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Presence of a class of chromophores as monitor of oxygen-linked conformational changes in hemocyanins

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Summary. Oxygenation of native hemocyanins β from *Helix pomatia* and *Panulirus interruptus* under conditions of cooperative binding, causes a change in the dynamic behaviour of the internal structure, leading to increased rotational mobility of a class of tryptophan residues emitting above 450 nm. This is associated with the complete depolarization of the emission on a time scale where the large hemocyanin is practically immobile. This class is thought to be very near the active site since it is strongly affected by the copper atoms. Moreover, fluorescence changes of the class of chromophores emitting above 450 nm is more marked in the molluscan *Helix* hemocyanin than in the arthropodan *Panulirus* hemocyanin, suggesting a possible difference in the structure of the active site or in the extent of the allosteric transition between the two species. This class of chromophores may by useful probes to monitor ligand-linked conformational change in hemocyanins.

Key words: Hemocyanin – *Helix pomatia* – Fluorescence – Conformational transition

Introduction

Hemocyanins are well characterized copper proteins having a bimetallic center at the active site, where O_2 and CO are bound reversibly. While CO binds to only one copper atom and the equilibrium isotherm is non-cooperative (Brunori et al. 1981), O_2 binds to both copper atoms as a bridge. In the latter case, binding is cooperative and the metals which are in the lower valence state, Cu(I), in the deoxy derivative become oxidized to Cu(II) in the oxygenated form (Loehr et al. 1974).

As in the case of hemoglobin, the classic two-state allosteric model (Monod et al. 1965; Perutz 1970), based on the assumption that a protein may exist in (at least) two functionally different quaternary states in chemical equilibrium, is often adequate to describe cooperative effects in the binding of O_2 to hemocyanins (van Hold and Miller 1982). Nevertheless, although two quaternary states of the protein are assumed to be present all the time, direct physical evidence supporting the presence of different conformations in equilibrium is limited. Spectroscopic studies have shown that, upon binding of O_2 at the active site of hemocyanin, the Cu–Cu distance decreases and this triggers the quaternary switch coupled to cooperative O_2 binding (Woolery et al. 1984). Generally, the optical properties of proteins in the near ultraviolet region reflect the environment of aromatic amino acid side chains, and thus provide a convenient intrinsic probe to detect local conformational changes. In *Limulus polyphemus* and *Panulirus interruptus* hemocyanins, in fact, it was found that the absorbance change around 290 nm is proportional to the degree of oxygenation (Makino 1986). Moreover in *Limulus* it was found that the polarization of the tryptophan fluorescence depends on the oxygenation state of the protein, being lower in the deoxygenated derivative (Ma et al. 1989).

In order to acquire direct experimental evidence on the ligand-linked conformational transition in hemocyanin we have applied autocorrelation spectroscopy to probe the internal mobility of tryptophans in the oxy and deoxy β-hemocyanins of *Helix pomatia* and *Panulirus interruptus*. The results show that in both hemocyanins one or more chromophores emitting above 450 nm increase significantly their rotational mobility when the protein binds oxygen, indicating that this probe could be very useful to monitor the ligand-linked conformational change in hemocyanins.

Materials and methods

Helix pomatia and *Panulirus interruptus* hemocyanins were isolated, stored and regenerated as previously described (Zolla et al. 1978; Kuiper et al. 1975). The apo form of β-hemocyanin from *H. pomatia* was prepared by removing copper from the protein by treatment with KCN (Zolla et al. 1984). For the excitation of tryptophan fluorescence a mode-locked and cavity-dumped dye laser

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(rhodamine 6G) was used with a frequency doubled by a KDP crystal ($\lambda_{exc}=300$ nm). The emitted fluorescence was observed through a cut-off filter and measured by time-correlated single-photon counting. In the present instrument (Rigler et al. 1984) an overall response time of 40 ps towards a laser pulse of 5 ps was achieved. Fluorescence intensities polarized parallel to the excitation polarization $I_{\parallel}(T)$ and at the magic angle $I_M(T)$ were measured together with the laser pulse in a double-beam spectrometer; they were collected in 2000 channels each.

