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Time-resolved fluorescence study of the single tryptophan in thiocyanate and azide derivatives of horseradish peroxidase: Implication for a *p*H-induced conformational change in the heme cavity

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Abstract. Detailed *p*H-dependent steady state and picosecond time-resolved tryptophan fluorescence studies on thiocyanate and azide complexes of horseradish peroxidase have been carried out. The fluorescence decay of the single tryptophan in these species was fitted to a discrete three exponential model. Maximum entropy method analysis also gave three distinct regions of lifetime distributions. The fast subnanosecond lifetime component was found to have > 97% amplitude contribution while other two longer lifetime components have small contributions. Small contributions from the nanosecond lifetime components possibly arise from apoprotein impurity or some small amount of disordered heme conformer of the protein. *p*H dependence of the fast picosecond lifetime components was found to show a systematic behavior which has been interpreted in the light of obligatory conformation change associated with activation of the enzyme at low *p*H.

Keywords. Horseradish peroxidase; tryptophan; hemeprotein; maximum entropy method; time-resolved fluorescence.

1. Introduction

Many of the hemeproteins and heme-containing enzymes show highly diverse biological functions while retaining an essentially unaltered prosthetic group made of an iron ligated to protoporphyrin IX. This prosthetic group hosts the vital redox reactions of the protein. The kind of redox reactions that these hemeproteins participate in, is dictated by the nature of the amino acid residues of the folded polypeptide matrix around the redox cavity and their interaction with the heme. The entry to the redox site of a substrate and its reactivity are delicately controlled by its interaction with the distal and proximal site residues and with the porphyrin side chains. Horseradish peroxidase (HRP, EC 1·11·1·7, donor H_2O_2 oxidoreductase) is an example of a wide class of plant hemeproteins involved in bacterial defence that primarily catalyzes reduction of H_2O_2 by substrates. The mechanism of function of this enzyme involves two redox intermediates called compound I and compound II by effecting heterolytic cleavage of O–O bond in peroxidases, with the result that the enzyme is promoted two equivalents above the resting state (Dunford and Stilman 1976). To facilitate this substrate-driven H_2O_2 decomposition process in peroxidases, various specific properties of HRP have been proposed. In cytochrome *c* peroxidase (CcP), which is believed to have many structural similarities to HRP, a H-bonding between proximal histidine to Asp-235 helps in

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stabilizing the higher oxidation states (Finzel *et al* 1984). An extensive H-bonding network among the residues in distal and proximal site in cyanide-ligated HRP has been observed. Molecular dynamics calculations performed on CcP and structural model of HRP revealed that extensive hydrogen bonding network among water molecules and protein residues around heme active site is very stable and are not broken even in solution (Banci *et al* 1994). Participation of proximal His-170 along with distal His-42 and Arg-38 has been found to be crucial for the H-bonding network, imparting significant imidazolate character to the axially bound histidine and hence stabilizing the heme in its higher oxidation states (Thanabal *et al* 1988). Previous studies using various metal substituted analogs of HRP and metmyoglobin showed that although both of these heme proteins contain the same protoheme active site in the native state, their reactivities towards H_2O_2 are quite different, indicating the importance of the amino acid residues in the heme cavity in regulating the function of the enzyme (Modi *et al* 1990; Mondal *et al* 1993). Various substrates of HRP have been found to bind at different positions near the equatorial plane of the heme. NMR studies as well as computer-aided simulations proposed that the most probable binding site for aromatic donor molecules like resorcinol, is a pocket composed of Tyr-185, Arg-183 and heme peripheral 8-CH_3 group and binding is stabilized by hydrophobic interaction with Tyr-185 and hydrogen bonding with nearby amino acid groups (Sakurada *et al* 1986). However, in case of indole-3-propionic acid, it has been proposed from 2-D NMR studies that it probably binds at a site to interact with two phenyl-alanine residues close to heme (Veitch and Williams 1990). Binding of inorganic anions also, exhibits variation in their binding site. Cyanide inhibits the enzyme activity of HRP by ligating strongly to heme iron. Cyanide, fluoride and azide anions bind at Fe(III) predominantly in protonated form (Dunford and Stilman 1976; Thanabal *et al* 1988). Like CN, azide also inhibits peroxidase activity of HRP (Keilin and Mann 1937, 1951) at low *p*H region (*p*H < 6) through complex formation and a fast chemical exchange of azide between native enzyme and the azide complex (Morishima *et al* 1977). The azide complex was found to be predominantly in ferric low spin state where lifetime of azide in the iron coordination position was proposed to be very short ($\sim 0{\cdot}1$ ms). In many cases, the *p*H-dependent ligand binding reactions in hemeproteins are facilitated by a heme-linked ionizable group (Ellis and Dunford 1968; Yamada and Yamazaka 1975) or by some ionizable amino acid residue(s) around the heme cavity. Thiocyanate, the oxidizable inorganic substrate of HRP, binds only at lower *p*H and binding is accompanied by protonation of an acid group with a $pK_a \sim 4$. Consequently, HRP-catalyzed thiocyanate oxidation by H_2O_2 gives maximum oxidation product at *p*H $\leqslant 4$ (Modi *et al* 1991).

