Heterotropic Effectors Exert More Significant Strain on Monoligated than on Unligated Hemoglobin

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ABSTRACT The effect of allosteric effectors, such as inositol hexakisphosphate and/or bezafibrate, has been investigated on the unliganded human adult hemoglobin both spectroscopically (employing electronic absorption, circular dichroism, resonance Raman, and x-ray absorption near-edge spectroscopies) and functionally (following the kinetics of the first CO binding step up to a final 4% ligand saturation degree). All data indicate that the unliganded T-state is not perturbed by the interaction with either one or both effectors, suggesting that their functional influence is only exerted when a ligand molecule is bound to the heme. This is confirmed by the observation that CO dissociation from partially liganded hemoglobin ($\bar{Y} \leq 0.04$) is strongly altered by the presence of either effector, and the effect is enhanced whenever the two effectors are simultaneously present. Altogether, these data are a direct demonstration of the occurrence of a strain induced by the presence of a ligand molecule bound to the heme, and for the first time there is a clear indication that the expression of the functional heterotropic effect by these non-heme ligands requires this strain, which is not present in the unliganded molecule.

INTRODUCTION

In order to efficiently play its physiological role, human adult hemoglobin (Hb) captures as much oxygen as possible in the lungs, where pO_2 is relatively high, releasing it to oxygen-depleted tissues. This function depends crucially on the cooperative transition between (at least) two quaternary conformations in rapid equilibrium, one being characterized by a low O_2 affinity and prevailing in the unliganded molecule (i.e., T state), and the other one displaying a high ligand affinity and prevailing in the fully oxygenated tetramer (i.e., R state) (Monod et al., 1965). Since the O_2 affinity of the R state tetramer is similar to that of isolated subunits (Mills et al., 1976), cooperative ligand binding is expected to arise from structural restraints that stabilize the deoxygenated T state, reducing its O_2 affinity. Ligand binding to a heme in a T state tetramer brings about tertiary conformational change(s), affecting the heme and its surrounding (Paoli et al., 1996), which decrease(s) the structural restraints, thus reducing the interaction free energy required for the conformational transition toward the R state (Perrella et al., 1992). This mechanism underlies the existence of a hierarchy of tertiary and quaternary conformations, expressed as "nesting" by Wyman (1984) and as "allosteric core" by Karplus and co-workers (Gelin et al., 1983). Furthermore, this hierarchy has been observed by resonance Raman spectroscopy to affect the Fe-imidazole stretching frequency (Scott and Friedman, 1984), which may then represent an important spectroscopic marker to detect tertiary and quaternary structural alterations at the level of the heme pocket.

Heterotropic effectors, such as inositol hexakisphosphate (IHP) and bezafibrate (BZF), are known to affect the quaternary conformational equilibrium toward the T state, and they have been shown to act synergistically (Benesch et al., 1968; Perutz and Poyart, 1983). Therefore, it seemed important to provide a detailed description of the mechanism by which these two non-heme ligands influence (either separately or together) the functional and structural properties of the unliganded and of monoliganded Hb, which is known to remain in a T liganded state (Liao et al., 1993). These data are compared with a previous investigation on the T state-bound HbCO, as obtained by the simultaneous presence of IHP and BZF (Marden et al., 1988), outlining that results obtained are in agreement with the pattern of structural changes described on the basis of crystallographic data (Paoli et al., 1996).

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MATERIALS AND METHODS

Human hemoglobin has been prepared from freshly drawn blood, as previously reported (Antonini and Brunori, 1971). Organic polyphosphates and other low-molecular-mass contaminants were removed as reported for "stripping" of hemoglobin (Riggs, 1981). Concentrations of oxyHb solution were determined spectrophotometrically employing an extinction coefficient $\epsilon = 13.8\ mM^{-1}\ cm^{-1}$ (per heme) at 541 nm.

