Circular dichroism spectroscopy of *Lucina I* hemoglobin

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Abstract The monomeric hemoglobin from the mollusc *Lucina pectinata* (HbI) represents an interesting model system for the study of heme-related circular dichroic (CD) bands in view of the highly asymmetric distribution of aromatic residues around the heme pocket revealed by the X-ray crystal structure. The CD spectra of both ferrous and ferric HbI derivatives exhibit negative CD bands in the Soret and ultraviolet region with an enhanced ellipticity of the heme N and L bands in the near-UV region. In contrast, the magnitude of the Cotton effect in the visible and Soret regions is comparable to that observed in other hemoproteins. The spectrum of the carbon monoxide derivative shows a surprising similarity with that observed for the soybean leghemoglobin carbon monoxide adduct. A common structural feature in the two proteins is the presence in the distal pocket of two Phe residues (B9 and B10) the aromatic rings of which are perpendicular to the heme plane.

Key words: Hemoglobin; Circular dichroism; Heme Cotton effect; Heme chirality

1. Introduction

The optical activity of hemoproteins has received special attention in view of the magnitude of the Cotton effect displayed in the heme absorption regions. Heme in solution is devoid of chirality but gives rise to a differential absorption of circularly polarized light when inserted in the globin moiety. However, the physical basis at the origin of the heme Cotton effect hemoproteins is still debated. The first model, proposed by Hsu and Woody [1] on the basis of the theory of Kirkwood [2] and Tinoco [3], provided a semiquantitative agreement with the rotational strength found experimentally in the visible and Soret regions. According to this model, the induced heme optical activity is determined mainly by a coupled oscillator interaction between the allowed electronic transition moments of the porphyrin ring (π–π^* transitions) and those of nearby aromatic residues (π–π^* transitions). More recently, the importance of other contributions has been recognized on the basis of the considerable optical activity displayed by the heme undecapeptide of cytochrome *c*, which is devoid of aromatic residues other than the proximal histidine [4]. Thus the inherent chirality in the heme has been postulated as arising in part from the protein-induced deformation of the porphyrin ring and in part by the contribution of polarizable groups in the vicinity of the heme.

In the present paper we report a circular dichroism (CD) investigation on the monomeric hemoglobin from the mollusc *Lucina pectinata* (HbI). *Lucina* HbI is characterized by a high affinity for hydrogen sulfide and by a unique physiological role which consists in facilitating the diffusion of oxygen and hydrogen sulfide between the host clam and the chemoautotrophic symbiotic sulfo-bacteria [5,6]. It represents an interesting model system for CD studies since it displays a unique pattern of aromatic residues that line the heme pocket and are expected to generate a highly asymmetric distribution of electronic transition moment vectors with respect to the heme plane [7]. This structural feature can be used to test the validity of the model proposed by Hsu and Woody which predicts a strong CD spectral intensity in the visible and Soret regions [1]. The CD spectra of both ferrous and ferric derivatives, including sulfide–HbI, indicate that the contribution of aromatic residues determines the rotational strengths of the heme mainly in the near-UV region rather than in the visible and Soret regions.

2. Material and methods

Lucina pectinata HbI was purified as described by Kraus and Wittenberg [5], and was obtained as an oxygenated derivative. Deoxy-HbI was prepared by degassing the protein in a tonometer and flushing extensively with pure nitrogen gas. The ferric derivatives were prepared by adding a slight molar excess of ferricyanide to the oxygenated protein. Cyanide and sulfide adducts were obtained by adding a 10-fold molar excess of KCN or Na_2S to the oxidized protein solution. All measurements were carried out in 50 mM phosphate buffer at pH 7.0 and 20°C. Protein concentrations were determined spectrophotometrically according to the extinction coefficients published previously [5]. A Jasco J-710 spectropolarimeter was used. A molar ellipticity scale on a heme basis was used, $[\theta]$ = degrees $dmol^{-1}$ cm^2.

