pH effects on the haem iron co-ordination state in the nitric oxide and deoxy derivatives of ferrous horseradish peroxidase and cytochrome *c* peroxidase

Paolo ASCENZI,* Maurizio BRUNORI,*† Massimo COLETTA* and Alessandro DESIDERI†

*C.N.R. Center for Molecular Biology and Department of Biochemical Sciences, University of Rome 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome, Italy, and †Department of Biology, University of Rome 'Tor Vergata', Via Orazio Raimondo 8, 00173 Rome, Italy

The spectral (e.p.r. and absorbance) properties of the NO and deoxy derivatives of ferrous horseradish peroxidase (HRP; EC 1.11.1.7) and baker's-yeast cytochrome *c* peroxidase (CCP; EC 1.11.1.5) were investigated between pH 7 and pH 2; over the same pH range the kinetics for CO binding were also determined. At neutral pH the e.p.r. and absorption spectra of the NO and deoxy derivatives of HRP and CCP are typical of systems in which the haem iron is in the hexaco-ordinated state and the pentaco-ordinated state respectively. By lowering pH, the e.p.r. and absorption spectra of HRP and CCP undergo reversible transitions, with pK_a values of 4.1 for the NO derivatives and ≤ 3 for the deoxy derivatives of the ferrous forms. By analogy with O_2 -carrying proteins and haem model compounds, the pH-dependent spectral changes of HRP and CCP were interpreted as indicative of the protonation of the $N_{(e)}$ atom of the proximal histidine residue and of the cleavage of the Fe– $N_{(e)}$ bond. However, the slow second-order rate constant ($0.003 \mu M^{-1} \cdot s^{-1}$) for CO binding to deoxy ferrous HRP and CCP does not increase substantially even at pH 2.6, suggesting that changes in the Fe–haem plane geometry, presumably associated with the cleavage of the Fe– $N_{(e)}$ bond, do not affect appreciably the observed ligand association rate constant.

INTRODUCTION

Spectroscopic and ligand-binding properties of haemoproteins are affected by the structure of the protein on the distal side of the haem pocket (Parkhurst *et al.*, 1980; Mims *et al.*, 1983; Brunori *et al.*, 1986), as well as by geometry of the iron atom–haem plane system and porphyrin contacts with the globin moiety (Friedman *et al.*, 1983; Coletta *et al.*, 1985; Brunori *et al.*, 1986). It is well known that protein–porphyrin interactions occur through the covalent bond between the iron atom and the proximal histidine residue (i.e., the fifth axial ligand of the Fe atom) and through van der Waals contacts between the protein and the side chains at the periphery of the haem group (Perutz *et al.*, 1976; Baldwin & Chothia, 1979; Fermi & Perutz, 1981; Friedman *et al.*, 1983; Coletta *et al.*, 1985).

In order to elucidate the role of the proximal histidine–iron bond in controlling ligand reactivity in haemoproteins, several years ago we initiated (Giacometti *et al.*, 1977) an investigation on the effect of pH, below neutrality, on the spectral and the CO-binding kinetic properties of monomeric O_2 -carrying proteins. These experiments were suggested by the findings obtained with model compounds that indicated that breakage of the Fe–proximal base bond was associated with a large increase in the CO-combination rate constant (Geibel *et al.*, 1975). In the course of these investigations it was shown that the NO and/or deoxy derivatives of several ferrous monomeric haemoproteins, notably sperm-whale Mb, horse Mb, *Dermochelys coriacea* Mb,