Resonance Raman spectra have been obtained at room temperature with CW excitation from an Ar^+ laser (Coherent, Innova 90/5). The backscattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG2S/2000) equipped with a cooled photomultiplier (RCA C31034A) and photon-counting electronics. The spectra were calibrated in frequency with indene and CCl_4 as standards to an accuracy of ± 1 cm^{-1} for the intense, isolated bands.

The Fe K-edge x-ray absorption near-edge (XANES) spectra were collected in the fluorescence mode at the LURE DCI synchrotron radiation facility (Orsay, France), operating at 1.8 GeV energy and a current of 270 mA. A Si(111) channel-cut single crystal was used as a monochromator. The energy resolution at the Fe K-edge is ~2 eV, and energy shifts of resolved absorption peaks of 0.5 eV can be detected (Pin et al., 1994). Harmonic contamination was rejected by using a total reflection mirror after the monochromator. Each spectrum represents a total signal averaging of 4 s/point, collected at room temperature by using a 7-element energy resolving Ge array detector from Canberra industries. The samples were mounted in a 1-mm-thick Teflon cell with Mylar windows, oriented with an angle of 45° with respect to the x-ray beam, with the detector positioned perpendicular to the beam direction. The specimen concentration was 8 mM heme in the absence and presence of allosteric effectors, and no protein damage has been detected after x-ray exposure. To keep the samples in the deoxygenated form during the collection of data, preliminary experiments were carried out by adding various amounts of sodium dithionite (between 4 mg/ml and 16 mg/ml) to six different Hb solutions (8 mM in heme). Spectra were collected on samples containing 5 mg/ml sodium dithionite in view of the evidence that over the concentration range observed the ratio of peaks C/D in XANES spectra was constant (see Fig. 1 *B*). Circular dichroism spectra were obtained on a Jasco-500 A spectropolarimeter equipped with a Jasco DP-500 processor. The molar ellipticity θ_M is expressed as deg · cm^2 · $dmol^{-1}$.

Kinetic experiments have been carried out on a Gibson-Durrum rapid-mixing instrument interfaced with a fast data collection system (On Line Instrument Service, Jefferson, GA). Experiments have been undertaken mixing deoxyHb (at various heme concentrations ranging between 0.05 and 0.4 mM after mixing), in the absence and presence of varying amounts of effectors, with a buffer solution where different amounts of a CO-saturated buffer were dissolved (to give after mixing a final heme/CO ratio of 25:1). This buffer solution contained the same amount of effectors and 20 mg/ml sodium dithionite. Such an experiment, followed at $\lambda = 569$ nm, allows determination of the pseudo-first-order rate (as a function of heme) for CO binding to deoxy Hb. The same procedure is used to obtain a hemoglobin solution that is 4% saturated with CO to follow the CO dissociation from a monoliganded molecule. The CO dissociation kinetics has been carried out mixing the partially CO saturated Hb solution with an equimolar solution of microperoxidase, and following the kinetics of CO-microperoxidase formation at $\lambda = 550$ nm (Sharma et al., 1976).

RESULTS AND DISCUSSION

It has been reported by several groups that IHP and BZF together exert a synergistic functional effect on ligand binding properties of human hemoglobin, which largely exceeds the individual one from either IHP and BZF alone (Perutz and Poyart, 1983; Perutz et al., 1986). This has been shown to be particularly evident in the case of CO binding to human Hb, such that the simultaneous addition of IHP and BZF appears to abolish any cooperative kinetic behavior for the CO association (Marden et al., 1988), leading to the conclusion that IHP and BZF together bring about a dramatic structural change of the whole molecule toward the T quaternary state. However, beside the evidence of the disappearance of any cooperativity in the CO binding kinetics, it is not clear from these data how this synergistic effect is exerted and which parameters are mostly affected.

