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Fluorescent effector as a probe of the allosteric equilibrium in methemoglobin

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Abstract

A fluorescent analogue of diphosphoglycerate (DPG), hydroxy-pyrenetrisulfonate (HPT), was used as a probe of the allosteric equilibrium of methemoglobin. Like DPG, HPT binds, one per tetramer, with a higher affinity to deoxyHb than to oxyHb. Once bound, the HPT fluorescence is quenched by energy transfer to the hemes. HPT can thus serve as a probe of the conformational state of the hemoglobin tetramer: a higher quenching indicates a stronger binding and therefore, more of the deoxy conformation. Since HPT binds to the same site as DPG, it can be displaced by DPG in order to determine the fluorescence intensity of the free HPT under the same conditions, to correct for the inner filter effect. The high spin ferric ligands, such as water and F, showed less fluorescence (more of the deoxy state) than low spin cyano-metHb. The aquo-metHb samples showed a reversion to the oxyHb conformation above pH 7, as expected due to the acid–alkaline transition forming hydroxy-metHb. Effectors such as bezafibrate, which do not bind to the same site as DPG, show an increase in the deoxy-like characteristics. © 1998 Published by Elsevier Science B.V.

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1. Introduction

The allosteric equilibrium in Hb is usually determined indirectly, from ligand binding studies. From the cooperative oxygen equilibrium curves, it is clear that Hb tetramers switch from a low affinity deoxy conformation to a high affinity structure [1–3]. Kinetics of ligand recombination also show resolved phases for the two conformational states [4]. However, estimations of the fraction of the two allosteric forms, at each ligation level, are based on model dependent simulations of the ligand binding data. Signals unique to the allosteric transition are difficult to isolate. One method is to study absorption changes near the isosbestic point for the ligand binding; a small absorbance change after ligand photolysis was observed on the time scale of 100 μs [5]. The absorption signals or Raman spectra [6,7] usually involve a superposition of the ligand binding and other tertiary and quaternary changes, making it difficult to quantify the fraction of the allosteric forms independently of ligand binding. After photodissociation, the fraction geminate recombination is also sensitive to the quaternary state, and can thus serve as a probe [8].

Another method is the use of a fluorescent analogue of the natural effector DPG. Although a weaker

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effector than DPG, HPT also shows a higher affinity for the deoxy form of Hb, with one binding site per tetramer [9,10]. Kinetic studies have shown that the fluorescence changes track mainly the allosteric transition, while the protein (heme) absorption changes are dominated by the ligand binding signal [5,10]. In this study we make static fluorescence measurements to quantify the allosteric equilibrium in metHb.

2. Materials and methods

Human hemoglobin was purified using whole blood from the local blood bank. Samples in the oxy form were frozen in liquid nitrogen until a few days before the experiments. Aquo-metHb was obtained by addition of ferricyanide; the excess ions were then stripped using a Sephadex G-25 column. The other ferric forms were obtained by addition of KF (20 mM) or KCN (2 mM).

Low protein concentrations are better to avoid inner filter effects for the fluorescence experiments. On the other hand, high concentrations insure more of the tetrameric form of Hb. However, for these studies of effector binding, the protein concentration is not a free parameter. One must work at a concentration where the difference in affinity of the oxy and deoxy conformations for the effector will result in an observable difference in the fraction bound. Thus too high a concentration will result in effector binding to both conformations. After trial and error experiments, we used 10 μM effector and 30 μM Hb (on a heme basis), although the best value is pH dependent. At this protein concentration, about 17% dimer is expected for the liganded form (with dissociation coefficient of about 1 μM); however, since oxyHb is used as reference state, the comparative value is less affected.

2.1. Fluorescent probe

The fluorescent effector, 8-hydroxy-1,3,6 pyrenetrisulfonate (Kodak, laser grade) was prepared in ethanol and kept in the dark until use. The concentration was determined at pH 10 from the absorption at 455 nm with $\epsilon = 25.3\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$. The 10 mM stock solution was successively diluted with buffer to obtain a 100 μM solution at the desired pH, and subsequently diluted in the sample solution at the same pH to a final effector concentration of 10 μM.

Fluorescence spectra were measured with an SLM-Aminco 8000 spectrometer. Spectra at 2 nm resolution were measured in several spectral regions. Both excitation and emission spectra were made at several wavelengths to determine the best conditions to avoid the inner filter effect. One series of measurements was made in quartz cuvettes of inner dimension 4×10 mm, which accept a serum cap to allow control of the gas phase. Excitation was along the 4 mm axis.