The heme-linked ionizable residue is likely to have interaction with the iron-bound ligand to perturb the electronic structure of the heme ring which is manifested in the hyperfine shifted proton NMR peaks of CN and N_3 complexes of HRP (Morishima *et al* 1977). Thus, a change in peak positions of the paramagnetically shifted proton signals may not always originate from conformational changes. However, physical entry of a ligand in a favourable situation to bind at the redox cavity, linked with gating behaviour of some ionizable group probably will be associated with conformational change. The aspects of conformational changes associated with protonation in heme cavity of HRP or HRP-ligand complexes are not clearly known. We have studied therefore, the features of *p*H-dependent binding of external ligands to HRP by means of time-resolved fluorescence spectroscopy.

Fluorescence spectroscopy has been shown to be a very useful technique for studying structure and dynamics in proteins (Beechem and Brand 1985). The intrinsic fluorescence emission from tryptophan residue in proteins is an 'in-built' probe and is sensitive to the microenvironment surrounding the fluorophore residue. Application of tryptophan fluorescence for studying heme proteins, has however been restricted mainly by their low quantum yield due to energy transfer to heme. However, despite low fluorescence quantum yield in heme proteins, tryptophan fluorescence has been shown to provide much interesting information regarding the structure and conformation of the protein. Time-resolved fluorescence spectroscopic techniques have been utilized for studies of hemeproteins like hemoglobin (Szabo *et al* 1984), myoglobin (Willis *et al* 1990), cytochrome *c* peroxidase (Fox *et al* 1993), cytochrome *c* oxidase (Das and Mazumdar 1993, 1994) and HRP (Das and Mazumdar 1995).

The lifetimes of tryptophan fluorescence decay in hemeproteins lie in the picosecond range mainly due to large efficiency of energy transfer from tryptophan to the heme center. In case of myoglobin and hemoglobin, the emission is dominated by picosecond lifetime components with very small contributions from nanosecond lifetimes (Hochstrasser and Negus 1984; Albani *et al* 1985). It was suggested from energy transfer and trajectory calculations that the tryptophan fluorescence in hemeproteins would be dominated by a fast picosecond lifetime component and longer lifetime components would have very small contribution because of conformational restrictions (Hochstrasser and Negus 1984; Janes *et al* 1987). The single tryptophan in HRP, thus appears to be a potential amino acid residue for photophysical studies which can provide structural information on the enzyme. However, very few such studies (Ugarova *et al* 1981; Brunet *et al* 1983; Pappa and Cass 1993), limited only to the steady state fluorescence of the enzyme have been reported so far. Steady state studies have been shown to be often inadequate to understand several important aspects of the molecular origin of the fluorescence process (Livesey and Brochon 1987). Various time-resolved techniques, specially those coupled to time-correlated single photon counting device are able to measure very short fluorescence decay times. In a recent report (Das and Mazumdar 1995) we have shown the sensitivity of tryptophan fluorescence in native HRP, to respond to conformational changes on alkaline transition of the protein (pK_a of transition $\sim$ 10·8). It was also observed that at lower *p*H region (*p*H < 6) another inflection occurred in the lifetime value, which is clearly indicative of a conformational opening in the redox cavity. Cyanide-ligated HRP also showed a similar conformational transition at low *p*H-region which matches with an earlier report (Ellis and Dunford 1968), even in presence of a stable H-bonding network acting in the heme cavity (Thanabal *et al* 1988).

Apart from understanding the structural properties associated with ligand binding in HRP, time resolved fluorescence of a single tryptophan containing heme protein has significant photophysical interest. The present report describes fluorescence studies on thiocyanate and azide binding to HRP at different *p*H as binding of these ligands are *p*H-dependent. The results were compared with native HRP and cyanide ligated HRP to understand the conformational gating of different ligands in the light of photophysics of tryptophan fluorescence.

2. Materials and methods

HRP (Grade VI, $Rz \sim 3$) was purchased from Sigma Inc. as a salt-free lyophilised powder. The protein is predominantly isoenzyme C which was further purified for the

fluorescence measurements by passing through Sephadex G-25 column equilibriated with 50 mM phosphate buffer, *p*H 0·7. The *p*H titration was carried out in acetate, phosphate and Tris buffers to cover the range of 3·4–9·0. Absence of any metal ion impurity was assured by passing the buffers through Chelex-100 (BioRad) column. HRP samples for fluorescence measurements were typically 15 μM in 50 mM buffer with 50–80 mM of thiocyanate or azide. The concentration of HRP was determined spectrophotometrically using the extinction coefficient of 102 $mM^{-1}\,cm^{-1}$ at 403 nm for the native enzyme (Veitch and Williams 1990).