We have thus carried out a set of experiments trying to characterize from the spectroscopic and kinetic standpoint the structural and functional effects related to the presence of IHP and BZF either alone or together. The first step has been dealing with the unliganded T state, employing several spectroscopic techniques, such as electronic absorption, circular dichroism, resonance Raman, and XANES. Fig. 1 reports the effect of the addition of IHP and/or BZF to deoxyHb at pH 7.0 on resonance Raman (*panel A*) and on

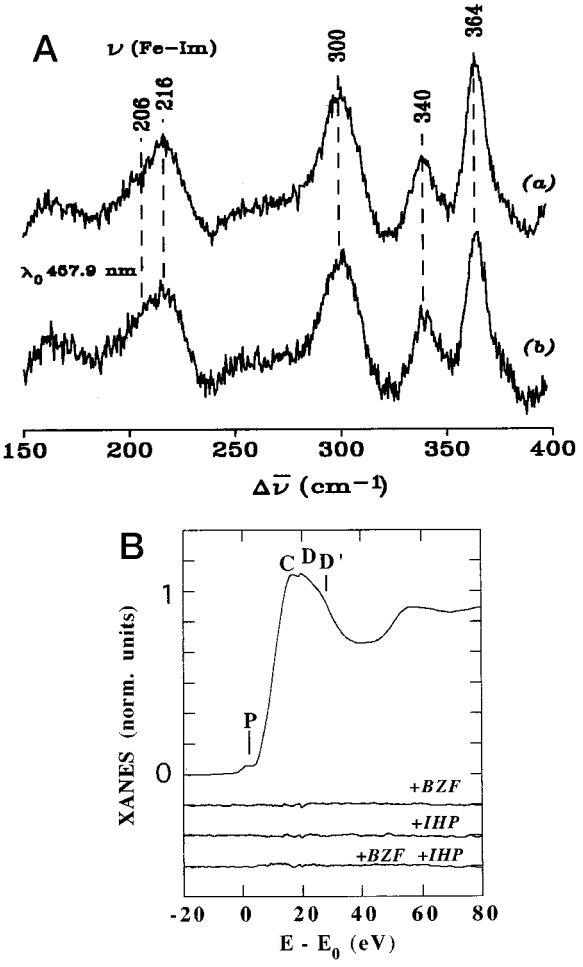

FIGURE 1 (*A*) Resonance Raman spectra of 0.2 mM heme human deoxyHb at 20°C in 100 mM Hepes buffer pH 7, in the absence (*spectrum a*) and in the presence of 1 mM IHP and 10 mM BZF (*spectrum b*). Experimental conditions: 457.9 nm excitation, 5 cm^{-1} resolution, 25 mW laser power at the sample, 4 s/0.5 cm^{-1} (*spectrum a*) or 12 s/0.5 cm^{-1} (*spectrum b*) integration time. A baseline was subtracted from both spectra. (*B*) XANES spectrum of human deoxyHb (8 mM in heme), at 20°C in 0.1 M Hepes pH 7.0. The main experimental features (P, C, D, D_1) due to multiple scattering of the photoselection at the absorbing atom are also reported (for their physical meaning, see Pin et al., 1994). Differential XANES spectra between deoxy Hb in 0.1 M Hepes pH 7.0 and deoxy Hb in 20 mM BZF (+*BZF*), in 20 mM IHP (+*IHP*) and 20 mM IHP + 20 mM BZF (+*BZF* +*IHP*) are also displayed, showing that no relevant spectral changes are detected upon addition of allosteric effectors. For further details, see text.