Coryphaena hippurus Mb, *Dicrocoelium dentriticum* haemoglobin, *Chironomus thummi thummi* erythrocyruorin (type III) and *Aplysia limacina* Mb, undergo e.p.r. and optical-absorbance changes upon lowering pH from 8 to 2. Such common pH-dependent spectroscopic changes, with pK_a values ranging between 5.5 and 2.5, have been interpreted as indicative of the protonation of the $N_{(e)}$ atom of the proximal histidine residue and of the cleavage of the Fe– $N_{(e)}$ bond (Giacometti *et al.*, 1977; Ascenzi *et al.*, 1981, 1985; Traylor *et al.*, 1983; Desideri *et al.*, 1984; Coletta *et al.*, 1985). The pH-induced cleavage of the proximal histidine–haem iron bond has been correlated for these haemoproteins with the observed marked increase, at low pH, of the second-order rate constant for CO binding, consistent with a more planar haem geometry derived from the tetraco-ordination of the deoxy ferrous form (Giacometti *et al.*, 1977; Traylor *et al.*, 1983; Coletta *et al.*, 1985). Although such an increase was not observed for *Chironomus thummi thummi* erythrocyruorin (type III), which displays a very fast pH-independent second-order rate constant for CO binding ($30 \mu M^{-1} \cdot s^{-1}$), this behaviour was considered not to be inconsistent with the model outlined above (Traylor *et al.*, 1983; Coletta *et al.*, 1985), as from high-resolution crystallographic data a planar haem geometry had already been observed for the pentaco-ordinated deoxy ferrous erythrocyruorin (Steigemann & Weber, 1979).

Among all haemoproteins known, horseradish peroxidase and baker's-yeast cytochrome *c* peroxidase display an extremely slow second-order combination rate

Abbreviations used: HRP, horseradish peroxidase; CCP, baker's-yeast cytochrome *c* peroxidase; Mb, myoglobin.

† To whom correspondence should be addressed.

constant for CO ($0.003 \mu\text{M}^{-1}\cdot\text{s}^{-1}$; Kertesz *et al.*, 1965; Iizuka *et al.*, 1985), which makes them very interesting proteins to investigate, especially in view of the availability of the three-dimensional structure of CCP (Finzel *et al.*, 1984). Thus, in order to increase our knowledge on the role of haem co-ordination on the spectral and ligand-binding properties of monomeric haemoproteins, the spectral properties of the NO and deoxy derivatives of the ferrous forms of HRP and CCP were measured in the pH range from 7 to 2; over the same range, kinetics for CO binding were also determined. These results were analysed with reference to the above-mentioned molecular control of reactivity for O_2 -carrying proteins and haem model compounds.

MATERIALS AND METHODS

Horseradish peroxidase (HRP; EC 1.11.1.7) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein concentration was determined spectrophotometrically at pH 7.0 on the ferric derivative by using $\epsilon = 93.5 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 402 nm (Keilin & Hartree, 1951).

Baker's-yeast cytochrome *c* peroxidase (CCP; EC 1.11.1.5) was kindly given by Dr. T. Yonetani (Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA, U.S.A.). Protein concentration was determined spectrophotometrically at pH 7.0 on the ferric derivative by using $\epsilon = 99.0 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 408 nm (Yonetani *et al.*, 1972).

CO was obtained from Caraccioloossigeno (Rome, Italy), and its concentration in solution was calculated from solubility data ($0.1 \text{ MPa CO} = 1 \text{ mM-CO}$ solution in water at 20°C) (Antonini & Brunori, 1971).

All the other reagents were from Merck (Darmstadt, Germany).

All chemicals were of analytical grade and used without further purification.

The NO derivatives of HRP and CCP were obtained, under anaerobic conditions, by the sequential addition of KNO_2 and $\text{Na}_2\text{S}_2\text{O}_4$ (final concentrations 10 and 5 mg/ml respectively) to the ferric form of the haemoprotein in 1 mM-sodium phosphate buffer, pH 7.0 (Yonetani *et al.*, 1972; Ascenzi *et al.*, 1981).

The deoxy derivatives of HRP and CCP were prepared, under anaerobic conditions, by addition of $\text{Na}_2\text{S}_2\text{O}_4$ (final concentration 5 mg/ml) to the ferric form of the haemoprotein in 1 mM-sodium phosphate buffer, pH 7.0 (Yonetani *et al.*, 1972).

pH-jump rapid-freezing e.p.r. experiments were carried out by essentially following the procedure of Bray (1961), by mixing the NO derivatives of the ferrous forms of HRP and CCP in 1 mM-phosphate buffer, pH 7.0, with 0.3 M acidic buffers in cold (110 K) isopentane and collecting the frozen material in an e.p.r. tube, where X-band spectra were recorded. At the various pH values examined, the integrated value of the e.p.r. signal accounted for $> 90\%$ of the expected value.

pH-jump absorbance changes were monitored by mixing the NO and deoxy derivatives of ferrous HRP and CCP in 1 mM-phosphate buffer, pH 7.0, with 0.3 M acidic buffers, and then collecting the spectra (Ascenzi *et al.*, 1981; Coletta *et al.*, 1985). The absorption spectra of the deoxy ferrous forms of HRP and CCP, at pH 2.6 and pH 2.3 respectively, were obtained from the

amplitude of the denaturation process monitored (at several different wavelengths) by mixing the deoxy derivatives of HRP and CCP in 1 mM-phosphate buffer, pH 7.0, with 0.3 M-phosphate buffer, pH 2.6 and 2.3 respectively (Coletta *et al.*, 1985).