3. Results and discussion

The general features of the iron–porphyrin absorption spectra in hemoproteins have been elucidated and most of the spectral absorption bands have been assigned to specific electronic transitions [8]. Four plane polarized π–π^* transitions have been identified which correspond to the Q (470–600 nm), B (380–450 nm), N (300–350 nm) and L (250–320 nm) bands. Each band exhibits a characteristic vibronic structure due to the coupling of the optical electron with the vibronically active modes of the appropriate symmetry. In particular, the electronically forbidden Q_0 (α) band has a distinct vibronic component, the Q_1 (β) band, which is due to the coupling of the 0–0 transition with a series of 0–1 transition built on asymmetric stretching vibrations of the macrocycle, while the electronically allowed B_0 (Soret) band envelopes the totally symmetrical modes of vibration of the heme. In contrast, the heme L and N bands have not been characterized in detail as they overlap in part with the UV absorption of protein aromatic residues. In hemoproteins, all these transitions give a prominent contribution to the observed CD spectra in that they can give rise to a different absorption of left versus right circularly polarized light [9].

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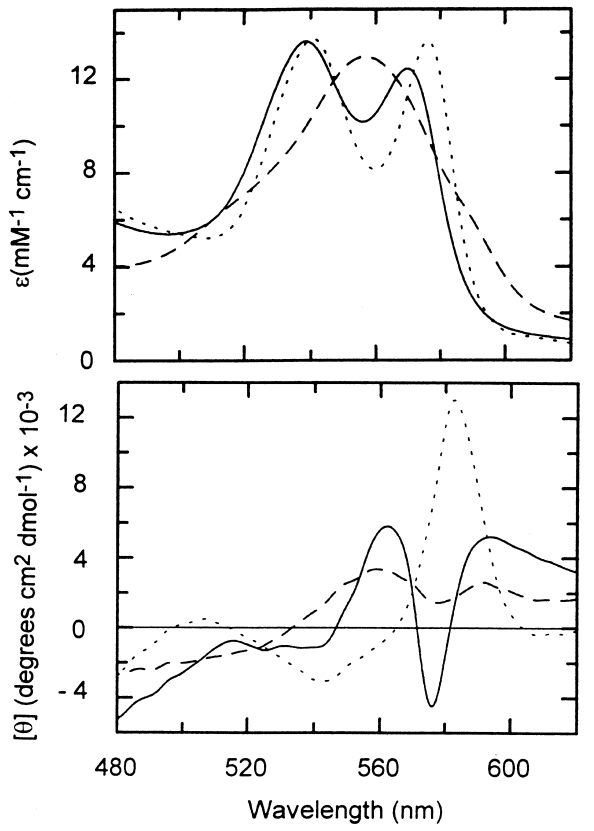

Fig. 1. Visible absorption (top) and CD (bottom) spectra of ferrous *Lucina pectinata* HbI hemoglobin derivatives. Deoxy- (—), Oxy- (···), carbonmonoxy-HbI (———). Protein concentrations were 4×10^{-5} M heme, in 50 mM phosphate buffer, pH 7.0.

3.1. Visible region

The CD spectra of the oxy-, deoxy-, and carbonmonoxy-HbI derivatives in the region 620–470 nm are reported in Fig. 1 in parallel with their respective absorption spectra. The CD spectrum of the deoxy derivative is characterized by two distinct positive peaks at 558 and 590 nm, corresponding to the Q_1 and Q_0 absorption bands, respectively. The value of the Q_0 ellipticity with respect to the Q_1 one has often been correlated to the asymmetry in the proximal bond. In fact, disruption of the C_4v symmetry leads to an enhanced intensity of the Q_0 band through the vibronic coupling mechanism [10]. In the case of HbI, the low value of the Q_0 ellipticity and the very small shoulder at 590 nm in the absorption spectrum point to a relatively symmetric heme structure. The oxy derivative displays a very sharp Q_0 peak, similar to that observed in many vertebrate hemoglobins and myoglobins and a negative Q_1 band which shows a vibronic structure. The CD spectrum of carbonmonoxy-HbI displays a split Q_0 band which results in a negative peak at 576 nm and an inflexion point at 570 nm, in correspondence with the absorption band. Interestingly, the overall shape of this spectrum shows a striking similarity to that of carbonmonoxy soybean leghemoglobin (legHb) [11]. In both HbI and legHb the Q_0 peak displays a large 'wing' on the red side which extends to 650–660 nm. This feature has been tentatively ascribed to the presence of a d–d transition around 625 nm with an enhanced rotational strength due to the intrinsically allowed magnetic transition dipole moment [8,11,12]. However, the CD profile may tend to level out slowly because the shape of the curve is intrinsically 'non-Gaussian'. Thus, the presence of the wing could be ascribed to a conformational heterogeneity in the geometry of the bound ligand which gives rise to a distribution of species with different rotational strength. Lastly, the origin of the wing could be ascribed to an anomalous polarization effect, i.e. an (apparent) increase in the damping factor of the electronic transition and hence in the line width of the CD band when the exciton splitting mechanism is operating. Though it is not uncommon to observe CD bands with a non-Gaussian profile, this feature has not been taken into account theoretically [13].

