BioMetals **12:** 219–225, 1999.

Fluorescence and circular dichroism spectroscopic studies on bovine lactoperoxidase*

M. Sharique Zahida W. Deva & Digambar V. Behere
Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Navy Nagar, Bombay 400 005, India (E-mail behere@tifr.res.in)

Received 23 November 1998; accepted 5 February 1999

Key words: lactoperoxidase, fluorescence, energy transfer, circular dichroism, guanidine hydrochloride, urea

Abstract

Intrinsic steady-state fluorescence of lactoperoxidase (LPO) and its ligand-bound complexes has been characterized as a structural probe of its structure in solution. On excitation at 295 nm, a broad emission maximum is observed around 338 nm for LPO and for its ligand-bound complexes. The quantum yield is 0.0185 ± 0.0005 for LPO and indicates tryptophan $\rightarrow$ heme energy transfer. Tryptophan residues are located away from heme and are approximately equally distributed among hydrophobic and hydrophilic environments. From Förster resonance energy transfer equations, the "average" distance between tryptophans and heme within the enzyme is computed to be 25.1 ± 0.2 Å. These fluorescence properties are consistent with the recent theoretical three-dimensional model for LPO and reveal that Trp337 and Trp404 dominate the intrinsic fluorescence, and together contribute $\sim$ 64% of the observed intensity. The effects of the denaturing agents guanidine hydrochloride and urea on the intrinsic fluorescence of LPO and CD of the backbone amide chromophores have been examined. The considerably red shifted emission maximum at 356 nm indicates that tryptophans, buried in the hydrophobic environment, are exposed to the solvent on denaturation. A simple two-state transition between the native and denatured forms of the protein has been used to explain the results. $[Denaturant]_{1/2} \sim 5.5$ M, determined from both these experiments, indicates that LPO is relatively stable toward the denaturing agents. Quenching studies using. I^-, Cs^+ and polar neutral acrylamide are consistent with this picture. Acrylamide can penetrate the protein matrix. It is an efficient quencher and the quenching process is essentially homogeneous with all the tryptophans being accessible. Cs^+ ion is a very inefficient quencher but the iodide ion shows the quenching process to be predominantly heterogeneous with widely differing tryptophan accessibility. The Stern–Volmer constants deduced are $K_{sv} = 8.4 \pm 1.4$ M^{-1} and $K_{sv} = 4.05 \pm 0.65$ M^{-1} for acrylamide and iodide quenching, respectively. The fractional accessibility, f_a, deduced is $f_a = 0.52 \pm 0.03$ for iodide quenching.

Introduction

Lactoperoxidase (LPO) (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) is a redox enzyme with antibacterial properties, found in several biological fluids such as milk, tears, saliva etc. It is a member of the mammalian peroxidase family (Pruitt & Reiter 1985). The enzyme catalyzes oxidation of thiocyanate and iodide ions to generate highly oxidizing and toxic products which can kill viruses, gram positive and gram negative bacteria, fungi, parasites, tumour cells and also mycoplasmas (Pruitt & Reiter 1985). LPO consists of a single polypeptide chain of 612 amino acid residues (Dull *et al.* 1990; Cals *et al.* 1991), a molecular mass of 78 500 Da, a covalently bound heme and about 10% carbohydrate content (Calstrom 1969). There are 15 tyrosine and 15 tryptophan residues in the enzyme (Dull *et al.* 1990; Cals *et al.* 1991). An x-ray structure for LPO is not available; however, recently, a theoretical three-dimensional model, built on the scaffold of an MPO

*Part of this work was presented at the fourth European Conference on Biological Inorganic Chemistry, Seville, Spain, July 20–25, 1998

x-ray structure, has been proposed for the LPO molecule (Gioia *et al.* 1996). The model shows that the polypeptide is a nearly spherical molecule with an average diameter of ≈ 64 Å and that the heme is deeply buried inside the peptide fold located approximately at the center.