Picosecond kinetics of tryptophan fluorescence was performed using the tunable dyelaser pulse, from a synchronously pumped cavity-dumped dye-laser (Rhodamine 6G) driven by frequency-doubled output (532 nm) of the CW mode-locked Nd-YAG laser system described elsewhere (Das *et al* 1993b). Fluorescence decay profiles were collected using a time-correlated single photon counting device coupled to a micro-channel plate photomultiplier. Width of the dye-laser pulse was typically 4–6ps and the half-width of the instrument response function was about 80 ps. The tunable output of the dye laser was further frequency-doubled to generate the UV beam at 295 nm which was used to excite the samples. Excitation wavelength was chosen so as to eliminate the contribution from fluorescence of five tyrosines present in HRP (Pappa and Cass 1993). The instrument response functions were obtained from a purely scattering medium. Emission profiles were collected at the magic angle (54·7°) of emission polarizer to avoid any contribution from anisotropy. A Schott made WG 320 cut-off filter was used prior to the monochromator to remove any scattering from samples at wavelengths below 320 nm (Das and Mazumdar 1993). Data were collected in 512 channels and total count for each decay profile was typically $1-2\times10^5$ (peak count $\sim$ 10,000). Absorption spectra were taken before and after each laser data collection of the HRP samples to ensure photochemical stability of the sample.

Fluorescence decay curves were analysed by a best fit to the reconvolution of instrument response function with a theoretical decay function consisting of a sum of exponentials.

$$I(t)=\sum_k \alpha_k \exp(-t/\tau_k), \quad k=1-3, \tag{1}$$

where α_k and τ_k are kth component of amplitude and decay lifetime in the multiexponential model. The goodness of fit was confirmed from random residual distribution with χ^2 value close to unity.

Analyses of the flurescence decay profiles were also carried out by maximum entropy method (MEM) using a uniform distribution of the lifetime components in logarithmic time scale. This method is based on choosing a distribution of amplitudes, $\alpha(\tau)$ that yields maximum value of the entropy parameter (called Shannon–Jaynes entropy) (Jaynes 1983; Livesey and Brochon 1987; Das and Mazumdar 1993) at the optimized χ^2. The optimized amplitude distribution $\alpha(\tau)$ recovered by MEM represent maximum probable distribution of amplitudes among the lifetime components. The final outputs of MEM analysis were displayed as amplitude distribution of lifetime components in a logarithmic scale.

Fluorescence energy transfer

The major mechanism of decrease in fluorescence lifetimes in hemeproteins has been shown to be energy transfer to the heme group (Hochstrasser and Negus 1984).

Förster's theory of resonance energy transfer can be used to estimate distance of the fluorophore from the acceptor group. The efficiency of energy transfer, E from the donor to the acceptor chromophore is given by the Förster's theory:

$$E = 1 - \tau_{da}/\tau_d, \tag{2}$$

where τ_{da} and τ_d represent the lifetime values of the fluorophore in the presence and absence of acceptor group respectively. Magnitude of energy transfer efficiency is related to the distance R between the centres of the fluorophore and the acceptor (Cheung 1992) by the following equation:

$$E = R_0^6/(R^6 + R_0^6), \tag{3}$$

where R_0 is the distance at which the energy transfer efficiency is 50%. R_0 is defined by the equation,

$$R_0 = (Jk^2 Q_{d^n}^{-4})^{1/6} \times 9{\cdot}79 \times 10^3 \text{ Å}, \tag{4}$$

where J is the spectral overlap integral, k^2 is the orientation factor for the dipole–dipole interaction and a value of 0·67 was used for it. n is the refractive index of the medium separating the donor and acceptor groups and it is taken to be 1·4. The quantum yield, Q_d of the donor in absence of acceptor, is equal to 0·13 (Lehrer 1971). The value of the overlap integral (Cheung 1992) between the donor emission and acceptor absorption is given by the equation:

$$J = \frac{\int F(\lambda)\varepsilon(\lambda)\lambda^4 \mathrm{d}\lambda}{\int F(\lambda)\mathrm{d}\lambda}, \tag{5}$$

where $F(\lambda)$ and $\varepsilon(\lambda)$ are the observed fluorescence intensity of the donor and extinction coefficient ($\text{cm}^{-1}\,\text{M}^{-1}$) of the acceptor at a wavelength λ respectively. The value of J was determined numerically (Campbell and Dwek 1984) over the region 300 nm to 450 nm for tryptophan emission in apoHRP and heme absorption in HRP. The magnitude of J was found to depend on the absorbance in the near UV region part of the heme Soret band of HRP. The value of the overlap integral was calculated to be $6{\cdot}8 \times 10^{-14}\,\text{cm}^3\,\text{M}^{-1}$ and $6{\cdot}0 \times 10^{-14}\,\text{cm}^3\,\text{M}^{-1}$ for thiocyanate and azide complexes of HRP respectively.