The spectra of the ferric aquomet, cyanide and sulfide derivatives are reported in Fig. 2. The shape of the high-spin aquomet CD spectrum is similar to that of the absorption spectrum, it is entirely positive and is characterized by a low ellipticity. The two low spin CD spectra exhibit a qualitatively similar behavior in that both display a positive Q_0 band, at 592 and 576 nm (the optical absorption peaks are at 577 and 568 nm) for the H_2S and CN derivatives, respectively. The Q_1 bands are broad and negative and their peaks correspond roughly to the absorption maxima.

3.2. Soret and near-UV regions

The CD spectra of the HbI ferrous adducts (Fig. 3B) show negative dichroic bands in the spectral range 250–480 nm. This behavior has been observed also in soybean legHb, in Chironomus and Glycera hemoglobins [8,14,15]. In contrast, vertebrate hemoglobins and myoglobins exhibit large positive

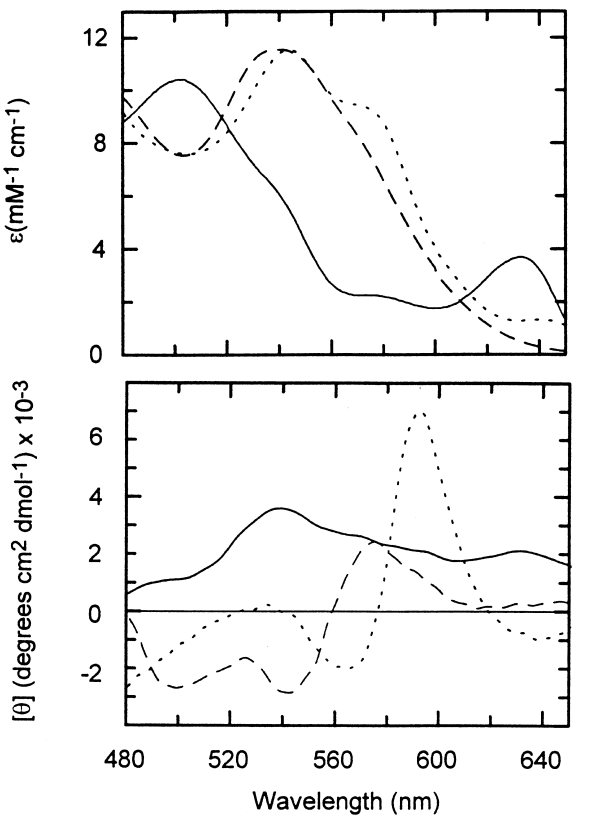

Fig. 2. Visible absorption (top) and CD (bottom) spectra of ferric *Lucina pectinata* HbI hemoglobin derivatives. Sulfide–HbI (···), cyanide–HbI (—), aquomet–HbI (———). Protein concentrations were 8×10^{-5} M heme, in 50 mM phosphate buffer, pH 7.0.

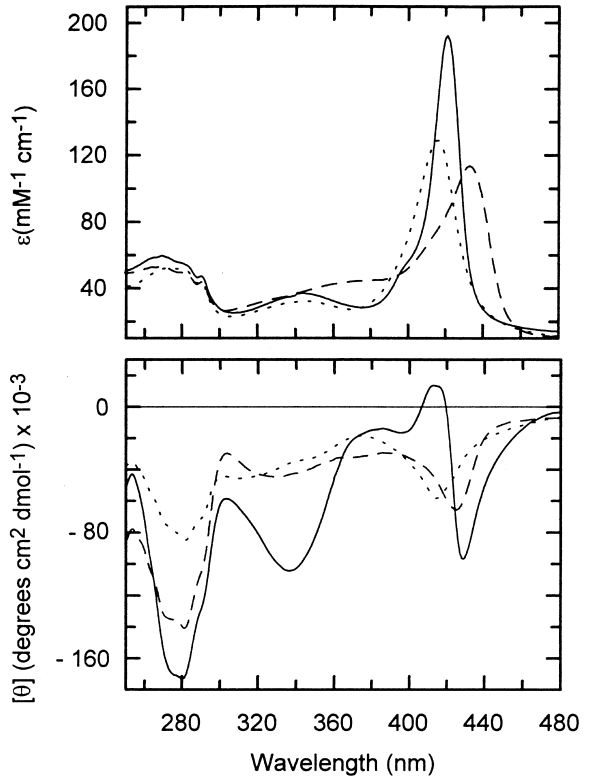

Fig. 3. Soret and UV absorption (top) and CD (bottom) spectra of ferrous *Lucina pectinata* HbI hemoglobin derivatives. Deoxy-HbI (—), Oxy-HbI (···), CO-HbI (———). Protein concentrations were 4×10^{-5} M heme, in 50 mM phosphate buffer, pH 7.0.