2.2. Capillary cuvette

Since heme protein samples cause large inner filter effects, we made a special capillary cuvette to minimize this effect. A block of plexiglass was first cut to fit snugly in the cuvette holder (square cross section of 13 mm on a side). A 1 cm diameter hole was drilled in the upper portion and the exterior made circular to accept a serum cap, for control of the atmosphere. The hole was continued with a smaller diameter (of 1 or 2 mm) for the sample. The exterior surfaces exposed to the excitation and emission light paths were reduced, to avoid scratching of these surfaces and to minimize absorption of the excitation light. The reflection and light dispersion was not a major problem: the ratio of HPT fluorescence to that of buffer alone was over 100. Using a glass capillary tube caused problems with scattered light, and with alignment of the tube. The plexiglass cuvette used in the present study reduces these effects since the exterior surfaces are flat as for normal optical cuvettes. With this cuvette, higher concentrations of Hb could be used without a high inner filter effect.

2.3. Effector replacement

Since HPT binds to the DPG site on Hb, but with a lower affinity, one can add an excess of DPG or inositol hexakisphosphate (IHP) to displace the HPT. For each sample, DPG or IHP was added to determine the fluorescence intensity with and without bound HPT under the same absorbing conditions. Thus the ratio of HPT fluorescence with and without IHP provides an additional control of the inner filter effect. After several series of experiments, it became

apparent that addition of DPG may be more reliable; while IHP has a higher affinity, it also may induce a larger perturbation of the system. This is apparent for oxyHb samples exposed to the air, where IHP may lower the oxygen affinity enough to induce a significant fraction of deoxyHb which changes the absorption spectrum; this causes a change in fluorescence through the inner filter effect. For each Hb species, studies were also made versus protein concentration to calibrate the inner filter. With both HPT and IHP present, addition of Hb decreases the fluorescence due to the inner filter effect. This curve could be used as a first order correction to the inner filter effect, which (at 100 μM on a heme basis) was about 20% for the capillary cuvette. For normal cuvettes, the inner filter effect can exceed a factor of 10.

After addition of a second effector, a correction may be necessary for the dilution of the sample. This dilution can be kept small ($< 2\%$), but note that the correction depends on the inner filter effect, and should be calibrated by adding an equivalent volume of water. For normal (1 cm) cuvettes, addition of buffer decreases the inner filter effect which may compensate for the lower fluorophore concentration.

3. Results

3.1. Fluorescence emission

As shown in Fig. 1, HPT fluoresces with a maximum emission near 510 nm. This is near a relative minimum for oxyHb absorbance (Fig. 1), but even the weak bands near 540 and 580 nm can produce significant inner filter effects when using normal optical cuvettes.

Choice of the excitation wavelength is more complicated; as seen in Fig. 2, there are several bands in the excitation spectrum. The band near 460 nm avoids the Soret absorption band of Hb, but this HPT band is highly pH dependent.

As previously observed, there is a higher level of fluorescence quenching for HPT in solutions of deoxyHb relative to the liganded forms (Fig. 2). This indicates that HPT binds preferentially to the deoxy conformation, as does the natural effector DPG; the fluorescence properties of this analogue can thus be used to study the allosteric equilibrium of Hb [9,10].

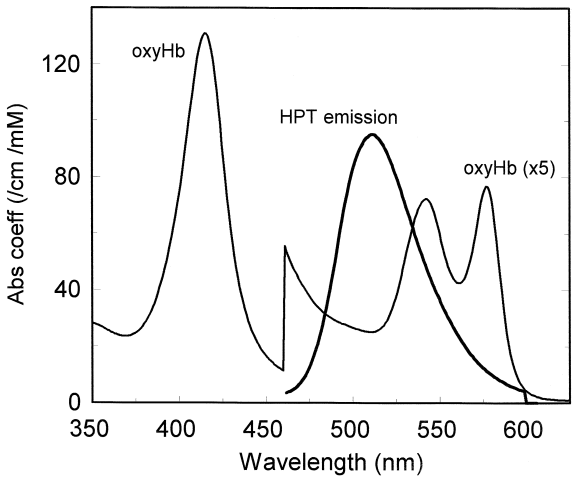

Fig. 1. Absorption spectrum of oxyHb (for a 30 μM sample, on a heme basis) and the fluorescence emission spectrum of hydroxypyrene-trisulfonate (HPT). Experimental conditions: 25°C, Tris buffer at pH 6.8, excitation of 10 μM HPT at 368 nm.