CO binding was monitored at 423 nm by mixing the deoxy derivatives of ferrous HRP and CCP in 1 mM-phosphate buffer, pH 7.0, with 0.3 M-phosphate buffer, pH 7.0 or 2.6, equilibrated with CO (Giacometti *et al.*, 1977; Traylor *et al.*, 1983; Coletta *et al.*, 1985).

All measurements were carried out in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ under anaerobic conditions.

The sodium phosphate (pH 2.0–3.5 and 6.0–7.0), sodium acetate (pH 3.0–6.0) and sodium citrate (pH 3.0–6.5) buffer systems (final concentration 0.15 M after mixing) were used for pH-jump rapid-freezing e.p.r. experiments, pH-jump optical-absorbance measurements and CO-binding kinetics between pH 7 and pH 2. No specific ion effects were found with different buffers at the same pH.

X-band e.p.r. spectra were recorded at 110 K on a Varian E-9 spectrometer. Absorption spectra were collected at 20°C on a Varian Cary 219 double-beam spectrophotometer. Rapid-mixing kinetic experiments were undertaken at 20°C with a Durrum–Gibson rapid-mixing stopped-flow apparatus equipped with a 2 cm-pathlength observation cell.

The pH value of the samples was determined after each experiment at 20°C with a Radiometer model 51 pH-meter. Under all the experimental conditions addition of third components (i.e. KNO_2 , $\text{Na}_2\text{S}_2\text{O}_4$ and NO) to the peroxidase/buffer systems employed did not affect the pH of the sample.

RESULTS AND DISCUSSION

The e.p.r. and/or optical-absorption spectra of the NO derivatives of ferrous HRP and CCP at pH 7.0, 4.06 and 3.2 and at pH 7.0, 4.0, 3.25 or 3.2 respectively are shown in Fig. 1. At pH 7.0 the e.p.r. spectra of the NO derivatives of ferrous HRP and CCP display a rhombic shape, with a good resolution of the nine-line superhyperfine structure in the $g_z (= 2.004)$ region (see Fig. 1). This feature seems to be a peculiarity of the NO derivatives of ferrous HRP and CCP (Henry & Mazza, 1974; Yonetani *et al.*, 1972). In fact, in the case of typical O_2 -carrying proteins, e.g. sperm-whale Mb, the nine-line superhyperfine structure in the g_z region is poorly if at all resolved (Yonetani *et al.*, 1972; Ascenzi *et al.*, 1981, 1985; Bartnicki *et al.*, 1983; Desideri *et al.*, 1984, 1985; Spagnuolo *et al.*, 1986). The different degrees of resolution of the superhyperfine structure in the g_z region as observed in different haemoproteins have been tentatively related to the degrees of interaction of the unpaired-electron spin density with the iron-bound proximal N_{O} atom (Yonetani *et al.*, 1972; Yoshimura *et al.*, 1979; Blumberg, 1981; Ascenzi *et al.*, 1985). Thus in peroxidases the well-resolved superhyperfine structure might stem either from the hydrogen bond between the proximal histidine residue and the carboxylate groups located, differently from O_2 -carrying proteins, in close proximity, i.e. glutamate in HRP and aspartate in CCP (Poulos *et al.*, 1980; La Mar & de Ropp, 1982), or alternatively from the interaction between the partially negatively charged NO molecule and the distal side of

Fig. 1. X-band e.p.r. spectra (*a* and *c*) and absorption spectra (*b* and *d*) of the NO derivatives of ferrous HRP (*a* and *b*) and of ferrous CCP (*c* and *d*)

The spectra were obtained at pH 7.0, 4.06, 4.0, 3.25 and/or 3.2 in a 0.15 M-phosphate buffer system. X-band e.p.r. and absorption spectra were recorded at 110 K and at 20 °C respectively. HRP and CCP concentrations ranged between 0.02 and 1.0 mM. Setting conditions for X-band e.p.r. spectra: 9.14 GHz microwave frequency; 20 mW microwave power; 0.2 mT modulation amplitude. For additional experimental details see the text.

the haem pocket of peroxidases, which is more positively charged than in other haemoproteins (due to the presence of Arg-48; Finzel *et al.*, 1984).