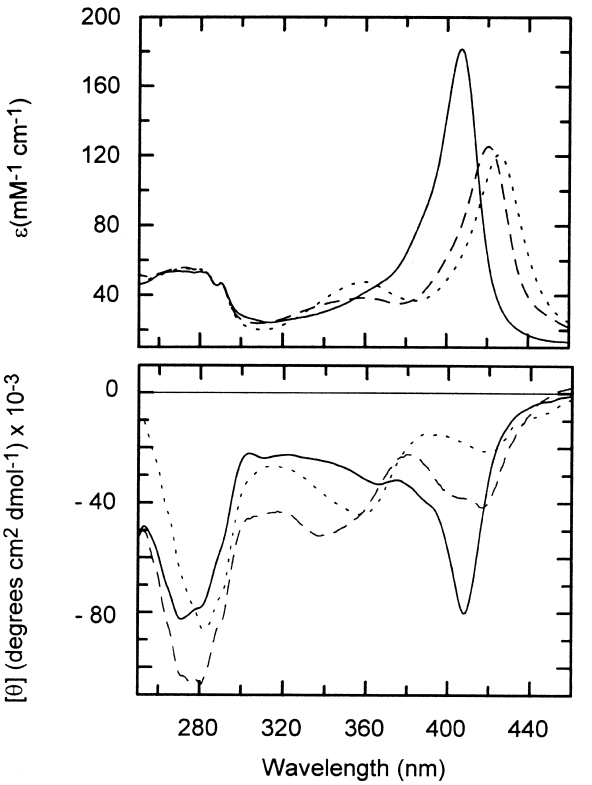

Fig. 4. Soret and UV absorption (top) and CD (bottom) spectra of ferric *Lucina pectinata* HbI hemoglobin derivatives. Sulfide–HbI (···), cyanide–HbI (—), aquomet–HbI (———). Protein concentrations were 8×10^{-5} M heme, in 50 mM phosphate buffer, pH 7.0.

ellipticities in the Soret region. Deoxy- and oxy-HbI display negative Soret peaks at 432 and 414 nm, roughly in correspondence with their respective absorption peaks. The dichroic band of the CO derivative is split into a sharp negative band peaked at 438 nm and a smooth positive band around 416 nm, the inflection point being at the CO absorption maximum, at 421 nm. Very large changes in the ellipticity values are observed upon ligand binding in the aromatic absorption region, around 280 nm. It is interesting that oxygen and CO have opposite effects: oxygen decreases the overall ellipticity dramatically with respect to the deoxygenated derivative ($>60\%$) whereas CO increases it. Such large changes have no counterpart in the absorption spectrum where only a minor peak shift is observed in the aromatic region (Fig. 3A). Both CD and absorption spectra in the aromatic region are relatively featureless probably due to the overlap of the contributions from the large number of tryptophan and phenylalanyl residues present. Another interesting feature is the large enhancement of the heme N band around 340 nm, which is even more negative than the Soret band in the CO, ferric cyanide and sulfide derivatives (Figs. 3 and 4B). The intensity of the N band appears to be strongly coupled to the intensity of the aromatic residues and heme L dichroic bands, with the exception of the high spin aquomet derivative. This finding points to the existence of a 'resonant interaction' between the electronic transition dipole moment vectors of the aromatic residues and that of the heme N and L transitions. In other words, the coupling between the optical electrons of the aromatic residues and those of the heme would be enhanced when both electrons are simultaneously excited. The spectra of the sulfide and cyanide ferric derivatives exhibit a quenched ellipticity of the Soret band, a feature which is observed com-

Table 1
Distances from the center of mass of the aromatic rings and the heme iron in the ferric sulfide *Lucina pectinata* hemoglobin I as compared to soybean leghemoglobin