As expected the intensity for aquo-metHb is intermediate to the oxy and deoxy samples.

The spectra in Fig. 2 show the uncorrected fluorescence intensities. At pH 6.8 there is little binding of HPT to oxyHb; the observed decrease for the oxyHb sample is due to the inner filter effect, about 20% for the capillary cuvette. This inner filter effect varies slightly for the different Hb forms, since they have

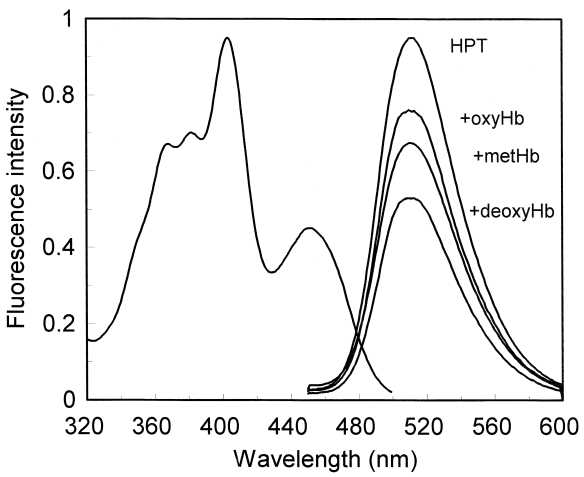

Fig. 2. Fluorescence excitation (emission at 510 nm) and emission (excitation at 368 nm) spectra of HPT. The emission spectra of HPT in solutions of oxyHb, metHb, and deoxyHb are also shown: conditions were 10 μM HPT, 30 μM Hb (on a heme basis), pH 6.8, 25°C.

unique absorption spectra. The observed intensities were corrected for the inner filter effect, before estimation of the fraction of the quaternary forms.

3.2. pH dependence

The overall peak emission versus pH is shown in Fig. 3. For these measurements with the capillary cuvette, the data were corrected for the inner filter effect using the reference curves versus Hb concentration; similar results were obtained by taking the ratio of the observed intensity (without other effectors) to that after addition of DPG or IHP. The overall difference between the oxy and deoxy was also similar for measurements with the larger cuvette (corrected by addition of a second effector), but in this case the sample to sample variation was larger, and the emission spectra were distorted since the inner filter effect is wavelength dependent. The data for deoxyHb show the least fluorescence (Fig. 3 and 4); the increase in the fluorescence above pH 7 corresponds to the decrease in affinity between HPT and deoxy Hb [9]. At the other extreme is oxyHb which corresponds to the liganded (or R-state) Hb conformation; the pH dependence again follows the change in affinity of oxyHb for HPT as previously determined [9]. HbCO and cyano-metHb show similar results to oxyHb. At pH 7, these three liganded forms (oxy, CO, and CN) showed little HPT binding; slight variations could be attributed to the inner filter effect. For the normal optical cuvette, the ratio of the emission spectra, with and without IHP, showed some wavelength dependence - apparently due to a small shift in absorbance induced by IHP; excitation at 330 nm provided the least distortion of the emission curves.

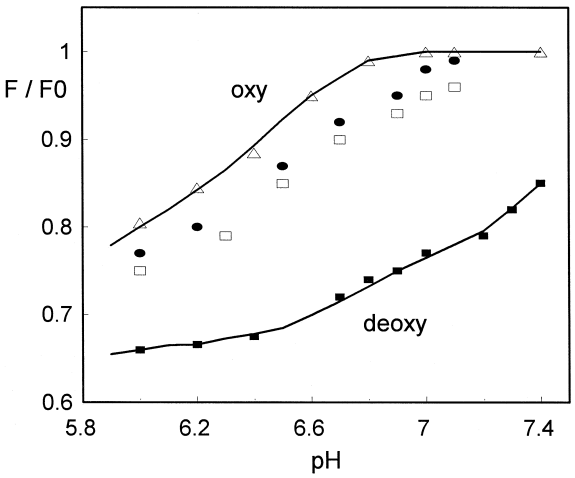

Fig. 3. Fluorescence intensity of HPT versus pH, for the various Hb solutions: (●) aquometHb, (□) fluoro-metHb. The intensity was corrected for the inner filter effect. Conditions were 10 μM HPT, 30 μM Hb (on a heme basis), 25°C, with Bis Tris (pH < 6.5) or Tris–HCl buffer.