XANES (*panel B*) spectra. It is immediately obvious that in the case of unliganded Hb in the T state, no effect whatsoever is observed with these two spectroscopic approaches upon addition of the two effectors either alone or together (see Fig. 1), and the same is true for absorption and circular dichroism (data not shown). In particular, for resonance Raman spectra in the low-frequency region, the Fe-imidazole stretching frequency displays, as already reported by others (Nagai and Kitagawa, 1980; Scott and Friedman, 1984), a splitting which has been attributed to the two types of subunits (i.e., α- and β-chains). In Fig. 1 *A* it appears evident that the two stretching frequencies remain unperturbed even after the simultaneous addition of the two effectors, clearly indicating that the structural arrangement of the heme pocket in the two subunits is not influenced by these two effectors in the unliganded state. This appears in line also with very recent observations (Peterson and Friedman, 1998), which have also shown that there is a close correlation between this stretching frequency and any structural alteration at the level of heme pocket and/or subunit interface. Altogether, spectroscopic data suggest that in the unliganded T state, IHP and/or BZF do not bring about significant structural alterations of the tertiary structure neither around the heme (as from electronic absorption, XANES, circular dichroism in the Soret region, and resonance Raman spectroscopy), nor in other regions carrying UV chromophores associated with quaternary transition(s), such as Trp37β located at the $\alpha_1\beta_2$ interface (as from circular dichroism spectra in the near-UV region, data not shown). Therefore, unlike the binding of 8-hydroxy-1,3,6-pyrenetrisulfonate (Marden et al., 1986), the association of IHP and/or BZF with deoxyHb does not seem to bring about a different conformation of the unliganded T state. Closely similar results have been obtained for dromedary Hb (data not shown), suggesting that this statement is likely valid for all Hb's. This evidence is in line with crystallographic results. In fact, 1) no significant effect is observed on the conformation of β subunits upon addition of polyphosphates (Richard et al., 1993), only small distortions being located at the side chains of two residues of the binding pocket between the two β subunits; 2) the structure of deoxyHb is the same with and without BZF (Lalezari et al., 1990), except for small shifts of atoms of the protein moiety surrounding the effector binding cleft; and 3) the very limited x-ray intensity differences observed upon addition of polyphosphates are consistent with small conformational changes associated to the replacement of well-ordered water molecules by the effector (Richard et al., 1993), suggesting that the strain exerted by a non-heme ligand on the deoxyHb does not spread far from their binding site.

Further support for this conclusion based on all spectroscopic results also comes from functional studies on the kinetics of CO binding to deoxyHb. Thus, the pseudo-first-order rate constant for the first binding step, concerning the process of CO ligation to a final 4% of hemes, does not display any significant variation upon the addition of IHP and BZF either alone or together (Fig. 2 *A*). A correlation between CO binding kinetics and alterations of Fe-imidazole stretching frequency in resonance Raman spectroscopy has been also shown in the case of site-directed mutants of Trpβ37 (Peterson and Friedman, 1998), and it appears confirmed also in our case. Therefore, in agreement with spectroscopic results, we can conclude that the interaction energy, retained in the protein when one or both non-heme ligands (i.e., IHP and BZF) are bound to deoxyHb, produces strain(s) in the protein that do(es) not propagate to the heme stereochemistry and to regions with UV chromophores.