Conserved residues		Distance (Å)		Non-conserved residues					
		LegHb	HbI			LegHb			HbI
$His^{96,97}$	(F8)	3.4	3.4	His^{63}	(E7)	5.8	Phe^{92}	(F4)	6.0
$Phe^{29,28}$	(B9)	7.8	7.2	His^{106}	(G6)	6.5	Trp^{21}	(B2)	12.5
$Phe^{30,29}$	(B10)	7.7	7.4	Phe^{107}	(G7)	9.6	Trp^{75}	(E18)	10.5
$Phe^{44,43}$	(CD1)	6.1	6.1	Tyr^{38}	(C3)	10.5	Phe^{110}	(G9)	10.4
Phe^{46}	(CD4)	10.1	11.4				Phe^{40}	(C5)	13.7
Phe^{68}	(E11)	11.2	7.8						

Only the residues within 14 Å from the heme iron are considered.

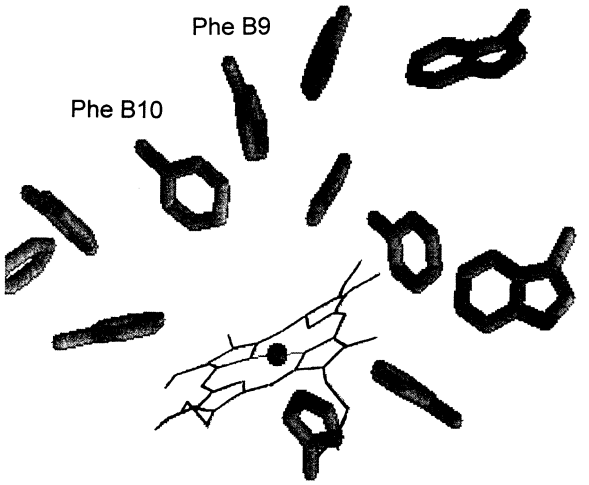

Fig. 5. Detail of the crystallographic structure of ferric sulfide *Lucina pectinata* I hemoglobin. The aromatic rings within 14 Å from the heme iron were selected (data from Brookhaven protein data bank).

monly in other hemoproteins and thus seems to be an intrinsic characteristic of the ferric low spin derivatives.

The comparison of the HbI CD spectra with those reported for other myoglobins and hemoglobins brings out an unforeseen similarity between HbI and soybean legHb, given the disparate behavior of the CD intensities and shapes in all other hemoproteins investigated to date [1,9,11,14–16]. The aromatic cores of HbI and legHb (Table 1) are characterized by additional residues within 14 Å from the heme iron with respect to horse heart myoglobin [1]. A calculation of the rotational strengths in terms of the coupled oscillator mechanism is beyond the scope of the present paper. However, it is immediately evident that an enhancement of the overall ellipticities would be expected in HbI and in legHb, due to the additional aromatic residues in the vicinity of the heme, since the contribution to the rotational strength is roughly proportional to the square of the distance between the heme core and the centers of the aromatic rings [1]. An enhancement is in fact observed only for the heme N and L bands, but not for the visible and Soret peaks suggesting that the coupled oscillator mechanism is operating only under resonance (heme L band) or pre-resonance (heme N band) conditions. The similarity of the visible and Soret CD spectra in HbI and soybean legHb, which is particularly striking for the CO derivative, has a structural counterpart in one specific feature, i.e. the presence of two Phe rings with very similar stereochemistry in positions B9 and B10 (Fig. 5). These two residues line the inner side of the distal pocket and are in Van der Waals contact with the bound ligand. In fact, a stabilizing effect toward the bound ligand has been proposed for the B10 residue, based on the increased oxygen affinity in the LeuB10→Phe sperm whale Mb mutant [17]. Thus, the B9 and B10 phenyl rings could be effective in determining the stereochemistry of the ligand and its relative orientation with respect to the heme plane. This specific feature, taken together with the high polarizability of the bound CO molecule may account for the split bands and the anomalous polarization effect in the carbonmonoxy derivatives. As suggested by Blauer et al. [4], a strong contribution to the overall rotational strength should arise from highly polarizable groups in the vicinity of the chromophore.

In conclusion, the behavior of *Lucina* HbI brings out that the role of the stereochemistry of the heme-ligand complex may determine the shape and intensity of the CD bands in the visible and Soret region whereas the distribution of aromatic residues contributes to the rotational strength of the heme bands only in the near-UV regions.

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