In this study, the intrinsic fluorescence of LPO and its ligand-bound derivatives has been examined. Fluorescence changes occurring during denaturation, in the presence of guanidine hydrochloride (GdnHCl), have been studied in the hope that it would help to provide information concerning the stability and reconstitution of LPO. The results have been compared with circular dichroism (CD) spectra of the amide backbone. Relative accessibility of fluorophores in a protein can be determined by the sensitivity of intrinsic fluorescence to a variety of freely diffusing quenchers. Steady-state fluorescence quenching with ionic quenchers, I^- and Cs^+, has been used to determine the amount of fluorophores accessible to the surrounding solvent water. Quenching by the uncharged polar quencher acrylamide has been examined to probe the efficiency of a quencher, which can penetrate, to some extent, the hydrophobic interior of LPO (Eftink & Ghiron 1976).

Experimental

LPO was isolated and purified from 30L of cows' unpasturized, raw milk (Dumantet & Rousset 1983). A purified enzyme having $R_z = A_{412}/A_{280} = 0.94$ was used for spectroscopic studies. Concentration of LPO was measured using $\epsilon_{412} = 112$ mM^{-1} cm^{-1} (Calstrom 1969). Steady-state fluorescence spectra were recorded at $\lambda_{ex} = 295$ nm on a SPEX-1681-0.22m spectrometer equipped with dM 3000 software for data acquiring and processing. Emission was scanned in the wavelength region between 300 and 550 nm. All measurements were made on a 2 ml sample in a 5 mM sodium phosphate buffer pH = 7.0 and at 23 °C. The fluorescence emission was corrected for inner-filter effects. The quantum yield (φ_p) of the proteins was calculated using tryptophan standard, *n*-acetyl tryptophan amide (*AcTrpNH*$_2$).

Förster-type nonradiative resonance energy transfer occurs as a result of dipole-dipole coupling between donor and acceptor (Stryer 1978). The efficiency of the energy transfer (E) between the donor (Trp) and the acceptor (heme) is related to the separation, r, between excited donor dipole and the acceptor dipole groups:

$$E = \left(1 - \frac{\varphi_p}{\varphi_a}\right) = \frac{r^{-6}}{r^{-6} + R_o^{-6}}, \qquad (1)$$

where φ_a, the quantum yield for tryptophan in absence of acceptors, is estimated to be 0.2 (Hill *et al.* 1986). R_o is the donor-acceptor separation at which the donor fluorescence is quenched by 50%. It is given by $R_o = 9.79 \times 10^3(\kappa^2 \varphi_a J n^{-4})^{1/6}\kappa^2$, the orientation factor of donor-acceptor transition dipoles, was assumed to be $2/3$ for random orientations between the transition dipole moments (Haas *et al.* 1978), since the tryptophan residues possess two perpendicular emission dipoles (Lakowicz *et al.* 1983); the heme also contains electronic transitions along two perpendicular axes, respectively. Under these circumstances, any error in R_o introduced by orientation is expected to be negligible (Stryer 1978). n is the refractive index of the intervening buffer medium of the protein solution. For very dilute protein solutions, n is generally given a value of 1.4 (Stryer 1978). Since the sixth root of n^{-4} is involved, 15% variation in the value n introduces less than 5% error in R_o. J is the spectral overlap integral between the tryptophan emission and heme absorption. It was numerically estimated over the spectral region from 300 to 550 nm, to be $J = 8.63 \times 10^{-14}$ cm^{-3} M^{-1}. For LPO, $R_o = 36.8$ Å and in a multi tryptophan lactoperoxidase, the "average" tryptophan-heme separation, $r = 25.1 \pm 0.2$ Å were computed using Equation 1.