3. Results

Steady state fluorescence of HRP shows emission maxima at 326 nm with excitation at 295 nm. The presence of 5 tyrosines in HRP makes a significant contribution to the observed fluorescence when excited at wavelengths < 290 nm (Pappa and Cass 1993). Excitation at 295 nm avoids simultaneous excitation of tyrosines and thus emission only from tryptophan fluorescence was observed. The steady state tryptophan fluorescence of HRP complexes was monitored at 330 nm (λ_{ex} = 295 nm), for *p*H titration in the range 3·4–9·0. A distinct change in fluorescence intensity was observed both in the thiocyanate and azide complexes of HRP in the *p*H range 6–3·4 (data not shown). Fluorescence intensity becomes minimum in the *p*H range of 6·5–7.

Binding of thiocyanate to HRP causes small change in the optical absorption spectrum which is detectable from the difference spectra (Modi *et al* 1989). However, on lowering the *p*H to 3·4, HRP-SCN complex shows major changes (figure 1) both in the visible region and in Soret band. Soret band was largely blue-shifted from 403 nm to

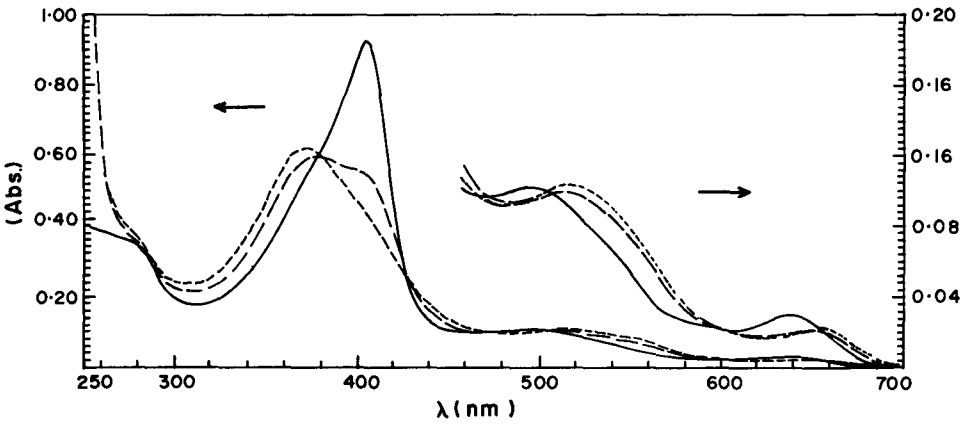

Figure 1. Optical spectra of HRP (8·8 μM in 50 mM acetate buffer) in absence (———) and in presence of thiocyanate (80 mM) at *p*H 3·4, within 2 min of mixing (– – –) and after 30 min (----).

372 nm. This change, however, was not very rapid and with the typical sample condition used here, it took around 30 mins for the complete conversion of the electronic spectrum. The control experiments were done to confirm the effect of thiocyanate on HRP spectrum at *p*H 3·4. Native HRP was incubated in acetate buffer (100 mM, *p*H 3·4) for one hour and no change in optical spectrum was found. However, when thiocyanate was added to this (final concentration of thiocyanate ~ 80 mM) the spectrum started changing as shown in figure 1. In case of azide complex also, the behaviour was very similar at the lower *p*H region. In presence of ~ 80 mM azide, on lowering the *p*H of the medium to 3·4, the spectral changes observed were very similar to that observed for HRP-SCN complex. The Soret band was highly broadened and shifted to 372 nm from 416 nm (figure 1). However, the rate of conversion was slower in case of HRP-N_3 than that observed in case of HRP-SCN complex.

Time-resolved fluorescence

The time-resolved fluorescence decay curves of the thiocyanate complex of HRP at *p*H 7·0 and *p*H 3·4 are shown in figures 2a & b respectively. The solid lines drawn through the curves represent triple exponential least square fits (see table 1). The weighted residuals distribution for three exponential fit is shown in figures 2c & d (for decays in figures 2a & b respectively). All data were best fitted to a sum of three exponentials as judged by residuals distribution, autocorrelation function and χ^2 minimization criteria. Table 1 shows that the maximum contribution (~ 97%) to the total decay of fluorescence comes from a picosecond lifetime component. Two other components in nanosecond time scale have very small amplitude contribution. The azide complex of HRP also showed three exponential fluorescence decay kinetics similar to the thiocyanate analog. Typical fluorescence decay profile for the azide complex has been shown in figure 3.