These changes in the e.p.r. spectra of the NO derivatives of HRP and CCP are paralleled by optical changes of the same adduct. Thus at pH 7.0 the molar absorption coefficient of the O₂-carrying proteins in the Soret band is approx. 140 mM⁻¹·cm⁻¹ (Antonini & Brunori, 1971; Perutz *et al.*, 1976; Ascenzi *et al.*, 1981), higher than those of the NO derivatives of the ferrous forms of HRP (110 mM⁻¹·cm⁻¹) and CCP (99 mM⁻¹·cm⁻¹) (see Fig. 1) (Yonetani *et al.*, 1972), whereas the value of λ_{max.} (≈ 419 nm) is the same for all haemoproteins.

As reported by Henry & Mazza (1974) for the NO derivative of HRP, the NO derivatives of HRP and CCP undergo a simple spectroscopic (both e.p.r. and optical) transition on lowering pH, being almost complete at pH 3.2, the lowest acidic condition where both nitrosyl ferrous haemoproteins are spectroscopically stable over time (approx. 20 min) without undergoing denaturation

(see Figs. 1 and 2). For both e.p.r. and optical transitions, the reversibility of the process is fully complete, as judged from the spectroscopic viewpoint.

The e.p.r. spectra of the acidic species of the NO derivatives of ferrous HRP and CCP are characterized by a three-line splitting [$A_z(\text{NO}) = 1.65$ mT] centred at $g_z = 2.01$ (see Fig. 1). Both e.p.r. and absorption spectra of the NO derivatives of ferrous HRP and CCP are identical with those obtained, at acidic pH, for the same derivatives of O₂-carrying proteins (Ascenzi *et al.*, 1981, 1985; Blumberg, 1981; Desideri *et al.*, 1984; Spagnuolo *et al.*, 1986). The transition shown in Figs. 1 and 2 is complete within the dead time of the pH-jump rapid-freezing experiment (about 0.05 s); for the NO derivative of ferrous *Aplysia limacina* Mb, the relaxation time of the corresponding pH-dependent transition was found to be approx. 0.3 ms at its pK_a (3.9) at 25 °C (Ascenzi *et al.*, 1981).

The observed pH-induced spectroscopic transition (see Figs. 1 and 2) is *a priori* consistent either with a perturbation of the iron 3d orbital energies in nitrosyl

Fig. 2. pH-dependence of the molar fraction of pentaco-ordinated NO derivatives of ferrous HRP ($\square$ and $\circ$) and ferrous CCP ($\blacksquare$ and $\bullet$) $\bar{Y}$ monitored by both e.p.r. ($\square$ and $\blacksquare$) and absorption ($\circ$ and $\bullet$) spectroscopy

The continuous line through the experimental points represents the best-fitting theoretical titration curve calculated according to eqn. (1) with pK_a 4.1. The data were obtained in 0.15 M-phosphate (pH 2.0–3.5 and 6.0–7.0), -acetate (pH 3.0–6.0) and -citrate (pH 3.0–6.5) buffer systems. X-band e.p.r. and absorption spectra were recorded at 110 K and at 20 °C respectively. For additional experimental details see Fig. 1 legend and the text.

ferrous haem complexes (as a consequence of the addition of a proton to the $N_{(1)}$ atom of the proximal histidine residue) (Chevion *et al.*, 1977, 1978; Blumberg, 1981) or else with the cleavage (or the severe weakening) of the Fe– $N_{(6)}$ proximal bond associated with the protonation of the $N_{(6)}$ atom of the proximal histidine residue (Kon, 1975; Maxwell & Caughey, 1976; Szabo & Perutz, 1976; Blumberg, 1981). The latter interpretation is at present preferable, being supported (among other considerations) by Raman and i.r. spectroscopy of nitrosyl ferrous haemoproteins and haem model compounds (Wayland & Olson, 1974; Maxwell & Caughey, 1976; Nagai *et al.*, 1980) and being consistent with theoretical calculations (Mun *et al.*, 1979).