The percent contribution of each tryptophan to the total fluorescence of LPO was calculated using Equation 2 (Fox *et al.* 1993).

$$\%\Phi = \frac{100\left(\frac{\varphi_p}{\varphi_a}\right)_i}{\sum\left(\frac{\varphi_p}{\varphi_a}\right)}, \qquad (2)$$

where $(\varphi_/\varphi_a)_i$ represents the relative quantum yield of i^{th} tryptophan. This was calculated for each tryptophan, using tryptophan-to-heme separations, computed from the three dimensional theoretical model for LPO (Gioia *et al.* 1996) and using Equation 1. $\sum \varphi_p/\varphi_a$ is the total relative quantum yield of fifteen tryptophans of LPO. It is related to the measured relative quantum yield, $(\varphi_p/\varphi_a)_{\text{measured}}$, which is an average of the relative quantum yield of all the fifteen tryptophans in LPO. Thus, it is $\sum(\varphi_p/\varphi_a) = 15 \times (\varphi_p/\varphi_a)_{\text{measured}}$. The % Φ computed for each tryptophan in LPO using Equation 2, shown in Table 2, thus depends on the tryptophan-to-heme separations.

Denaturation measurements were carried out on DTT-treated samples (Jocelyn 1987) as LPO contains

Table 1. Fluorescence of LPO derivatives & other heme proteins

Protein	λ_{em} (nm)	Soret λ_{max} (nm)	φ ($AcTrpNH_2$)	φ_p	Reference
LPO	338	412	0.142	0.0185	This work
LPO-CN	338	426	0.148	0.0193	This work
LPOII/S	343	412	0.0209	0.00273	This work
HRP	328	403	0.039	0.005	Ugarova *et al.* 1981
CCP	324	408	0.07	0.009	Fox *et al.* 1993
Cytochrome-C Oxidase	328	422	0.325	0.065	Hill *et al.* 1986

twelve disulfide bridges (Gioia *et al.* 1996). Relative fluorescence intensity (F/F_o) was measured as a function of concentration of the denaturing agent, guanidine hydrochloride (GdnHCl). F_o is the fluorescence intensity in absence of the denaturing agent. Unfolding of DTT-treated LPO was also studied, for comparison, by following the effect of the increasing concentration of a denaturant on the CD activity of the peptide backbone (amide) chromophore at 222 nm. Urea was preferred as a denaturant for CD measurements over GdnHCl as it gave a relatively very low baseline CD absorption of the buffer, containing equivalent urea samples. This was subtracted from the protein CD absorption.

The free energy of unfolding at each concentration of the denaturing agent was calculated, assuming two-state transitions between native (N) and unfolded (U) forms being in equilibrium, and using:

$$\Delta G = -RT \ln \frac{(X_n - X_i)}{(X_i - X_d)}. \tag{3}$$

X_i is the numerical value of the structure sensitive parameter (F/F_o). X_n, X_d in Equation 3 are the numerical values of the same parameter of the protein when in native state (n) and the denatured state (d), respectively. Variation of the calculated free energy change as a function of the concentration of the denaturing agent is given by (Pace 1986):

$$\Delta G = \Delta G^{\mathrm{H_2O}} - m[\text{denaturant}]. \tag{4}$$

Quenching of LPO fluorescence by the neutral quencher acrylamide showed the presence of a static component. The Stern–Volmer quenching constant (K_{sv}), a measure of the dynamic quenching, was determined using a modified Stern–Volmer equation (Eftink & Ghiron 1976):

$$\frac{F_o}{F \exp(V[L_o])} = 1 + K_{sv}[L_o], \tag{5}$$

where V is a parameter which takes care of the static quenching component. Fluorescence quenching data by ionic quenchers, however, showed differing accessibility. K_{sv} was determined by a modified Lehrer plot expressed as (Lehrer 1971):

$$\frac{F_o - F}{F_o} = \frac{f_a K_{sv}[L_o]}{(1 + K_{sv}[L_o])}, \tag{6}$$

where K_{sv} represents the dynamic Stern–Volmer quenching constant and f_{a} represents the fraction of the protein fluorophore accessible to the quencher.

