacoordinate spectral characteristics [57]. The crystal structure confirms the absence of a sixth ligand to the heme iron, the closest water molecule being at 3.1 Å from the iron atom. Binding of both SHA and BHA to MPO is accompanied by only very small changes in the optical absorption spectrum [26], and the crystal structure of the SHA-MPO complex showed that the aromatic ring is approximately parallel to the heme plane with the functional groups of SHA located inside the distal pocket and extending much further into the active site than for the cases of HRPC- and ARP-BHA complexes. The hydroxyl group is positioned above the heme iron, but not coordinated to it, and no water molecule on the distal side of the heme iron has been detected [27]. EPR studies at 77 K showed that the HS signals, characteristic of ferric MPO, were replaced by both HS and LS components upon SHA binding [28], suggesting that at low temperature the hydroxamic acid group provided a sixth ligand to the iron. Therefore, for the SHA-MPO complex, the absence of any water molecules in the distal cavity not only maintains the heme iron in its 5-c HS state upon complexation with the hydroxamic acids, but also facilitates the binding of the aromatic donor upon lowering the temperature, when the increased packing forces reduce the distance between the heme and the hydroxamic acid.

The present results clearly contradict the finding of a maximum distance of about 2.1 Å observed for the axial Fe-O bond of HS Fe(III) aqua complexes in model compounds [58]. The results also raise the question of why CCP and recombinant CCP (CCPMI), which have a water molecule at 2.4 Å and 2.7 Å, respectively [52, 59], are mainly 5-c species both in solution [48, 60, 61] and single crystal [53, 54]. Similarly, APX has the distal water molecule at 2.7 Å [62] and is mainly 5-c HS in solution [50]. It appears that the differences derive from the hydrogen-bonding partners available to the distal water molecule in these peroxidases as compared with peroxidases of class II and III. Peroxidases belonging to class I have a conserved Trp residue in the distal heme cavity which donates a hydrogen bond to the water molecule. This Trp is replaced by a non-hydrogen-bonding Phe residue in the other peroxidases [46]. In CCP, CCPMI, and APX (class I) the distal water molecule is directly involved in three hydrogen bonds, with two other water molecules and the nitrogen atom of the indole group of the Trp residue [52, 59, 62]. However, in class II and III peroxidases the distal water molecule appears to be involved in only two hydrogen bonds, with another water molecule and the distal His residue [20, 21, 63–68]. Moreover, considering that the N atom of the indole group of the Trp residue can only act as a H-donor, this might result in the occupation of both the lone pairs of the oxygen atom of the distal water molecule, which therefore cannot bind to the Fe atom. Removal of the hydrogen bond between the distal Trp and the water molecule in the Trp51Phe CCPMI mutant resulted (in the absence of methylpentenediol) in an enzyme with 6-c HS iron [53, 60]. This hypothesis and the present results reconcile the apparent inconsistencies concerning the coordination state, as revealed by the crystal structure determined distance between the water molecule and the Fe atom and the spectral features in solution, reported for some peroxidases [34]. In particular, two class II peroxidases, lignin peroxidase (LIP) and manganese peroxidase (MnP), whose electronic absorption spectra are typical of an aqua 6-c HS heme [69], show a water molecule at 2.7–2.8 Å from the Fe atom [63, 65]. Moreover, RR data have shown recently that MnP contains a mixture of 5-c and 6-c HS hemes at room temperature [70]. For these two proteins, RR experiments on single crystals are necessary to clarify the coordination state of the heme iron as compared to the solution sample.

The disagreement between our findings and the maximum Fe-O distance observed for a water molecule bound to the Fe atom in model compounds might derive from the particular polar cavity of peroxidases, which may favor the interaction between the iron atom and a distal water molecule. The distal cavity is particularly polar due to the presence of a positively charged

guanidinium group, which helps to keep in place the distal network of water molecules by hydrogen bonds. Substitution of distal Arg by the aliphatic residue Leu in CCPMI resulted in the disappearance of the distal water molecule from the heme cavity and the displacement of the iron atom by 0.4 Å toward the proximal side [71]. The mutant gave rise to almost exclusively 5-c HS species in solution [71, 72] and similar results were obtained for the corresponding mutants Arg38Leu HRPC [13] and Arg51Leu CIP [73].