The effect of pH on the NO derivatives of ferrous HRP and CCP was examined quantitatively by e.p.r. and/or absorption spectroscopy at different proton concentrations (Fig. 2). The degree of pentaco-ordination of the NO derivatives of ferrous HRP and CCP ($\bar{Y}$) on lowering pH was determined (i) from X-band e.p.r. spectra, from both the progressive appearance of the three hyperfine splitting over the range 322–325 mT and the disappearance of the shoulder at 327.5 mT, and (ii) from absorption spectra in the Soret region, from both the progressive increase of the absorption coefficient at 419 nm and its decrease at 435 nm. The absolute values were fitted independently in accordance with a single ionization, allowing for each parameter the extrapolation to the value corresponding to 100% of pentaco-ordination. Next, from the comparison between the extrapolated value at low pH and that obtained at pH 7.0, the percentage of pentaco-ordination was obtained independently for each e.p.r. and absorption spectrum at a given pH, as shown in Fig. 2. After this, the continuous line given in Fig. 2 was obtained according to eqn. (1) (Thamer & Voigt, 1952):

$$\bar{Y} = 1/(1 + K_a/[H^+]) \quad (1)$$

where K_a is the apparent proton-dissociation equilibrium constant. At the pK_a of the transition (4.1; see Figs. 1 and 2) the two neutral and acidic species involved are equally contributing to both e.p.r. and absorption spectra.

As shown in Figs. 1 and 2, the absorbance changes observed in the NO derivatives of ferrous HRP and CCP on lowering pH from 7.0 to 3.2 parallel the pH-dependent transitions monitored by e.p.r. spectroscopy, in both cases reflecting the perturbation of the haem iron coordination state (see Blumberg, 1981). As previously reported (Ascenzi *et al.*, 1981), this finding suggests that e.p.r. measurements, although performed at 110 K, correspond to the equilibrium condition prevailing at 20 °C, where absorption spectra were recorded.

The absorption spectra of the deoxy derivatives of ferrous HRP and CCP at pH 7.0, 4.0 and 2.6 or 2.3 respectively are shown in Fig. 3; spectra obtained at pH 7.0 and 4.0 are superimposable. On lowering pH from 4.0 to 2.6 or 2.3, deoxy derivatives of HRP and CCP undergo a spectroscopic transition in the visible region; such a process appears to be completely reversible, as judged from spectroscopic, CO-binding and enzyme-activity viewpoints (see also Loo & Erman, 1975). By analogy with O_2 -carrying proteins and haem model compounds (Coletta *et al.*, 1985), the observed pH

Fig. 3. Absorption spectra of deoxy ferrous HRP (a) and deoxy ferrous CCP (b) at pH 7.0, 4.0 and 2.6 or 2.3

The experimental points ($\circ$) were obtained, at 20 °C, from the absorbance changes between time zero (following an acidic pH-jump) and the final time, corresponding to the acidic denatured deoxy derivative (at equilibrium) of ferrous HRP and CCP at pH 2.6 and 2.3 respectively; such a procedure allows construction of the deoxy transient spectra of HRP and CCP at pH 2.6 and 2.3 respectively (Coletta *et al.*, 1985). For additional experimental details see Figs. 1 and 2 legends as well as the text.

effects (see Fig. 3) may be attributed to the appearance of a tetraco-ordinated haem, below neutrality, coupled to the protonation of the N_{ϵ} atom of the proximal histidine residue. Although the pH range explored is insufficient to yield an accurate determination of the pK_a , this may be estimated to be equal or lower than 3 (see Fig. 3); such a value is roughly the same as that observed for other monomeric haemoproteins (Coletta *et al.*, 1985).