Lifetime distributions from maximum entropy method (MEM) analysis performed on large number of data sets show one principal component in picosecond region with two other components contributing little to the integrated amplitude (figure 4). The baricenter of the distribution band in the short lifetime region matches well to the

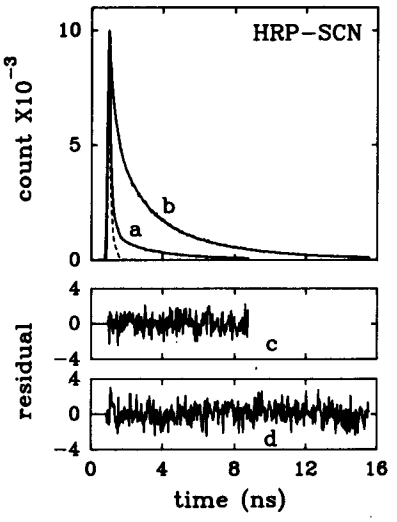

Figure 2. Fluorescence decay profile of HRP (15 μM) in presence of 80 mM potassium thiocyanate, (a) at *p*H 5·8 and (b) at *p*H 3·4 (λ_{ex} = 295 nm, λ_{em} = 330 nm). Solid lines through the decay curves correspond to three exponential fits. The dotted profile is the instrument response function. The weighted residuals for three exponential fits are shown in lower traces (c) residual for trace a and (d) residual for trace b.

Table 1. Fluorescence decay lifetimes and amplitudes for heme proteins.

Protein	λ^{em}_{max} (nm)	τ_1 (ps)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3	No. of Trp	Reference
YET metMb	320	33	1·00	—	—	—	—	1	a
Tuna metMb	320	31	0·90	0·132	0·08	2·17	0·02	1	b
SW metMb pH 7·0	325	21·5	0·45	0·112	0·55	—	—	2	a
SW metMb	325	16	0·53	0·135	0·39	2·22	0·08	2	b
HRP-C pH 3·8	326	61	0·96	1·42	0·03	4·8	0·01	1	c
HRP-C pH 6·6	326	45	0·97	1·4	0·02	4·6	0·01	1	c
HRP-CN pH 7·0	327	56	0·96	1·46	0·03	4·8	0·01	1	c
HRP-CN pH 3·7	327	76	0·95	1·48	0·03	4·9	0·02	1	c
HRP-SCN pH 7·0	327	50	0·97	1·41	0·02	4·44	0·01	1	d
HRP-SCN pH 3·4	327	168	0·60	1·46	0·25	4·6	0·15	1	d
HRP-N_3 pH 7·0	327	42	0·975	1·31	0·015	4·6	0·01	1	d
HRP-N_3 pH 4·0	327	60	0·97	1·42	0·02	4·75	0·01	1	d

[a]Willis *et al* (1990); [b]Hochstrasser and Negus (1984); [c]Das and Mazumdar (1994, 1995); [d]this work

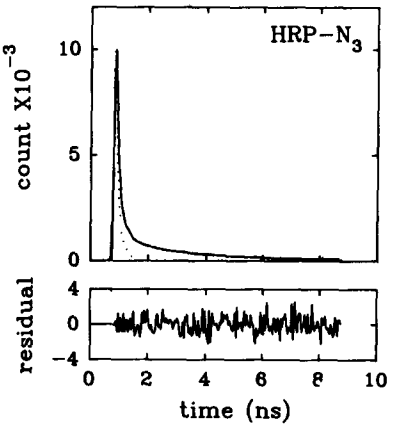

Figure 3. Fluorescence decay profile of HRP (15 μM) in presence of 80 mM sodium azide at *p*H 4·1. (λ_{ex} = 295 nm, λ_{em} = 330 nm). Solid lines through the decay curve correspond to three exponential fits. The dotted profile is the instrument response function. The weighted residuals for three exponential fits are shown in lower traces.

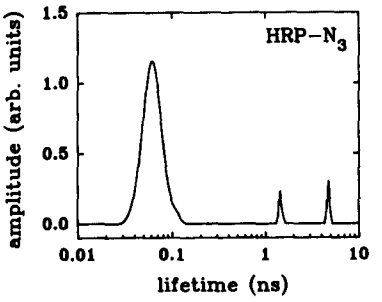

Figure 4. Amplitude profile of fluorescence lifetimes for azide derivative of HRP at *p*H 4·1 as recovered by MEM analysis. Three baricentres in the distribution profile were at 0·061, 1·42 and 4·76 ns.

lifetime value as obtained from discrete exponential analysis. It is evident from the distribution of amplitudes that the picosecond region forms the majority of the decay (Jaynes 1983; Livesey and Brochon 1987; Das and Mazumdar 1994, 1995). The distribution in the higher lifetime region was found to vary in different data sets. This variation was observed probably because the contribution of the counts in the higher nanosecond time scale was very small. The negligible contribution of higher lifetimes in the MEM recovered lifetime profile strongly indicates that the single-tryptophan fluorescence decay in HRP complexes is predominantly single exponential.