Acknowledgements This work was supported by the Italian Consiglio Nazionale delle Ricerche (CNR) and Ministero della Università e della Ricerca Scientifica e Tecnologica (MURST ex 60% and ex 40%, Cofin. MURST 97 CFSIB) (to G.S.), and the EU BIO4–97–2031 (to G.S.). We thank Prof. K. G. Welinder (Aalborg University, Denmark) for many helpful discussions.

References

1. Veitch NC (1995) Biochem Soc Trans 23:232–240
2. Gilfoyle DJ, Rodriguez-Lopez JN, Smith AT (1996) Eur J Biochem 236:714–722
3. Schonbaum GR (1973) J Biol Chem 248:502–511
4. Maltempo MM, Ohlsson PI, Paul KG, Petersson L, Ehrenberg A (1979) Biochemistry 18:2935–2941
5. Gupta RK, Mildvan AS, Schonbaum GR (1979) Biochem.Biophys Res Commun 89:1334–1340
6. Teraoka J, Kitagawa T (1980) J Phys Chem 84:1928–1935
7. Kitagawa T, Hashimoto S, Teraoka J, Nakamura S, Yajima H, Toichiro H (1983) Biochemistry 22:2788–2792
8. Sakurada J, Takahashi S, Hosoya T (1986) J Biol Chem 261:9657–9662
9. Smulevich G, English A, Mantini AR, Marzocchi MP (1991) Biochemistry 30:772–779
10. La Mar GN, Hernández G, de Ropp JS (1992) Biochemistry 31:9158–9168
11. de Ropp JS, Mandal P, Brauer SL, La Mar GN (1997) J Am Chem Soc 119:4732–4739
12. Smulevich G, Feis A, Focardi C, Tams J, Welinder KG (1994) Biochemistry 33:15425–15432
13. Howes BD, Rodriguez-Lopez JN, Smith AT, Smulevich G (1997) Biochemistry 36:1532–1543
14. Veitch NC, Gao Y, Smith AT, White CG (1997) Biochemistry 36:14751–14761
15. Smith AT, Sanders SA, Sampson C, Bray RC, Burke JF, Thorneley RNF (1993) In: Welinder KG, Rasmussen SK, Penel C, Greppin H (eds) Plant peroxidases: biochemistry and physiology. University of Geneva, Geneva, pp 159–168
16. Rodriguez-Lopez JN, Smith AT, Thorneley RNF (1996) JBIC 1:136–142
17. Reimann L, Schonbaum GR (1978) Methods Enzymol 52:514–521
18. Itakura H, Oda Y, Fukuyama K (1997) FEBS Lett 412:107–110
19. Henriksen A, Schuller DJ, Meno K, Welinder KG, Smith AT, Gajhede M (1998) Biochemistry 37:8054–8060
20. Kunishima N, Amada F, Fukuyama K, Kawamoto M, Matsunaga T, Matsubara H (1996) FEBS Lett 378:291–294
21. Gajhede M, Schuller DJ, Henriksen A, Smith AT, Poulos TL (1997) Nature Struct Biol 4:1032–1038
22. Edwards SL, Poulos TL (1990) J Biol Chem 265:2588–2595
23. Takano T (1977) J Mol Biol 110:537–568
24. Evans SV, Brayer GD (1990) J Mol Biol 213:885–897
25. Smith AT, Veitch NC (1998) Curr Opin Chem Biol 2:269–278
26. Ikeda-Saito M, Shelley D, Lu L, Booth KS, Caughey WS, Kimura S (1991) J Biol Chem 266:3611–3616
27. Davey CA, Fenna RE (1996) Biochemistry 35:10967–10973
28. Hori H, Fenna RE, Kimura S, Ikeda-Saito M (1994) J Biol Chem 269:8388–8392
29. Dalbøge H, Jensen EB, Welinder KG (1992) Patent No.WO 92/16634
30. Zoppi A (1998) Thesis, University of Florence
31. Dunford HB, Stillman JS (1976) Coord Chem Rev 19:187–251
32. Tamura M (1971) Biochim Biophys Acta 243:249–258
33. Maltempo MM (1976) Biochim Biophys Acta 434:513–518
34. Smulevich G (1998) Biospectroscopy 4:S3–S17
35. Feis A, Howes BD, Indiani C, Smulevich G (1998) J Raman Spectrosc 29:933–938
36. Smulevich G, Paoli M, Burke JF, Sanders SA, Thorneley RNF, Smith AT (1994) Biochemistry 33:7398–7407