Results and discussion

On excitation at 280, 290 and 295 nm respectively, the fluorescence emission for LPO shows a single, broad maximum at 338 nm (bandwidth $\approx$ 52 nm). However, the fluorescence, at $\lambda_{ex} = 295$ nm, is relatively less intense than observed at $\lambda_{ex} = 280$ and 290 nm. In addition, the excitation spectrum shows a peak at 280 nm with shoulders around 290 and 294 nm, respectively. Tyrosine residues in protein are not excited at 295 nm (Lakowicz 1983). The observed less intense fluorescence emission in LPO at $\lambda_{ex} = 295$ nm therefore arises nearly entirely from the tryptophan residues, and the contribution from the tyrosine residues, if any, must therefore be negligibly small.

The considerably red shifted emission maximum, as compared to other hemeproteins (Table 1) suggests that in LPO, the tryptophan residues are approximately symmetrically distributed in hydrophobic as well as hydrophilic environment. Emission maxima for LPO derivatives also occur essentially at the same wavelength (Table 1).

Table 2. Percent quantum yield (%Φ) for Tryptophan residues in LPO

Tryptophan	r Å	% Φ
Trp163	18.77	1.25
Trp178	23.20	4.26
Trp220	14.93	0.32
Trp337	31.18	19.50
Trp404	39.59	43.81
Trp441	21.81	3.00
Trp485	26.15	8.22
Trp501	22.39	3.48
Trp569	18.26	1.06
Trp610	9.970	0.03
Trp646	20.93	2.36
Trp647	26.12	8.17
Trp2 + Trp91 + Trp119	–	4.59
SUM		100

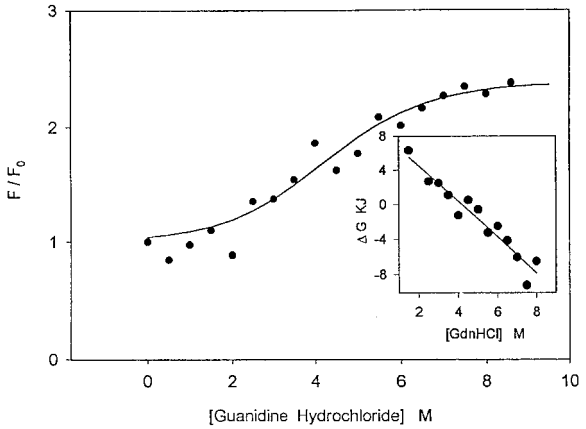

Figure 1. Effect of GdnHCl concentration on the relative fluorescence intensity, (F/F_0) of LPO during denaturation. The excitation wavelength was 295 nm. Measurements were made at 23 °C and *p*H = 7.0 in 5 mM, sodium phosphate buffer. GdnHCl stock solutions were made in 5 mM sodium phosphate buffer pH = 7.0. Smooth curve was calculated using Equation 3. Inset shows the ΔG as function of guanidine hydrochloride concentration.

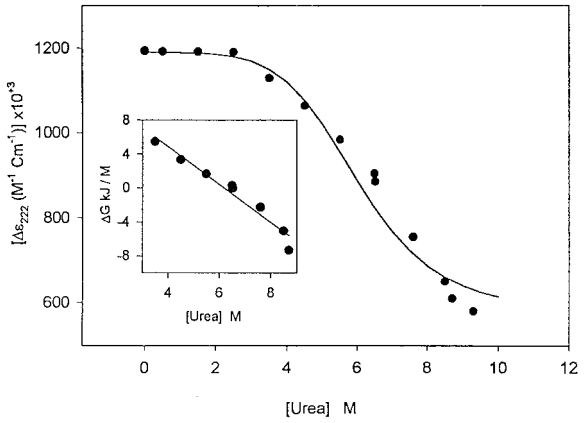

Figure 2. Dependence of amide backbone CD of LPO on urea concentration during denaturation. The structure sensitive $\Delta\epsilon_{222}$ M^{-1} cm^{-1}, values at 222 nm plotted against urea concentration. Smooth curve was obtained using fitted parameters in Equations 3 & 4.