Results

Fig. 1 reports the room-temperature emission spectra of native and apo-B-hemocyanin from *H. pomatia* recorded upon exitation at 300 nm in the presence and in the absence of oxygen. It may be observed that the emission spectrum of native oxygenated hemocyanin shows a reduced peack at 330 nm when compared to the deoxy form and a broad tail over 500 nm (Fig. 1A). In contrast, the emission spectra of apo-hemocyanin in the absence or presence of oxygen display only a small difference at 330 nm (Fg. 1B). Thus, while the latter difference at 330 nm may be attributed to the well known quenching of oxygen (Ma et al. 1977), the extended tail of emission observed in oxy-hemocyanin at long wavelengths seems to be characteristically related to O_2 binding.

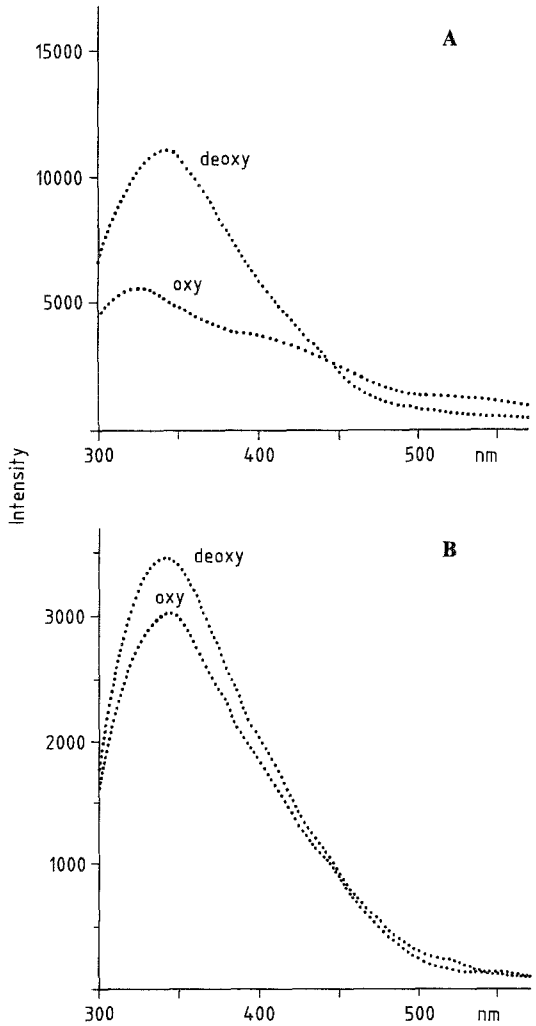

Fig. 1 A, B. Emission spectra of native (**A**) and apo (**B**) *Helix pomatia* β-hemocyanin in the presence and in the absence of oxygen upon excitation at 300 nm