37. Choi S, Spiro TG, Langry KC, Smith KM, Budd DL, La Mar GN (1982) J Am Chem Soc 104:4345–4351
38. Spiro TG, Li X-Y (1988) In: Spiro TG (ed) Biological applications of Raman spectroscopy. Wiley, New York, pp 1–37
39. Feis A, Marzocchi MP, Paoli M, Smulevich G (1994) Biochemistry 33:4577–4583
40. Ravichandran KG, Boddupalli SS, Hasemann CA, Paterson JA, Deisenhofer J (1993) Science 261:731–736
41. Hu S, Smith KM, Spiro TG (1996) J Am Chem Soc 118:12638–12646
42. Hildebrandt P, Heibel G, Anzenbacher P, Lange R, Krüger V, Stier A (1994) Biochemistry 33:12920–12929
43. Eaton WA, Hochstrasser RM (1968) J Phys Chem 49:985–995
44. Smulevich G, Neri F, Marzocchi MP, Welinder KG (1996) Biochemistry 35:10576–10585
45. Neri F, Kok D, Miller MA, Smulevich G (1997) Biochemistry 36:8947–8953
46. Welinder KG (1992) Curr Opin Struct Biol 2:388–394
47. Yonetani T, Anni H (1987) J Biol Chem 262:9547–9554
48. Smulevich G, Mantini AR, English AM, Mauro JM (1989) Biochemistry 28:5058–5064
49. Smulevich G, Neri F, Willemsen O, Choudhury K, Marzocchi MP, Poulos TL (1995) Biochemistry 34:13485–13490
50. Nissum M, Neri F, Mandelman D, Poulos TL, Smulevich G (1998) Biochemistry 37:8080–8087
51. Andersson LA, Renganathan V, Loehr TM, Gold MH (1987) Biochemistry 26:2258–2263
52. Finzel BC, Poulos TL, Kraut J (1984) J Biol Chem 259:13027–13036
53. Smulevich G, Wang Y, Edwards SL, Poulos TL, English A, Spiro TG (1990) Biochemistry 29:2586–2592
54. Smulevich G, Wang Y, Mauro JM, Wang J, Fishel LA, Kraut J, Spiro TG (1990) Biochemistry 29:7174–7180
55. Smulevich G, Spiro TG (1993) Methods Enzymol 226:397–408
56. Sun J, Fitzgerald M, Goodin DB, Loehr TM (1997) J Am Chem Soc 119:2064–2065
57. Stump R, Oliver J (1985) Biophys J 47:82A
58. Kastner ME, Scheidt WR, Mashiko T, Reed WR (1978) J Am Chem Soc 100:666–667
59. Wang J, Mauro JM, Edwards SL, Oatley SJ, Fishel LA, Ashford VA, Xuong N, Kraut J (1990) Biochemistry 29:7160–7173
60. Smulevich G, Mauro JM, Fishel LA, English AM, Kraut J, Spiro TG (1988) Biochemistry 27:5486–5492
61. Dasgupta S, Rousseau DAH, Yonetani T (1989) J Biol Chem 264:654–662
62. Patterson WR, Poulos TL (1995) Biochemistry 34:4331–4341
63. Poulos TL, Edwards SL, Wariishi H, Gold MH (1993) J Biol Chem 268:4429–4440
64. Piontek K, Glumoff T, Wintehalter K (1993) FEBS Lett 315:119–124
65. Sundaramoorthy M, Kishi K, Gold MH, Poulos TL (1994) J Biol Chem 269:32759–32767

66. Petersen JWFW, Kadziola A, Larsen S (1994) FEBS Lett 339:291–296
67. Schuller DJ, Ban N, van Huystee RB, McPherson A, Poulos TL (1996) Structure 4:311–321
68. Henriksen A, Welinder KG, Gajhede M (1998) J Biol Chem 273:2241–2248
69. Glenn JK, Gold MH (1985) Arch Biochem Biophys 242:329–341
70. Kishi K, Kusters-van Someren M, Mayfield MB, Loehr TM, Gold MH (1996) Biochemistry 34:13176–13182
71. Vitello LB, Erman JE, Miller MA, Wang J, Kraut J (1993) Biochemistry 32:9807–9818
72. Smulevich G, Miller MA, Kraut J, Spiro TG (1991) Biochemistry 30:9546–9558
73. Neri F, Indiani C, Welinder KG, Smulevich G (1998) Eur J Biochem 251:830–838
74. Spiro TG, Stong JD, Stein P (1979) J Am Chem Soc 101:2648–2655