Lowering of *p*H below ~ 7·0 showed an increase in the value of the shortest lifetime component (τ_1) for both thiocyanate and azide ligands. *p*H dependence of the fast lifetime component (τ_1) of various HRP species are shown in figure 5. In presence of thiocyanate, at *p*H 7·0, the shortest lifetime is around 50 picosecond and it is very similar to that of native HRP (~ 45 picosecond) at *p*H 7·0 (table 1). On lowering the *p*H below ~ 6·0 τ_1 increases slowly upto *p*H ~ 4. However, further lowering of *p*H causes

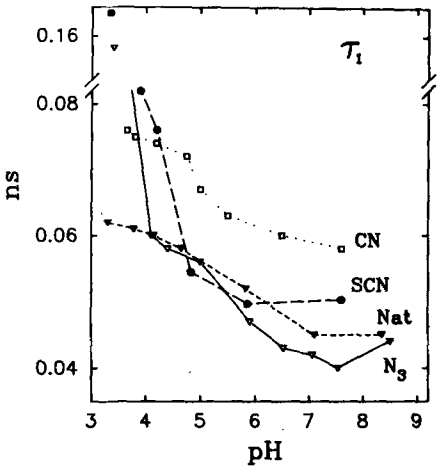

Figure 5. The fastest lifetime component (τ_1) in the fluorescence decay, as a function of *p*H for native HRP and its cyanide, thiocyanate and azide derivatives.

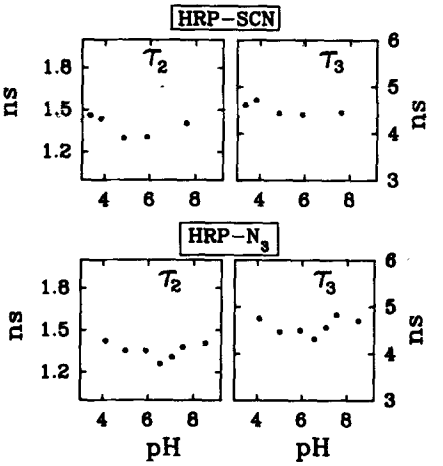

Figure 6. *p*H dependence of fluorescence lifetimes τ_2 and τ_3 for thiocyanate and azide derivatives of HRP.

a large enhancement in the magnitude of this lifetime component (τ_1). Analogous to thiocyanate binding, azide binding also shows inflection in τ_1 value on lowering the *p*H upto ~4 and this *p*H dependence behaviour shows marked similarity with that of native HRP in the lower *p*H region (figure 5). Although native HRP does not show much change in τ_1 value on lowering *p*H from 4·0 to 3·4, this fast lifetime component both in HRP-SCN and HRP-N_3 showed sharp increase in this *p*H region. It is interesting to note that native HRP as well as the various complexes of HRP studied here, showed increase in the major lifetime value (τ_1) at lower *p*H region, while the nanosecond lifetime components (τ_2 and τ_3) in cases of both HRP-SCN and HRP-N_3 showed only slight changes on *p*H variation (figure 6). This suggests that longer

components with small amplitude might be due to presence of small amounts of impurity of apoprotein or different conformers of the protein.

Fluorescence energy transfer

The efficiency of energy transfer for tryptophan to heme was estimated and the calculated value was used to determine the distance of sole tryptophan residue in HRP from the heme centre using (2)–(5). The Förster energy transfer distance between the tryptophan and heme of HRP in presence of different ligands have been calculated assuming a random orientation ($k^2 = 0{\cdot}67$ in (4)) of the fluorophores (Hochstrasser and Negus 1984). The picosecond lifetime component (τ_1) arises solely due to fast energy transfer from tryptophan to heme, hence the distance values were calculated from the fast picosecond lifetime component. The calculated distance of the tryptophan residue was found to be 12·2 Å from the heme for thiocyanate and 11·7 Å for azide at *p*H 7·0. These values are very close to that of native HRP (12·2 Å; Das and Mazumdar 1995) showing that additions of these anions possibly do not cause any significant change in the heme environment at *p*H 7·0.

*p*H dependence of lifetimes may arise either from change in distance between the fluorophores or from change in the orientation factor, k^2 (see (4)). Both these changes would be associated with significant conformational change in the Trp site. Calculation of the Förster energy transfer distance assuming a random orientation of the fluorophores ($k^2 = 0{\cdot}67$) showed that lowering of *p*H from 7·0 to 4·0 in case of the azide complex increases the distance between heme and tryptophan by $\sim 0{\cdot}7$ Å; very similar behaviour was found in the native as well as in the cyanide modified enzyme (Das and Mazumdar 1995). The thiocyanate complex of HRP also showed similar changes on lowering the *p*H from 7·0 to 4·0 and an increase in distance of $\sim 0{\cdot}9$ Å was observed. However, binding of thiocyanate or azide induced a large change in distances on further lowering of *p*H below 4·0. The quantum yield of tryptophan fluorescence of these two complexes at *p*H 3·4 increased by several orders of magnitude. The shortest lifetime component of HRP-SCN as well as HRP-N_3 is increased to around ~ 170ps with a concomitant decrease in its amplitude at *p*H 3·4. The higher lifetimes of these species, however, remained almost unchanged at this *p*H, while their contribution to the amplitude increased significantly (table 1). It is to be noted that native HRP does not show this type of drastic change in lifetimes (figure 4) upon lowering the *p*H to $\sim 3{\cdot}4$. The decay characteristics of HRP-SCN and HRP-N_3 at *p*H 3·4 closely resembled that of apoHRP (unpublished results) which indicates that these complexes of HRP most probably undergo a severe weakening of heme coordination at this stage.