If the interpretation of the pH-dependent e.p.r. and optical-absorption transitions observed in the NO and deoxy derivatives of ferrous HRP and CCP is correct, the protonation of the N_{ϵ} atom of the proximal histidine residue of the NO derivatives of ferrous HRP and CCP (pK_a 4.1; see Fig. 2) as well as of O_2 -carrying proteins (pK_a values ranging between 5.5 and 3.9; Ascenzi *et al.*, 1981, 1985; Blumberg, 1981; Desideri *et al.*, 1984; Spagnuolo *et al.*, 1986) is shifted to low pH as compared with a solvent-exposed histidine residue (pK_a 6.8; Windol, 1976). This corresponds to a free-energy change of about 12–13 kJ/mol for the binding of the proximal histidine residue to NO-ferrous porphyrin adducts. This free-energy value is comparable with that determined, under similar assumptions, for the interaction of the proximal histidine residue with the iron atom in the pentaco-ordinated ferric *Aplysia limacina* Mb and its adduct with azide (Giacometti *et al.*, 1981). Conversely, the pK_a values for protonation of the N_{ϵ} of the deoxy derivative of HRP and CCP ($pK_a \leq 3$), as well as of monomeric O_2 -carrying proteins, range between 4.0 and 2.5 (Coletta *et al.*, 1985), indicating that in this derivative free-energy values of about 20–25 kJ/mol are involved in the Fe– N_{ϵ} bond. The smaller Fe– N_{ϵ} bond energy of the NO-ferrous haemoprotein adducts can be tentatively related to the partially dipolar nature of the NO-ferrous iron complexes, in which the Fe(II) serves as a π donor and NO acts as a σ donor, with a net donation of electron density from Fe(II) to NO (Maxwell & Caughey, 1976). This appears to be consistent with a correlation between the free energy involved in the Fe– N_{ϵ} bond and the electron-donation property from the Fe atom to the sixth ligand, which in the case of NO is comparable with that of ferric complexes, both being lower than that observed for deoxy ferrous adducts.

Next, the cleavage of the Fe– N_{ϵ} bond has been correlated in monomeric haemoproteins, such as sperm-whale Mb, *Coryphaena hippurus* Mb, *Dermochelys coriacea* Mb and *Aplysia limacina* Mb (Giacometti *et al.*, 1977; Traylor *et al.*, 1983; Coletta *et al.*, 1985), to the observed marked increase at acidic pH values of the second-order rate constant for CO binding, postulating a more planar haem geometry as a consequence of the tetraco-ordination of the deoxy form (Coletta *et al.*, 1985). As previously reported for HRP (Coletta *et al.*, 1986), such an increase has not been observed for deoxy ferrous HRP and CCP, which, even at pH 2.6, display the unusually slow second-order rate constant observed at pH 7.0 ($0.003 \mu M^{-1} \cdot s^{-1}$; Kertesz *et al.*, 1965; Iizuka *et al.*, 1985). As reported (Coletta *et al.*, 1986), such a behaviour seems not to be attributable to solvent third components (i.e. 2 M-guanidinium chloride), which, on the other hand, control the CO dissociation process. At pH 2.6, the pseudo-first-order rate of CO binding to ferrous HRP and CCP is always faster, by at least 10-fold, with respect to the denaturation process of both peroxidases ($t_{1/2} > 5$ s); this finding ensures that the measured value of the CO-combination rate constant is

characteristic of molecules that, albeit spectrophotometrically different (see Fig. 3), are still folded.

The CO-binding behaviour reported for HRP and CCP seems exactly the opposite of that observed in *Chironomus thummi thummi* erythrocyruorin (type III), which displays a very fast pH-independent second-order rate constant for CO binding ($30 \mu M^{-1} \cdot s^{-1}$) even at low pH, where the iron centre is tetraco-ordinated (Coletta *et al.*, 1985). In the latter case, this behaviour was interpreted on the basis of the structural evidence (Steigemann & Weber, 1979) that in the deoxy pentaco-ordinated form the iron atom of *Chironomus thummi thummi* erythrocyruorin (type III) is in the plane of the porphyrin, whereas in sperm-whale Mb (and other O_2 -carrying proteins) the iron atom is out of the plane and doming towards the proximal site (Takano, 1977). Thus in *Chironomus thummi thummi* erythrocyruorin (type III) the cleavage of the proximal bond would not bring about any decrease of the activation free energy for CO binding (Coletta *et al.*, 1985). In the case of HRP and CCP, the slow pH-independent second-order rate constant for CO binding suggests that the foregoing explanation does not hold, and that some other event(s) is (are) limiting the rate of ligand binding (see also Doster *et al.*, 1987). A possible candidate is represented by the distal side and in particular by the invariant side chain of Arg-48 (see Welinder, 1976; Takio *et al.*, 1980), which lies very close to the iron atom and brings about severe restriction to the access of the ligand in the proximity of the haem (Finzel *et al.*, 1984). Thus preliminary experiments by site-directed mutagenesis on CCP (M. Coletta, J. Kraut & T. G. Traylor, unpublished work) indicate that substitution(s) of this residue bring(s) about a marked enhancement of the CO-binding process. Therefore, differently from other haemoproteins, a hindered ligand pathway towards the haem pocket might represent the rate-limiting step of the CO-binding process to ferrous HRP and CCP. This feature would impair the observation of a pH-dependent enhancement of the rate constant due to the cleavage of the Fe– N_{ϵ} bond, which in the case of other haemoproteins has a major effect on the rate of binding (Coletta *et al.*, 1985). This interpretation is in complete agreement with the low-temperature data of Doster *et al.* (1987).