The tryptophan-to-heme distances in Table 2 show Trp337 and Trp404, located far away from the ferric heme at 31.18 Å and 39.59 Å, respectively, dominating together the steady-state fluorescence of LPO, and contributing almost ≈ 64% of the observed intensity. The fluorescence intensity of all other tryptophan emission is quenched differently, depending on their proximity to the heme group and their relative orientation. It can be seen (Table 2) that three tryptophan residues, Trp2, Trp91 and Trp119, omitted from the model together contribute only ≈ 4.6% to the observed intensity. If they are assumed to contribute equally, it is then estimated that they are located around 19.5 Å from the heme center. It may be noted that the tryptophan-to-heme distances in Table 2, are only approximate average distances, as they are based on the model. Therefore, the percent contributions of each tryptophan, (%Φ), to the observed fluorescence intensity calculated here are also approximate. It is fair to expect, however, that their relative contributions would remain nearly the same.

The emission maximum for LPO in the presence of 9M guanidine hydrochloride is observed at 356 nm. Considerable red shift in the emission maximum (Table 1) indicates that tryptophans, buried in the interior hydrophobic environment in the native folded state and inaccessible to solvent water, get all exposed to aqueous environment. Figure 1 shows the plot of the observed relative fluorescence intensity as a function of guanidine hydrochloride concentration. It shows a typical sigmoid nature of the unfolding process. The sigmoid nature is characteristic of a simple two-state transition between the native (N) and unfolded (U) forms of the protein in equilibrium. The free energy of unfolding at each concentration of the denaturant was calculated by using Equation 3. The inset in Figure 1 shows that the free energy change varies linearly with the denaturant concentration.

The CD spectrum is absence of the denaturant shows two maxima at 208 and 222 nm, which are typical of a polypeptide in the α-helix and β-sheet conformations (Sievers 1980). Figure 2 shows that

the CD activity ($\Delta\epsilon_{222}$ M^{-1} cm^{-1}) is initially insensitive at low urea concentrations. At high urea concentrations however, the variation of CD activity has a sigmoid profile. This behavior is consistent with a simple two-state transition between the native and denatured state of LPO. The inset in Figure 2 also shows that the free energy change varies linearly with the denaturant concentration.

Table 3 shows the estimated Δ^{H_2O} and *m* values by the least squares fit of ΔG Vs [*denaturant*] data to Equation 4. The coefficients of determination, r^2, are the measure of closeness of the fit. The value of ΔG^{H_2O} determined from the CD measurements (Table 3) is of larger magnitude than that obtained from the analysis of the fluorescence emission data. Figure 2 shows that even up to 9.5M urea, the CD activity at 222 nm does not attain constancy as compared to the relative fluorescence intensity up to 9M GdnHCl (Figure 1). This indicates the existence of some residual secondary structure at 9.5 M urea. Neutral urea is known to be 1.5 to 2.5 times less effective as denaturant than GdnHCl (Pace 1986). The unfolding by urea in the CD experiment is somewhat less complete up to 9.5M urea concentration and may be partly responsible for the difference in the ΔG^{H_2O} values in the two experiments. In addition, fairly large extrapolations of ΔG^{H_2O} values from 9M of urea and 8M GdnHCl (see inserts Figures 1 and 2), up to zero denaturant concentration have to be effected in order to obtain an estimation of ΔG^{H_2O}. This can also introduce differences in the computed values of the parameter (Ahamad *et al.* 1982). In absence of the denaturant, ΔG^{H_2O} is a measure of stability of the protein conformation (Pace 1986). For many globular proteins, the stability values are in the range of 21–41.8 kJ/mole (Pappa & Cass 1993). The low values determined in the present study indicate that at neutral *p*H LPO is not a very stable protein.