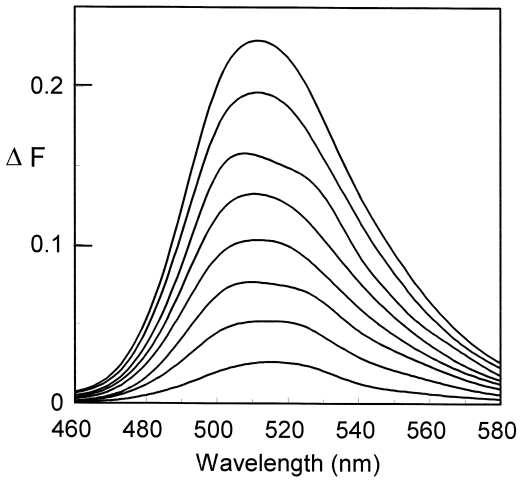

Fig. 4. Fluorescence emission spectra of HPT at varying concentrations of bezafibrate, a drug which binds to a site different from the DPG site. From top to bottom are 0 to 3.2 mM bezafibrate, in steps of 0.4 mM. The HPT concentration was 10 μM in solutions of 30 μM (in heme) metHb at pH 6.8. The decrease in fluorescence corresponds to a shift towards the Hb T-state conformation.

3.3. Spin transition in metHb

The high spin ligands H_2O and F show intermediate values for the fluorescence intensity. The aquometHb sample showed about 20% T-state behaviour at low pH, which disappears above pH 7, probably due to the transition to the low spin hydroxy metHb form. The fluoro-metHb sample showed about 25% T-state conformation at acid pH.

Since HPT is a weak effector of Hb; the allosteric equilibrium determined should, therefore, correspond to a value intermediate to those for Hb with and without DPG. The shift in allosteric equilibrium due to the presence of DPG or IHP cannot be determined by this method, since they compete for the same binding site with HPT.

3.4. Temperature dependence

Measurements were also made at 15°C, but the difference in HPT fluorescence between the oxy and deoxy forms was not increased. The measurements at lower temperature revealed additional experimental difficulties. First, as for most fluorophores, the emission intensity is temperature dependent, and a long equilibration time is necessary. Second, the pH is temperature dependent for Tris buffers; phosphate buffers are less temperature dependent, but diminish the effect of effectors like DPG. This pH effect is especially sensitive for excitation of the HPT band near 450 nm; the inner filter effect was lower in this region of excitation (see Fig. 1), but the results are less consistent due to the pH problems.

3.5. Double effector: HPT and bezafibrate

Since bezafibrate does not bind to the same site as DPG [11], its influence could be tested. The HPT fluorescence emission in metHb solutions decreased with increasing concentrations of bezafibrate (Fig. 4), suggesting a shift towards the T-state. However control experiments with HPT alone also showed a decrease in fluorescence. The actual shift in allosteric equilibrium is smaller, in agreement with ligand binding studies [12].

4. Discussion

Previous absorption studies indicated a transition in metHb upon addition of IHP [13,14]; however, it was difficult to determine the extent of the transition. For metHb there is no guarantee of having pure R or T reference states. In fact use of multiple effectors (IHP plus bezafibrate) showed larger absorption changes than for IHP alone, indicating that IHP alone did not induce a full conversion to the T-state [14].

Studies with partially oxidized samples helped determine the allosteric parameters for the tetrameric forms with 1 to 3 oxidized subunits, but an extrapolation is then required to estimate the properties of the fully oxidized forms [3]. Since each ligand accounts for approximately a 100-fold change in the allosteric equilibrium, the extrapolation can easily have an error of a factor of two. While the extrapolation estimated an IHP induced transition of 44% (from 28% to 72% T-state) for fluoro-metHb [3], Raman studies indicate a change of about 66% [6]. These values are probably in agreement within the precision of the two methods, both corresponding to a shift in allosteric equilibrium by over an order of magnitude.