4. Discussion

Multiexponential decay of tryptophan fluorescence have been observed in many tryptophan-containing proteins, and probably arises due to the multiple conformers seen by the fluorophore (Beechem and Brand 1985). In some cases, fluorescence decay in even single tryptophan proteins has been described as comprising two distinct lifetimes (Szabo *et al* 1983; Kim *et al* 1993) arising from two microconformers interacting differently with the tryptophan residue. However, in case of hemeproteins, due to extremely efficient energy transfer to heme from excited states of tryptophan, picosecond decay lifetimes are observed and, since the rate of energy transfer is very fast, it would suppress all other deactivation pathways (Janes *et al* 1987). Hence each

tryptophan in a hemeprotein has been proposed to show monoexponential dynamics. Results from several reports strongly support this view in case of myoglobin and hemoglobin (Hochstrasser and Negus 1984; Szabo *et al* 1984, 1989; Albani *et al* 1985; Janes *et al* 1987; Willis *et al* 1990). The single tryptophan (Trp-117) in HRP showed a picosecond lifetime component which accounts for 97% of the fluorescence decay (table 1, Das Mazumdar 1993b). The results showed that the fast picosecond lifetime component represents a conformation in which the heme and the tryptophan residues are aligned in such a way that energy transfer to heme is most efficient. Very small contributions from nanosecond lifetime components possibly arise due to small amounts of impurity apo protein or disordered conformers where tryptophan interactions are different with its neighbours. Fluorescence lifetimes and amplitudes of various single and double tryptophan containing heme proteins have been given in table 1. Table 1 shows that fluorescence decay of various single tryptophan heme proteins such as tuna metmyoglobin, YFT metmyoglobin and various HRP are characterized by a major picosecond lifetime component. The fluorescence decay in SW myoglobin has two predominant lifetime components in the picosecond time range which have been assigned to Trp-14 and Trp-7 (Willis *et al* 1990). It was also shown that effects of ligand binding and *p*H changes on myoglobin structure could be traced by fluorescence decay kinetics. The small contribution of nanosecond lifetime components in hemeproteins might arise due to presence of small amount of non-hemeprotein or structurally altered protein (Willis *et al* 1990; Gryczynski *et al* 1993).

From model considerations based on patterns of sequence homology between peptide back bones of yeast cytochrome *c* peroxidase and HRP, Weilander (1985) proposed presence of eight helical segments A to J, in the plant peroxidase (figure 2 of Weilander 1985). According to this model, the Trp-117 residue is located in a loop connecting D & E α-helixes (nomenclature by Weilander 1985). Pappa and Cass (1993) predicted on the basis of this model, a distance of 13 Å between the Trp-117 and heme in native HRP and this prediction was supported by our earlier findings of a distance of $\sim$ 12·2 Å (Das and Mazumdar 1995). The present results suggest a distance of $\sim$ 12·2 Å and $\sim$ 11·7 Å for thiocyanate and azide complexes of HRP respectively at *p*H $\sim$ 4. Pappa and Cass (1993) proposed that the Trp-containing loop is more rigid in HRP than that in apoHRP, indicating that the heme group in HRP might be responsible for imparting this extra rigidity of the Trp-117 containing loop. The extra stability may come from the formation of salt bridge (Modi *et al* 1994) and the hydrogen-bonding network (Thanabal *et al* 1988). Salt bridges are found to have distinct roles in the organization of multimeric proteins, proper alignment of hemes in cytochrome complexes (Rodgers and Sligar 1991), substrate binding and transition state stabilization by enzyme (Wada and Nakamura 1981; Luzhkov and Warshed 1991). The free energy of these interactions can be significantly high where the common environment of the salt bridge is restricted and interactions are provided by the protein's local, largely hydrophobic structure (Anderson *et al* 1990). The pentacoordinate heme structure in HRP with the proximity of several hydrophobic residues may lead to strong stabilization of the heme core which hinders the binding of substrates at neutral *p*H (Modi *et al* 1994). The existence of a salt bridge between heme propionic acid group and a distal residue was earlier shown to hinder the entry of substrate into the heme crevice (Modi *et al* 1994). A change in *p*H of the medium would affect the salt bridge and hydrogen bonding, which might decrease the rigidity of the loop containing the tryptophan residue. A significant increase in the picosecond lifetime at *p*H 4·0 (table 1) suggests that

decrease in *p*H causes a conformational change near the heme cavity of HRP which decreases the efficiency of fluorescence energy transfer from the Trp residue to heme. This change in conformation is probably associated with protonation of one or more ionizable groups situated in the distal side of heme.