The *m* value depends on the size and composition of the protein chain exposed to solvent upon unfolding. The values of 2.1 and 2.2 kJ.[mol protein]$^{-1}$.M [denaturant]$^{-1}$ respectively determined here are low compared to 8.7 kJ.[mol protein]$^{-1}$. M [denaturant]$^{-1}$ reported for myoglobin (Ahamad *et al.* 1982). The low values are consistent with the three-dimensional model. They essentially indicate that only a small amount of the additional peptide surface of LPO is exposed to the solvent, upon unfolding. The ratio, m (GdnHCl)/m (urea), has been suggested to be related to the polarity of the unfolding (Greene & Pace 1974). The ratio higher than 2.4 indicates the predominantly

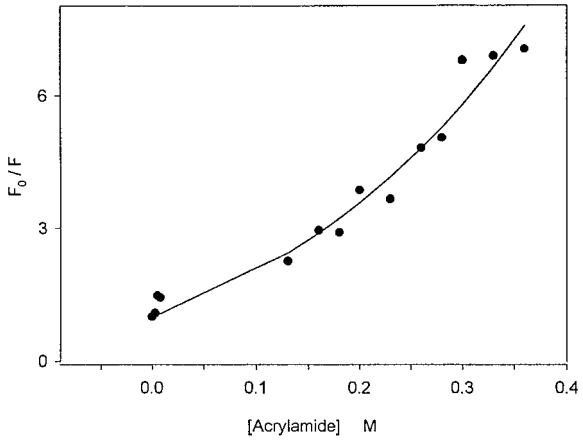

Figure 3. Steady-state fluorescence quenching of LPO by acrylamide. The fluorescence was measured in 5 mM phosphate buffer *p*H = 7. The ionic strength of the sample was maintained at 1 M by addition of appropriate quantity of NaCl from the stock solution made in the same buffer. The fluorescence intensity is expressed as the fraction relative to unquenched fluorescence, $(F/F_o) \cdot \lambda_{ex} = 295$. $\lambda_{ex} = 295$ nm and $\lambda_{em} = 338$ nm. Solid circles are the data points and the smooth curve was obtained using equation 5, and using fitted parameters.

polar nature of the unfolding unit. The low value of ≈ 1 observed here shows that unfolding residues in LPO are predominantly non-polar in character.

The transition mid-point is defined by the concentration of the denaturant required to effect 50% of the total change, ([*denaturant*] $_{\frac{1}{2}}$). It is a measure of the stability of the protein towards the denaturing agent. The values of transition mid-point in Table 3 show that LPO is more stable toward the denaturing agents, as compared to myoglobin (1.7 M GdnHCl) (Bismuto *et al.* 1983) and horse heart cytochrome-c (2.8 M GdnHCl) (Mines *et al.* 1996).

Figure 3 shows the fluorescence quenching by acrylamide. The upward curving plot indicates that all the tryptophan residues are nearly accessible, and a dynamic quenching process, having a static component (Eftink & Ghiron 1976). Magnitude of $K_{sv} = 8.4 \pm 1.4$ M^{-1} was determined for acrylamide using Equation 5. K_{sv} in multi-tryptophan proteins essentially denotes the weighted average of the individual quenching constants.

Iodide ions also quenched LPO fluorescence. Steady-state quenching by iodide ion did not give a satisfactory linear plot of F_o/F Vs [L_o]. However, $((F_o - F)/F_o)$ is seen to smoothly follow the quencher concentration (Figure 4), although data show some more scatters than is generally observed. Data have been analyzed using the modified Lehrer equation

Table 3. Thermodynamic data on the Denaturation of LPO

Probe	ΔG^{H20} kJ/m	m $kJ.[moleprotein]^{-1}$ $M[denaturant]^{-1}$	r^2	$[denaturant]_{1/2}$ M
Tryptophan Fluorescence	8.7	2.1	0.93	5.5
Backbone CD	13.9	2.2	0.95	5.6