In conclusion, a relevant role appears to be played by the ionization of the N_{ϵ} atom of the proximal histidine residue in determining the co-ordination state of the haem iron as well as the spectroscopic properties of NO and deoxy derivatives of ferrous HRP and CCP, and CO-combination kinetics. On the other hand, the charged state of haem propionate groups appears to control the spectroscopic and kinetic properties of the CO derivative of ferrous HRP (Coletta *et al.*, 1986).

This work was supported by grants from the Ministero della Pubblica Istruzione of Italy to P. A. and M. B.

REFERENCES

- Antonini, E. & Brunori, M. (1971) Hemoglobin and Myoglobin in their Reactions with Ligands, pp. 31–33. North-Holland Publishing Co., Amsterdam
- Ascenzi, P., Giacometti, M. G., Antonini, E., Rotilio, G. & Brunori, M. (1981) *J. Biol. Chem.* **256**, 5383–5386
- Ascenzi, P., Coletta, M., Desideri, A. & Brunori, M. (1985) *Biochim. Biophys. Acta* **829**, 299–302
- Baldwin, J. & Chothia, C. (1979) *J. Mol. Biol.* **129**, 175–220

- Bartnicki, D. E., Mizukami, H. & Romero-Herrera, A. E. (1983) *J. Biol. Chem.* **258**, 1599–1602
- Blumberg, W. E. (1981) *Methods Enzymol.* **76**, 312–329
- Bray, R. C. (1961) *Biochem. J.* **81**, 189–195
- Brunori, M., Ascenzi, P. & Coletta, M. (1986) in *Supermolecules: Biological and Chemical Aspects* (Montanari, F., ed.), pp. 55–73, Accademia Nazionale dei Lincei, Rome
- Chevion, M., Blumberg, W. E. & Peisach, J. (1977) in *Metal-Ligand Interactions in Organic Chemistry and Biochemistry* (Pullman, B. & Goldblum, N., eds.), part 2, pp. 153–162, D. Reidel, Dordrecht
- Chevion, M., Stern, A., Peisach, J., Blumberg, W. E. & Simons, R. S. (1978) *Biochemistry* **17**, 1745–1750
- Coletta, M., Ascenzi, P., Traylor, T. G. & Brunori, M. (1985) *J. Biol. Chem.* **260**, 4151–4155
- Coletta, M., Ascoli, F., Brunori, M. & Traylor, T. G. (1986) *J. Biol. Chem.* **261**, 9811–9814
- Desideri, A., Meier, T., Winterhalter, K. H. & Di Iorio, E. E. (1984) *FEBS Lett.* **166**, 378–380
- Desideri, A., Chiancone, E. & Ascoli, F. (1985) *J. Inorg. Biochem.* **25**, 225–228
- Doster, W., Bowne, S. F., Frauenfelder, H., Reinisch, L. & Shyamsunder, E. (1987) *J. Mol. Biol.* **194**, 299–312
- Fermi, G. & Perutz, M. F. (1981) in *Atlas of Molecular Structures in Biology*, vol. 2: Haemoglobin and Myoglobin (Phillips, D. C. & Richards, F. M., eds.), pp. 26–29, Clarendon Press, Oxford
- Finkel, B. C., Poulos, T. L. & Kraut, J. (1984) *J. Biol. Chem.* **259**, 13027–13036
- Friedman, J. M., Scott, T. W., Stepnoski, R. A., Ikeda-Saito, M. & Yonetani, T. (1983) *J. Biol. Chem.* **258**, 10564–10572
- Geibel, J., Chang, C. K. & Traylor, T. G. (1975) *J. Am. Chem. Soc.* **97**, 5924–5926