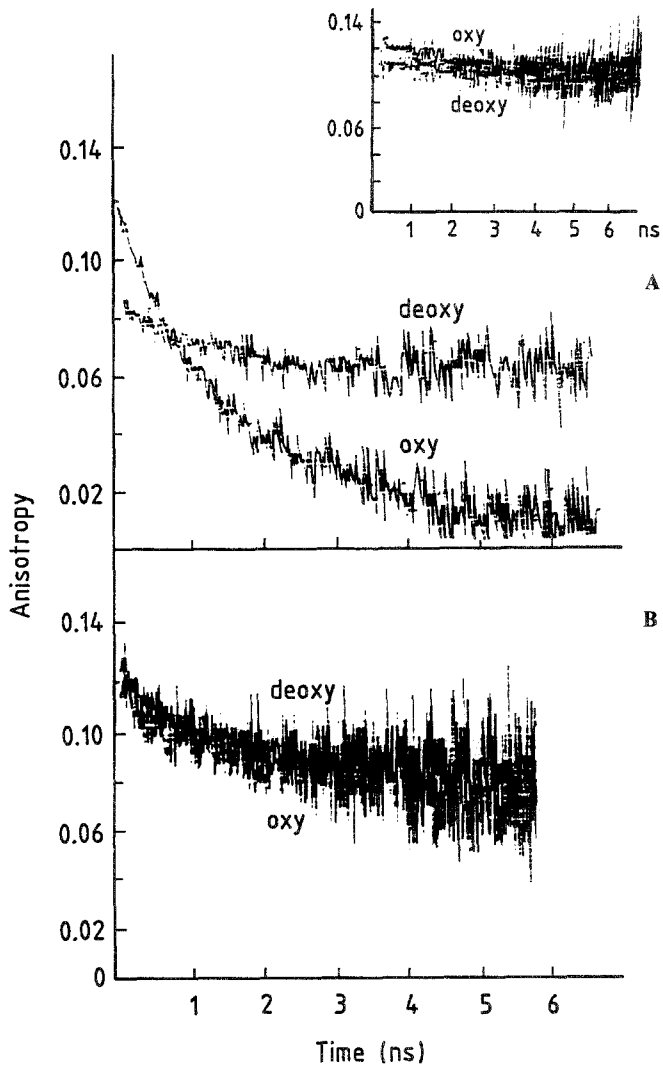

Fig. 2 A, B. Anisotropy decay of native (**A**) and apo (**B**) *Helix pomatia* hemocyanin recorded above 450 nm. Experimental conditions: 20° C, excitation at 300 nm, cut-off filter Schott KV418. The *inset* reports the anisotropy decay of native hemocyanin recorded above 400 nm (cut-off filter Schott KV370)

In order to determine whether the characteristic difference in emission is related to the binding of O_2 to the binuclear center or to the ligand-linked conformational change, we have measured the anisotropy decay of tryptophans to evalute their rotational motion and thus obtain information on the internal dynamics of the protein.

The anisotropy decay of native (Fig. 2A) and apo (Fig. 2B) hemocyanin were recorded in the presence and in the absence of O_2 on a nanosecond time scale; the decay of emission was recorded below and above 450 nm. It may be observed that the anisotropy decay of oxy and deoxy hemocyanin recorded in the range 335–400 nm (inset of Fig. 2A) takes place with same

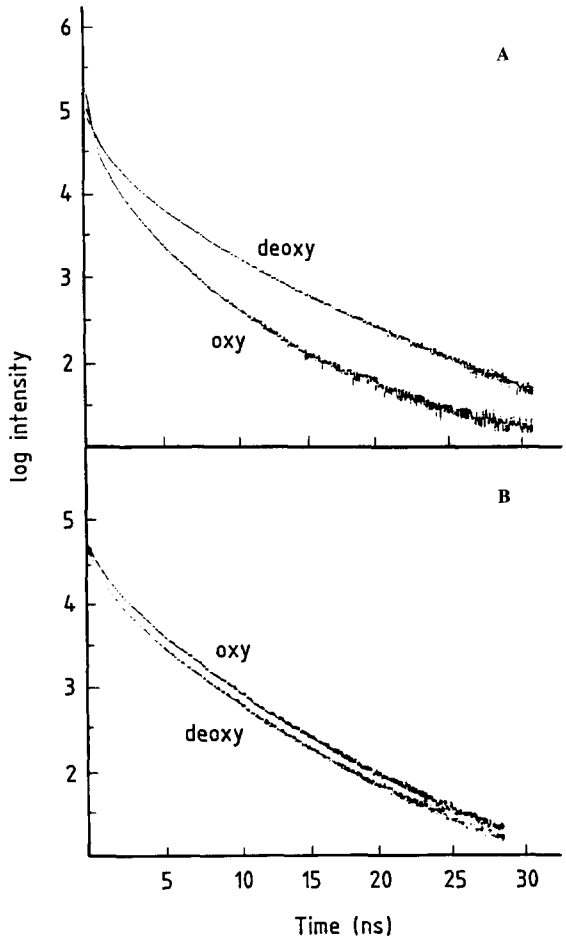

Fig. 3A, B. Fluorescence decay of native (**A**) and apo (**B**) *Helix pomatia* β-hemocyanin recorded above 450 nm. Experimental conditions: 20° C, excitation at 300 nm, cut-off filter Schott KV418