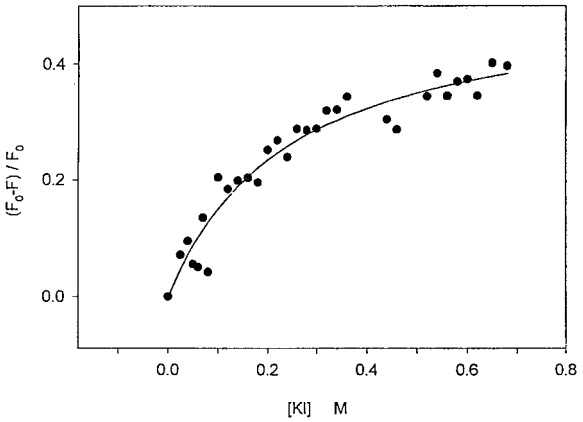

Figure 4. Modified Lehrer's plot of quenching of the steady state fluorescence of LPO by iodide ion. Fluorescence was measured in 5 mM phosphate buffer pH = 7. The ionic strength of the sample was maintained at 1 M by the addition of appropriate quantity of NaCl from the stock solution made in the same buffer. Relative decrease in the fluorescence, $(F_o - F)/F_o$ at $\lambda_{ex} = 295$ nm and $\lambda_{em} = 338$ nm plotted as a function of iodide ion concentration. Smooth curve calculated using parameters fitted to Equation 6.

(Equation 6). A unique set of parameters (K_{sv} f_a) are obtained with $K_{sv} = 4.05 \pm 0.64\ M^{-1}$ and $f_a = 0.52 \pm 0.03$. The Solid line in Figure 4 shows the curve calculated using fitted parameters. This indicates a dynamic quenching process due to free diffusion of iodide ions. Quenching, however, is essentially heterogeneous with tryptophan residues widely differing in their accessibility.

Thiocyanate ion is very similar to iodide ion in binding to LPO (Modi *et al.* 1989; Ferrari *et al.* 1997). However, the relative fluorescence intensity in the presence of thiocyanate anion does not shown any systematic variation with the quencher concentration. The ineffectiveness of the thiocyanate ion as a quencher may be due to its linear shape, it not being conducive enough for effective collisional quenching as a spherical iodide ion (Lakowicz 1983). Electronegativities of nitrogen and sulfur are different. The asymmetric charge distribution over the linear molecule may therefore also be responsible for the ineffectiveness of the thiocyanate as quencher.

Cs^+ does not quench LPO fluorescence up to 0.7 M. Cs^+ was only seen to produce a small downward curving Stern–Volmer plot with considerable scattering in the data (not shown). That indicated the Cs^+ ion to be a very inefficient quencher. LPO with $pI > 8$ (Cals *et al.* 1991) carries a net positive charge on the surface. This may possibly inhibit the diffusion of Cs^+ ions but may also have facilitated the diffusion and quenching by negatively charged iodide ions more effectively.

Fluorescence quenching by both acrylamide and iodide suggests that the surface of the LPO molecule is sufficiently conducive for interactions of substrates with both hydrophobic and hydrophilic characters.

References

Ahamad F, Bigelow C. 1982. Estimation of the free energy of stabilization of ribonulease A, lysozyme, α-lactoglobulin and myoglobin. *J Biol Chem* **257**, 12935–12938.

Bismuto E, Colonna G, Irace G. 1983 Unfolding pathway of myoglobin. Evidence of multistate process. *Biochemistry* **22**, 4165–4170.

Cals CM, Mailliart P, Brignon G, Anglade P, Dumas BR. 1991 Primary structure of bovine lactoperoxidase, fourth member of a mammalian heme peroxidase family. *Eur J Biochem* **198**, 733–739.

Calstrom A. 1969 Physical and compositional investigations of sub fractions of lactoperoxidase, *Acta Chem Scand* **23**, 171–213.

Dull T, Uyeda JC, Strosberg AD, Nedwin G, Seilhamer JJ. 1990 Molecular cloning of cDNA encoding bovine and human lactoperoxidase, *DNA Cell Biol* **9**, 499–509.