- Giacometti, G. M., Traylor, T. G., Ascenzi, P., Brunori, M. & Antonini, E. (1977) *J. Biol. Chem.* **252**, 7447–7448
- Giacometti, G. M., Ascenzi, P., Bolognesi, M. & Brunori, M. (1981) *J. Mol. Biol.* **146**, 363–374
- Henry, Y. & Mazza, G. (1974) *Biochim. Biophys. Acta* **371**, 14–19
- Iizuka, T., Makino, R., Ishimura, Y. & Yonetani, T. (1985) *J. Biol. Chem.* **260**, 1407–1412
- Keilin, D. & Hartree, E. F. (1951) *Biochem. J.* **49**, 88–105
- Kertesz, D., Antonini, E., Brunori, M., Wyman, J. & Zito, R. (1965) *Biochemistry* **4**, 2672–2676
- Kon, H. (1975) *Biochim. Biophys. Acta* **379**, 103–113
- La Mar, G. N. & de Ropp, J. S. (1982) *J. Am. Chem. Soc.* **104**, 5203–5206
- Loo, S. & Erman, J. E. (1975) *Biochemistry* **14**, 3467–3470
- Maxwell, J. C. & Caughey, W. S. (1976) *Biochemistry* **15**, 388–396
- Mims, M. P., Parros, A. G., Olson, J. S., Noble, R. W. & Peterson, J. A. (1983) *J. Biol. Chem.* **258**, 14219–14232
- Mun, S. K., Chang, J. C. & Das, T. P. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4842–4846
- Nagai, K., Welborn, C., Dolphin, D. & Kitagawa, T. (1980) *Biochemistry* **19**, 4755–4761
- Parkhurst, L. J., Sima, P. & D. J. Goss (1980) *Biochemistry* **19**, 2688–2692
- Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A. & Simons, R. S. (1976) *Biochemistry* **15**, 378–387
- Poulos, T. L., Freer, S. T., Alden, R. A., Edwards, S. L., Skogland, U., Takio, K., Eriksson, B., Xuong, N., Yonetani, T. & Kraut, J. (1980) *J. Biol. Chem.* **255**, 575–580
- Spagnuolo, C., Desideri, A., Chiancone, E. & Ascoli, F. (1986) in *Invertebrate Oxygen Carriers* (Linzen, B., ed.), pp. 101–105, Springer-Verlag, Berlin
- Steigemann, W. & Weber, E. (1979) *J. Mol. Biol.* **127**, 309–338
- Szabo, A. & Perutz, M. F. (1976) *Biochemistry* **15**, 4427–4428
- Takano, T. (1977) *J. Mol. Biol.* **110**, 569–584
- Takio, K., Titani, K., Ericsson, L. H. & Yonetani, T. (1980) *Arch. Biochem. Biophys.* **203**, 615–629
- Thamer, B. J. & Voigt, A. F. (1952) *J. Phys. Chem.* **56**, 225–230
- Traylor, T. G., Deardurff, L. A., Coletta, M., Ascenzi, P., Antonini, E. & Brunori, M. (1983) *J. Biol. Chem.* **258**, 12147–12148
- Wayland, B. B. & Olson, L. W. (1974) *J. Am. Chem. Soc.* **96**, 6037–6041
- Welinder, K. G. (1976) *FEBS Lett.* **72**, 19–23
- Windolz, W. (ed.) (1976) *The Merck Index*, 9th edn., p. 619, Merck and Co., Rahway
- Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S., Jr. & Reed, G. H. (1972) *J. Biol. Chem.* **247**, 2447–2455
- Yoshimura, T., Ozaki, T., Shintani, Y. & Watanabe, H. (1979) *Arch. Biochem. Biophys.* **193**, 301–313

Received 23 May 1988/29 September 1988; accepted 4 October 1988