The same Raman method applied to valency hybrids of Hb [7] indicated only a small change in allosteric equilibrium, while functional studies of CO binding still showed a large shift [15]. The CO binding studies showed that the rapid phase, characteristic of R-state tetramers, disappeared upon addition of IHP [15]; note that while IHP may induce new R' and T' states [16,3], the ligand binding properties for the two allosteric conformers are still easily separated, differing by over a factor of 50 for the rates for CO association and by a factor of 100 for the oxygen affinities.

Another technique that provides a probe of structural changes is the use of hydrogen exchange [17]. The kinetics for hydrogen exchange are sensitive to protein structure and can be used as a probe of the allosteric conformation. A partial shift towards the T-state induced by IHP was observed using this technique, as well as the synergistic effects of IHP with bezafibrate [17].

Evidence for a difference in liganded R-states was recently reported using the lifetime of the Hb bound HPT as probe [18]. These kinetic studies showed a short lifetime, indicating a weak fluorescence yield for the bound HPT. Their study also shows direct evidence that the effector binds not only to the deoxy form, but also to the oxy or CO forms of Hb.

In all cases, use of an effector as probe is a perturbing method. The observed fraction of T-state Hb is overestimated since the effector has a higher affinity for the deoxy conformation and shifts the allosteric equilibrium. There is thus a need for allosteric probes of Hb which show a good correlation with the functional studies. The technique presented here also has its limitations, and requires strict controls: slight variations in pH, temperature, or sample concentration all effect the observed fluorescence. However, once the best sample conditions and spectral wavelengths are found, a series of measurements gave reliable differences for the oxy and deoxy samples. The fluorescence intensities can easily be determined to within 1%, and the total difference between

oxy and deoxy is 30% (in the best case at pH 6.8). With a careful correction for the inner filter effects, this method provides a probe of the allosteric conformation of Hb.

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References

[1] E. Antonini, M. Brunori, Hemoglobin and Myoglobin in Their Reactions with Ligands, North-Holland Publ., Amsterdam, Holland, 1970
[2] R.G. Shulman, J.J. Hopfield, S. Ogawa, Q. Rev. Biophys. 8 (1975) 325–420.
[3] M.C. Marden, L. Kiger, J. Kister, B. Bohn, C. Poyart, Biophys. J. 60 (1991) 770–776.
[4] Q.H. Gibson, J. Physiol. 134 (1956) 123–134.
[5] C.A. Sawicki, Q.H. Gibson, J. Biol. Chem. 251 (1976) 1533–1542.
[6] V. Jayaraman, K.R. Rodgers, I. Mukerji, T.G. Spiro, Biochemistry 32 (1973) 4547–4551.
[7] I. Mukerji, T.G. Spiro, Biochemistry 33 (1994) 13132–13139.
[8] M.C. Marden, E.S. Hazard, C. Kimble, Q.H. Gibson, Eur. J. Biochem. 169 (1987) 611–615.
[9] R. MacQuarrie, Q.H. Gibson, J. Biol. Chem. 247 (1972) 5686–5694.
[10] M.C. Marden, E.S. Hazard, Q.H. Gibson, Biochemistry 25 (1986) 7591–7596.
[11] M.F. Perutz, C. Poyart, Lancet 15 (1983) 881–882.
[12] I. Lalezari, P. Lalezari, C. Poyart, M.C. Marden, J. Kister, B. Bohn, G. Fermi, M.F. Perutz, Biochemistry 29 (1990) 1515–1523.
[13] M.F. Perutz, J.K.M. Sanders, D.H. Chenery, R.W. Noble, R.R. Pennelly, L.W.M. Fung, C. Ho, I. Giannini, D. Pörschke, H. Winkler, Biochemistry 17 (1978) 3640–3652.
[14] R.W. Noble, A. DeYoung, S. Vitale, M. Cerdonio, E. DiIorio, Biochemistry 28 (1989) 5288–5292.
[15] R. Cassoly, Q.H. Gibson, J. Biol. Chem. 247 (1972) 7332–7341.
[16] R.D. Gray, Q.H. Gibson, J. Biol. Chem. 246 (1971) 7168–7174.
[17] R.E. McKinnie, J.J. Englander, S.W. Englander, Chem. Physics 158 (1991) 283–293.
[18] D.S. Gottfried, L.J. Juszczak, N.A. Fataliev, A.S. Acharya, R.E. Hirsch, J.M. Friedman, J. Biol. Chem. 272 (1997) 1571–1578.