Dumontet C, Rousset B. 1983 Identification, purification, and characterization of a non-heme lactoperoxidase in bovine milk. *J Biol Chem* **258**, 14166–14172.

Eftink MR, Ghiron CA. 1976 Exposure of tryptophanyl residues in proteins, quantitative determination by fluorescence quenching studies. *Biochemistry* **15**, 672–680.

Ferrari RP, Ghibaudi EM, Traversa S, Laurenti E, Gioia LD, Salmona M. 1997 Spectroscopic and binding studies on the inter-

action of inorganic anions with lactoperoxidase. *J Inorg Biochem* **68**, 17–26.

Fox T, Ferreira-rajabi L, Hill BC, English AN. 1993 Quenching of intrinsic fluorescence of yeast cytochrome c peroxidase by covalently and non-covalently-bound quenchers. *Biochemistry* **32**, 6938–6943.

Gioia LD, Ghibaudi EM, Laurenti E, Salmona M, Ferrari RP. 1996 A theoretical three-dimensional model for lactoperoxidase and eosinophil peroxidase, built on the scafold of the myleoperoxidase x-ray structure. *J Biol Inorg Chem* **1** 476–488.

Greene RF Jr, Pace CN. 1974 Urea, guanidine hydrochloride denaturation of ribonuclease, lysozyme α-cymotrypsin and β-lactoglobulin. *J Biol Chem* **249**, 5388–5393.

Haas E, Katchalski-Katzir E. 1978 Effect of the orientation of donor and acceptor on the probably of electronic transitions of mixed polarization. *Biochemistry* **17**, 5064–5070.

Hill BC, Horowitz PM, Robinson NC. 1986 Detection, characterization, and quenching of the intrinsic fluorescence of bovine heart cytochrome c oxidase. *Biochemistry* **25**, 2287–2292.

Jocelyn PC. 1987 Chemical reduction of disulfides. *Meth Enzymol* **143**, 246–249.

Lakowicz JR. 1983 Protein fluorescence In *Principles of Fluorescence Spectroscopy*. New York: Plenum; 341–381.

Lakowicz JR, Maliwal BP, Cherek H, Balter A. 1983 Rotational freedom of tryptophan residues in proteins and peptides. *Biochemistry* **22**, 1741–1743.

Lehrer SS. 1971 Solute perturbation of protein fluorescence. the quenching of tryptophenyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry* **10**, 3254–3263.

Mines GA, Pascher T, Lee SC jr., Winkler JR, Gray HB. 1996 Ctochrome folding triggered by electron transfer. *Chem Biol* **3**, 491–497.

Modi S, Behere DV, Mitra S. 1989 Binding of thiocyanate to lactoperoxidase: ^{1}H and ^{15}N nuclear magnetic resonance studies. *Biochemistry* **48**, 4689–4694.

Pace CN. 1986 Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Meth Enzymol* **131** 266–280.

Pappa HS, Cass AT. 1993 A step towards understanding the folding mechanism of horseradish peroxidase. *Eur J Biochem* **212**, 227–235.

Pruitt KM, Reiter B. 1985 Biochemistry of peroxidase system: antimicrobial effects. in Pruitt KM. Reiter Tenovuo JO (ed) *The Lactoperoxidase System*. New York: Marcel Dekker; 143–177.

Sievers G. 1980 Structure of milk lactoperoxidase; A study using circular dichroism and difference absorption spectroscopy. *Biochim Biophys Acta* **624**, 249–259.

Stryer L. 1978 Fluorescence energy transfer as a spectroscopic ruler. In: Snell EE, Boyer PD, Richardson C (eds) *Ann Rev Biochem* **47**, 819–846.

Ugarova NN, Savitski AP, Berzein IV. 1981 The protoporphyrin-apoperoxidase complex as a horseradish peroxidase analog a fluorometric study of the heme pocket. *Biochim Biophys Acta* **662**, 210–219.