However, quantitative analysis of O_2 binding isotherms in the absence and presence of IHP and BZF either alone or together shows that the first binding step (i.e., β_1) is indeed affected by these effectors (Gill et al., 1987, 1989). Although the mechanism of hemoglobin cooperativity is certainly more subtle than originally thought, there is a general agreement that values of β_1 obtained by fitting procedures indeed describe the equilibrium between unligated and monoligated hemoglobin. Therefore, variations observed for the value of β_1 strongly suggest that the monoliganded species may undergo an effector-linked structural alteration, and that it occurs only in subunits where the heme is occupied by a ligand. This possibility has been investigated carrying out kinetics of CO dissociation from a partially liganded molecule, such that only 4% of the hemes were CO-bound. It must be stated that, even though populations of intermediates may significantly depend on which heme ligand (i.e., O_2 or CO) is bound (Gill et al., 1987), the physical meaning of β_1 and its quantitative trend as a function of allosteric effectors is unequivocal and independent on the ligand. The rate of displacement of CO by microperoxidase turns out to be markedly influenced by the presence of the two effectors either alone or together (Fig. 2 *B*), allowing one to assess that the alteration of the equilibrium constant for the first ligand binding step is completely referrable to an effector-induced influence on the ligand dissociation rate constant. This behavior, which underlies the occurrence of a physical strain on the heme only when a ligand is bound, finds convincing support by crystallographic evidence. Thus, in contrast with what is observed for the fully liganded T state Hb stabilized by IHP and BZF together (Marden et al., 1988), in the partially ligated T state Hb atomic movements are restricted to tertiary changes involving the heme stereochemistry and the close proximity of the allosteric effector interaction site (Abraham et al., 1992). This suggests the occurrence of an energy pathway that directly transmits structural alterations between the heme-ligand bond and the heterotropic binding site such that interaction of effector(s) brings about a destabilization of monoligated species via a tertiary structural change of the liganded subunit. This mechanism is in agreement with a model proposed by Karplus and co-workers (Gelin et al., 1983), which suggests that the most meaningful strain in hemoglobin has to be sought not in the fully unligated species, but in the partially liganded T structure, underlying the existence of a hierarchy of tertiary arrangements within a single quaternary T state. In other words, the