Binding of exogenous ligands to HRP

Ligands like fluoride and cyanide react in their protonated form which is distinctly different from the usual binding of inorganic transition metal ions. This difference in reaction mechanism between the enzymatic and inorganic systems may be attributed to difference in coordination number. Cyanide binds in its protonated form and the proton is taken up by the distal histidine-42 which forms a H-bond to the nitrogen of bound cyanide (Thanabal *et al* 1988). Azide also binds in a similar fashion where hydrazoic acid is the reactive ligand (Holzwarth *et al* 1988) and the proton is stabilized by hydrogen bonding to a distal basic group. *p*H-dependent proton NMR spectra showed that azide binding is facilitated by proton transfer of the enzyme and a HRP-N_3 complex is predominantly formed at lower *p*H(*p*H $\sim$ 4·5). The shortest lifetime of tryptophan fluorescence of HRP in presence of azide at different *p*H above $\sim$ 6 shows marked similarity with that of native HRP at similar *p*H values. At *p*H > 6, azide does not bind to the ferric iron centre and it is expected that the lifetime value will be similar to that of the native one. However, on lowering the *p*H, rapid binding of azide to the iron centre becomes significant and this interaction should be observable in the time scale of the rate constants of the fluorescence decays studied here. As a result one would expect an altered value of lifetime on azide binding. However, due to the fact that the *p*H-dependent lifetime variation in azide-bound HRP is very much similar to that of native HRP, we propose that a conformational transition is obligatory in native HRP itself and this may be associated with so called 'gating' for the entry of different ligands. Many of the oxidizable inorganic substrates of HRP bind with maximum affinity only at lower *p*H(*p*H $\sim$ 4). This indicates that native HRP in absence of any exogenous ligand undergoes a conformational opening most probably associated with protonation of some group(s). When a ligand is present in the medium, the protonation of HRP makes ligand binding facile.

In case of thiocyanate too, the shortest lifetime shows enhancement on lowering the *p*H from 6·0, down to $\sim$ 4·0. The slow but significant change in lifetime around *p*H $\sim$ 3·4 for both thiocyanate and azide complexes reflects a large conformational change in the heme cavity. A large blue-shift of the Soret band in the optical spectrum (figure 1) also indicates that the heme linkage to the protein probably has been severely weakened. A similar kind of change in electronic spectrum was recently observed for micelle-induced heme release from nitric oxide complex of myoglobin at physiological *p*H (Das *et al* 1993a) by us.

Both myoglobin and HRP contain protoheme as their prosthetic group bound through the proximal histidine to the globin part. Extra stabilization of the heme group in the redox cavity comes from the extensive H-bonding network and presence of the salt bridge through the proximal and distal side amino acid residues and the heme group itself. In myoglobin, a salt bridge found between porphyrin carboxylate side chain, His-97 and Arg-45 is proposed to impart extra support to heme group (La Mar *et al* 1991). In native HRP, a protonation with $pK_a = 4{\cdot}2$ was attributed due to protonation of heme propionic acid from hyperfine-shifted proton NMR studies (Morishima *et al* 1977). Recently ^{15}N & ^{1}H-NMR studies on binding of thiocyanate to

chemically modified HRP have been utilized to demonstrate the existence of salt bridge between the heme propionic acid and distal side amino acid residues (Modi *et al* 1994). It was observed that thiocyanate binding to native HRP can occur only when the salt bridge structure is altered by protonation of the heme propionic acid group. Our observations indicate that the HRP-SCN complex is more prone to proton induced denaturation than native HRP. It shows that thiocyanate must have caused extra instability in the heme surroundings by interacting with the H-bonding network associated with proximal histidine and/or, with the salt bridge structure linked to the heme propionic acid side chain. Azide binding to heme iron of HRP, also, vitiates the H-bonding structure in the heme cavity. The observation that azide also causes heme destabilisation at *p*H $\sim$3·4, supports the proposition that azide is most probably connected to distal histidine via H-bonding interaction and hence is involved with the whole network of interaction, as is observed in case of cyanide ligation to HRP (Thanabal *et al* 1988). To show that heme destabilization in HRP-SCN and HRP-N_3 is not just a ionic strength induced phenomenon (salt effect), equimillimolar amount of NaCl was added to HRP solution at similar *p*H. Only very small amount of depletion of Soret peak was observed. Hence it can be concluded that thiocyanate and azide interact strongly with the hydrogen bonding network present in the heme cavity. These studies hence further support the importance of salt bridge in gating the entry of a ligand to heme cavity.

The fact that the conformational transition in HRP is obligatory, is further supported by cyanide binding (Das and Mazumdar 1995) to HRP. Cyanide can bind to HRP at substantially higher *p*H, and hence is not facilitated by protonation at low *p*H region. Hence, if the protonation of HRP associated with conformational change were essential only for ligand binding, one would not expect any *p*H dependent conformational change in the cyanide complex as well as in native HRP. However, the fact that cyanide also shows a similar conformational change, indicates that the conformational opening is obligatory in the lower *p*H region for HRP. The fact that cyanide binds to HRP at wider *p*H range, is a testimony to the stability of interaction between heme-iron-bound cyanide and the distal imidazolium side chain which serves as hydrogen-bond donor to the bound CN^- (Thanabal *et al* 1988).

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