time course ($\tau = 100$ ns), while the anisotropy decays recorded above 450 nm are significantly different, being faster by a factor 50 in oxy-hemocyanin. In contrast, in apo-hemocyanin (Fig. 2B), where no binding of oxygen occurs, the time course of the anisotropy decay above 450 nm, in the presence of absence of O_2, is essentially the same. This confirms that the difference observed upon oxygenation of native hemocyanin is indeed a consequence of O_2 binding to the active site. Moreover, in both native and apo-hemocyanin the initial anisotropy (τ_O) is about 0.12 while the initial anisotropy of proteins has been estimated to be 0.2–0.3. This suggests that only a fraction of the emitting chromophores is recorded under our experimental conditions.

Concomitantly with the anisotropy decay measurements, we have recorded the life-time decay (above and below 450 nm) of native and apo-hemocyanin in the absence and presence of O_2. In apo-hemocyanin (Fig. 3B), the life-time profiles of the tryptophans are largely independent of O_2, while a significant difference is observed in native oxy and deoxy hemocyanin (Fig. 3A). Moreover evaluation of the time-dependent fluorescence intensity, by non-linear least-square parameter fitting and appropriate deconvolution routines, yields a spectrum of decay times which is represented by at least three different decay processes (Table 1). The direct comparison shows that the life times of native hemocyanin are much shorter than those of their apo counterparts. Moreover the presence of oxygen has a very small effect on the life time of the apo form, while in the case of native hemocyanin, the presence of oxygen (which is known to quench the stationary emission) shortens significantly all the decay times and biases the relative populations toward shorter times.

As a final investigation we have carried out anisotropy and life-time decay measurements in *P. interruptus* hemocyanin, both on the hexameric form and on the purified subunit a. Upon exitation at 295 nm, the emission spectra shows a tail above 450 nm only in oxyhemocyanin. Moreover, the anisotropy decays, as well as the life times, are faster in the oxygenated derivative compared to the deoxy form, although the difference is less pronounced than that observed in *H. pomatia* hemocyanin. In contrast, the anisotropy and life-time decays of purified subunit a (which binds O_2 non-cooperatively) are similar in the absence and in the presence of O_2.

Discussion

The results reports above show unequivocally that oxygenation of native hemocyanins β of *H. pomatia* and *P. interruptus* under conditions of cooperative binding causes a change in the dynamic behaviour of the internal structure, leading to increased rotational mobility of a class of tryptophan residues emitting above 450 nm. This is associated with complete depolarization

Table 1. Fluorescence decay times (τ) and anisotropy decay times (τ_R) for β-hemocyanin and apo-β-hemocyanin at 20° C in presence and absence of oxygen

	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	A_1	A_2	A_3 (in relative %)	τ_R (ns)
β-hemocyanin							
Oxy	0.312±0.002	2.44±0.02	5.71±0.04	73.2	21.4	5.4	2.0±0.1
Deoxy	0.99 ±0.04	3.5 ±0.2	9.9 ±1.1	59.1	38.2	2.7	102±16
Apo-β-hemocyanin							
Oxy	1.01 ±0.03	3.65±0.09	8.5 ±0.2	37.0	49.3	13.7	35±2
Deoxy	0.70 ±0.02	3.17±0.05	8.08±0.09	36.1	48.0	15.9	33±2

of the emission on a time scale where the large hemocyanin is practically immobile. Concomitantly the life time spectrum is shifted to shorter times, as a consequence of dynamic quenching caused by increased rotational mobility. These conformational changes we believe to be unequivocal physical evidence for the ligand-linked allosteric transition of cooperative hemocyanin. It is interesting that some of the internal tryptophans of the deoxygenated derivative of hemocyanin display a reduced mobility. Studies of oxygen distribution and migration within hemoglobin have revealed that O_2 diffusion is reduced in deoxyhemoglobin (Jameson et al. 1984), correlating with the more compact structure of this state of the protein.

