Short Communication

Non-Quenching by Iodide of Fluorescence in Mesoporphyrin-Substituted Peroxidase

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Iodide is a donor substrate for peroxidases, reducing both compounds I and II. The reaction rate constants with horse-radish peroxidase (HRP) are inversely proportional to pH.[1,2] It is striking that iodide, like ferrocyanide but unlike aromatic donors, does not form an optically detectable complex with this peroxidase. NMR spectroscopy, however, demonstrated binding of iodide to HRP with $K_d \sim 100$ mM at pH 4.[3] A good model for some properties of HRP is obtained by replacing heme with mesoporphyrin (MP).[4,5] Unlike heme, which is non-fluorescent, MP is highly fluorescent and can serve as a reporter of events at the heme pocket. HRP isoenzyme C contains one tryptophan and five tyrosine residues. Based on the relatively high fluorescence yield, the single tryptophan is estimated to be ≧22 Å from the heme.[5] Iodide quenches singlet excited states by spin–orbit coupling; therefore, quenching occurs when electrons on iodide are in close contact with the π electrons of fluorescent molecules. The present study compares information from NMR spectroscopy with those from fluorescence measurements, with emphasis on the question of whether iodide is bound in a manner that permits quenching of MP or tryptophan fluorescence.

Materials and methods

Apo-HRP C was allowed to react for one h with an equivalent amount of MP at pH 8. Unbound MP was removed on DEAE-Sephadex A-50 at pH 5. Coproporphyrin was chosen as the reference free porphyrin in order to minimize the effect of aggregation. Fluorescence spectra were measured at 22 °C on a Perkin Elmer LS-5 spectrofluorimeter. MP HRP C in 0.1 M sodium acetate (pH 5.0) at a concentration of 5 μM was excited using 280 and 290 nm light for tryptophan and 395 nm for MP. Tryptophan emission was monitored at 350 nm and MP emission at 613 nm. Freshly prepared KI (1 M stock solution containing 1 mM $Na_2S_2O_3$) was added to give concentrations up to 0.3 M. Quenching constants were determined from eqn. (1),

$$F_0/F = 1 + k\,\tau_0[I^-] \quad (1)$$

where F_0 is the fluorescence intensity without

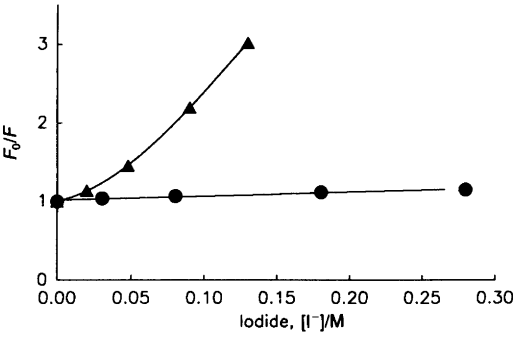

Fig. 1. Iodide quenching of 5 μM coproporphyrin (▲) or MP HRP C (●) fluorescence.

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iodide and F is the intensity at a given iodide concentration, τ_0 is the fluorescence lifetime (determined as described),[5] and k is the quenching constant.

MP is more loosely bound than protoheme to apo-HRP. The experimental conditions were confined by the risk of perturbations in the MP-protein relationship by hydrogen ions or the chaotropic properties of iodide ions.

Results and discussion

There was no change in the fluorescence intensity of tryptophan up to 0.3 M iodide. This is in keeping with the previous conclusion that tryptophan is ≧22 Å away from the heme.[6]

Coproporphyrin fluorescence showed a lifetime of 13.0 ns. The non-linear course of the quench plot for free porphyrin (Fig. 1) is to be expected if more than one iodide ion at the same time reaches within the quenching radius. The tangent at low iodide concentration yields a quenching constant of 5×10^8 M^{-1} s^{-1}. The fluorescence of MP HRP responded linearily but weakly to $[I^-]$ (Fig. 1). With τ_0 = 16.2 ns the quenching constant for MP HRP becomes 3×10^7 M^{-1} s^{-1}.

Benzhydroxamic acid is a high-affinity donor substrate to HRP, with K_d = 2.5 μM at pH 5.[7] At a concentration of 10 μM it shifted the emission maximum from 613 to 622 nm and decreased the lifetime from 16.2 to 14.7 ns. This suggests that MP has become more exposed to water; nevertheless, the quenching constant for the porphyrin fluorescence remained low, viz. 6×10^7 M^{-1} s^{-1}.

Quenching due to an 1:1 equilibrium between fluorogen and quencher will cause a linear increase in F_0/F, such as that seen in Fig. 1. The response to the iodide concentration is, however, weak, if any, which can be explained in three ways: (a) Loose binding of iodide. K_m for iodide with compound I is 4 mM^2, and K_d with HRP C itself is about 100 mM^3, both at pH 4. If K_d responds to pH in the same manner as the rate does,[1] it should attain a value of about 1 M at pH 5; with 0.3 M iodide 25 % of MP HRP C should carry iodide. The quenching was, however, very much weaker, and this explanation is unlikely. (b) Iodide is bound at a point beyond the influence of the MP π electrons, i.e. ≧5 Å. NMR measurements indicated binding most probably within 6 Å of the 1- and 8-methyl protons,[3] which is even further away from the annular conjugated system. (c) Equatorial binding of iodide, in the plane of the porphyrin disk, would be unfavourable for quenching.

The present results from fluorimetry corroborate those from NMR spectroscopy.[3] Neither technique seems to be able to distinguish between the binding alternatives (b) and (c). In effect, iodide in both positions is inaccessible to the π electrons. The biological function of HRP C is probably not that of an iodoperoxidase. A comparison with some rapidly reacting iodide peroxidase would reveal whether the distance between iodide and the ferryl-bound oxygen is related to the large K_m-value for interaction between HRP compound I and iodide.

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References

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