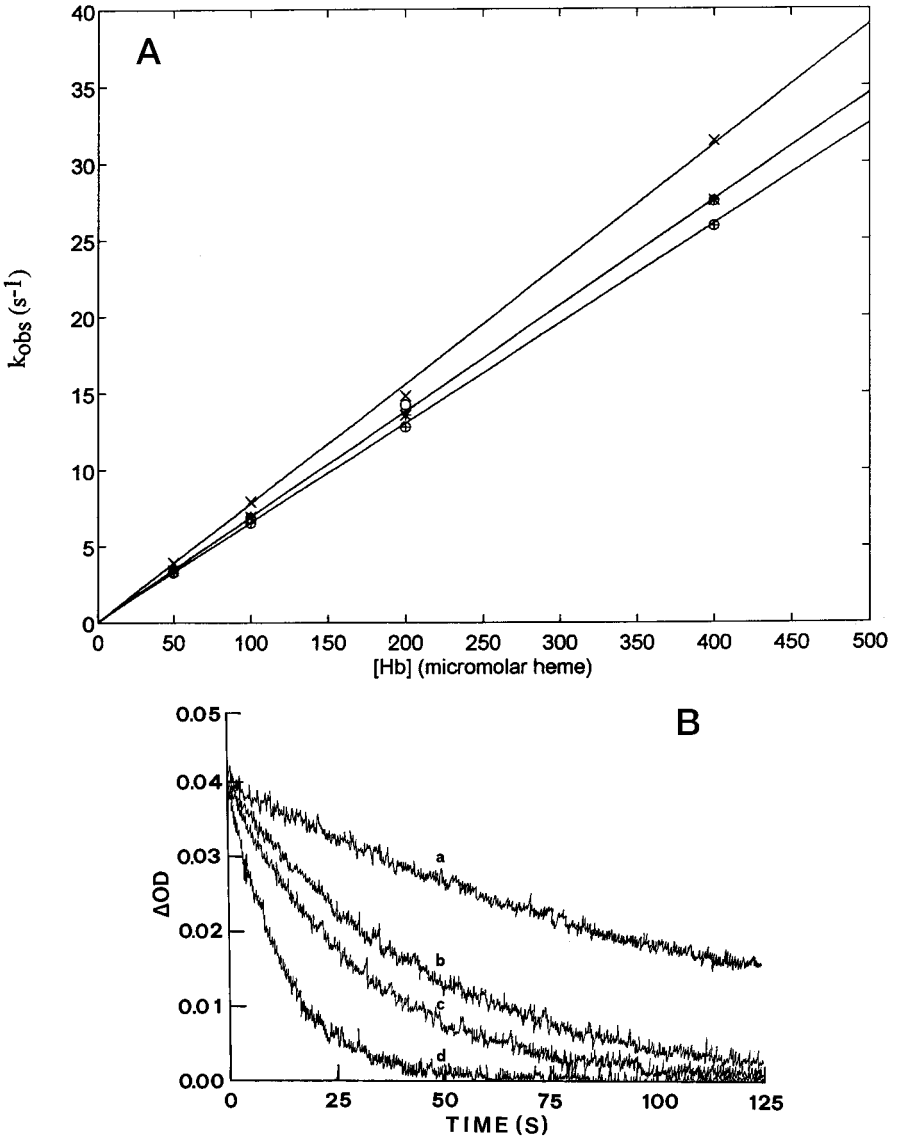

FIGURE 2 (*A*) Observed rate constants for CO binding to human Hb in the absence (×), in the presence of 10 mM IHP (○), 30 mM BZF (*), and 1 mM IHP and 10 mM BZF (⊕), at 20°C, in 0.1 M Hepes pH 7.0. The observations are carried out at different heme concentrations mixing with substoichiometric amounts of CO, such that the final saturation degree = $\bar{Y} \leq 0.04$. The continuous lines are obtained employing the following equation:

$$k_{obs}(s^{-1}) = k_{on} \cdot [\text{Heme}] + k_{off} \quad (1)$$

where $k_{on} = 7.8\ (\pm 1.4) \times 10^4\ M^{-1}\ s^{-1}$ and $k_{off} = 7.0\ (\pm 1.5) \times 10^{-3}\ s^{-1}$ in the absence of effectors, $k_{on} = 6.9\ (\pm 1.2) \times 10^4\ M^{-1}\ s^{-1}$ and $k_{off} = 1.5\ (\pm 0.3) \times 10^{-2}\ s^{-1}$ in the presence of 10 mM IHP, $k_{on} = 6.9\ (\pm 1.3) \times 10^4\ M^{-1}\ s^{-1}$ and $k_{off} = 2.0\ (\pm 0.5) \times 10^{-2}\ s^{-1}$ in the presence of 30 mM BZF, $k_{on} = 6.5\ (\pm 1.2) \times 10^4\ M^{-1}\ s^{-1}$ and $k_{off} = 6.0\ (\pm 1.5) \times 10^{-2}\ s^{-1}$ in the presence of 1 mM IHP and 10 mM BZF. Values of k_{off} are obtained from CO dissociation curves reported in (*B*). For further details, see text. (*B*) Kinetic progress curves from partially saturated HbCO ($\bar{Y} \leq 0.04$) in the absence of effectors (*curve a*), in the presence of 10 mM IHP (*curve b*), in the presence of 30 mM BZF (*curve c*), and in the presence of 1 mM IHP and 10 mM BZF (*curve d*), at 20°C in 0.1 M Hepes pH 7.0. The values of dissociation rate constants are reported in (*A*). For further details, see text.

presence of allosteric effectors strengthens the normal strain present in the T state, impairing the heme group and/or the protein moiety to adopt the liganded geometry. This outcome supports the idea that most of the free energy of cooperativity is not localized at the heme(s), but resides in several specific bonds throughout the protein, which are then reinforced by the association of IHP and BZF with Hb. Such a result is consistent with the observation of cooperative free energy for distal ligand binding within the T state (Ackers et al., 1992).

In conclusion, reported observations provide a clear evidence for 1) the existence of only one quaternary T state in the absence and presence of saturating amounts of IHP and/or BZF; and 2) the existence of more meaningful tensions in the monoligated T state Hb, with respect to the unliganded form, whenever heterotropic effectors are present. These features may account for the reduced ligand binding affinity of T-state Hb in the presence of IHP and/or BZF (as resulting from the variations observed for β_1), confirming that knowledge of thermodynamic and kinetic properties of monoligated species is essential for a better comprehension of the cooperative mechanism and of interplay between heme and non-heme ligands on the modulation of functional properties in human hemoglobin.

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