In contrast to that reported above, measurements of the translational diffusion coefficient in the absence and in the presence of oxygen yield the same value, indicating that the oxygen-linked conformational change(s) are not associated with large pertubations in the molecular assembly (Zolla et al. 1986). In turn, this suggests that the class of chromophores which increases significantly its mobility upon oxygen binding probably involves a limited domain within the protein. In native hemocyanin the life times of the tryptophans emitting above 450 nm are much shorter than those of their apo counterparts, indicating the effect of the copper atoms in speeding-up the decay. Similar results have been observed in comparing holo and apo azurin, a copper protein containing one atom metal/molecule (Petrich et al. 1987). Three different classes of tryptophan have been observed in *Octopus* hemocyanin by steady-state and time-resolved fluorescence spectroscopy (Ricchelli et al. 1987). One class is supposed to be very near the active site and is strongly affected by copper atoms, being totally quenched when the metals are in the higher oxidation state Cu(II), as it is in oxy, met and half-apo derivatives. The effect is not due to energy transfer to a Cu-O_2 complex, but to the paramagnetic properties of the metal cluster. It is important to note that the change of copper from Cu(I) in deoxy to Cu(II) in the oxygenated state (Loehr et al. 1974), causes a change in the radius of the copper ions, probably a change in Cu–Cu distance in the preferred mode of coordination and in the electric field generated (Reed 1985). Further support for the possible role played by the copper is the observation that in both hemocyanins investigated here, the chromophore(s) affected by the conformational transition displays a phosphorescent luminescence (emitting above 450 nm) in a time range of nanoseconds. Generally proteins display phosphorescence when tryptophans are contained in a β-laminar secondary structure, but the life time is in the time range of milliseconds to seconds (Strambini and Gabellieri 1984).

At present, the three-dimensional structure of oxyhemocyanin is not known, since the crystallographic studies on *Limulus* II and *P. interruptus* hemocyanins were not extended to the oxygenated derivative (Nakaschima et al. 1986; Gaykema et al. 1985). Consequently we cannot yet locate the chromophore(s) probing the allosteric quaternary change and any further structural interpretation of the data would be a matter of speculation. Moreover, we cannot establish whether this class of chromophore(s) is the same in both phyla. In the case of *P. interruptus* hemocyanin, the crystallographic data show that a few tyrosines are close (≈ 1.7 nm) to the copper pair and, upon oxygenation, one of these moves closer to the metal atoms (Gaykema et al. 1985). On the other hand, the marked fluorescence changes observed in the molluscan *Helix* hemocyanin compared to the arthropodan *Panulirus* hemocyanin suggests a possible difference between the two phyla. Similarly, the weak luminescence observed at low energy (attributed to emission from copper-to-imidazole charge-transfer state), was found to be different between molluscan and arthropodan hemocyanin, being more quenched in molluscan hemocyanin (Sorrel et al. 1988). Both these pieces of evidence suggest a possible difference in the structure of the active site or in the entity of the allosteric transition between the two species. The latter hypothesis agrees with the evidence that the allosteric conformational change observed in *Panulirus* upon oxygenation is limited, the protein being oxygenated with some dimers in the oxy orientations and with others in the deoxy orientation (Gaykema et al. 1985), and that oxygen binding by arthropod hemocyanin cannot be described by a two-state model (Rikey et al. 1985).

As a conclusion, in spite of difficulties in correlating spectroscopy to structure, these data reveal that one class of chromophores is a useful probe to monitor ligand-linked conformational changes in hemocyanins.

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