CD STUDIES ON THE HEME ENVIRONMENT IN ERYTHROCRUORINS AND CHLOROCRUORINS

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1. Introduction

The presence of a distinct heme environment in erythrocruorins and chlorocruorins with respect to hemoglobins and myoglobins, is indicated by the observation that the molecular weight on a heme basis (23 000–28 000) is consistently higher than the molecular weight of the polypeptide chains (12 000–18 000). This lack of one-to-one correspondence has led to the suggestion that either not all the chains are associated with a heme group or that the heme is interacting with more than one chain [1,2].

The CD spectra in the Soret region have been shown [3] to be strongly dependent on the immediate conformational environment of the heme group. Large differences in the CD spectra with respect to hemoglobins and myoglobins have been observed in studies on earthworm erythrocruorin [4]. Comparison of the Soret CD spectra of several erythro- and chlorocruorins appeared to be a useful tool to establish whether the heme environment has common characteristics in all the high molecular weight heme proteins. To this end, the study of the Soret CD spectra has been extended to the erythrocruorins of *Arenicola marina* and *Nereis diversicolor* and to chlorocruorin from *Spirographis spallanzanii*. The spectra of chlorocruorin have been compared with those of hemoglobin reconstituted with chloroheme (2-formyl, 4-vinyl-deuteroheme).

The results obtained have given clear indications of the conformational similarity of the heme environment in erythro- and chlorocruorins. Moreover they show that in these proteins the nature of the heme–protein interaction differs from that of the low molecular weight hemoglobins and myoglobins.

2. Materials and methods

Erythrocruorin from *Nereis diversicolor* was prepared from live worms purchased in a bait store in Cagliari (Sardinia); these were minced in I = 0.1 M Tris buffer, (pH 7.2) containing 10^{-5} M EDTA and 1 mg/ml of the proteolytic inhibitor phenyl methylsulphonylfluoride; the protein was purified by preparative ultracentrifugation. Erythrocruorin from *Arenicola marina* [5] was a kind gift of Dr J. Everaarts. Chlorocruorin from *Spirographis spallanzanii* was obtained as in [6]. Chloroheme-reconstituted hemoglobin was prepared from apohemoglobin and chloroheme [7,8]. Chloroheme was obtained from chlorocruorin during the preparation of the apoprotein by the acid–butanone method [9]. The organic layer was washed with a small volume of water, dried over anhydrous sodium sulfate and evaporated under vacuum. The brown residue of chloroheme was used directly for the reconstitution experiments.

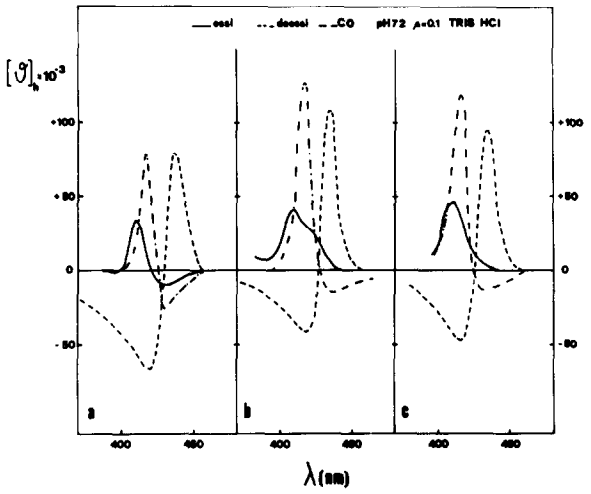
The heme content was determined with the pyridine hemochromogen method [10] taking ϵ_{mM} = 32 at 557 nm for the derivative of protoheme [10] and ϵ_{mM} = 28 at 583 nm for the derivative of chloroheme [11]. Protein concentrations are expressed on a heme basis; the following ϵ_{mM} for the oxygenated derivatives were used: *Nereis diversicolor* erythrocruorin, 13.2 at 540 nm; *Arenicola marina* erythrocruorin, 13.9 at 540 nm; chlorocruorin, 24.1 at

606 nm; chloroheme reconstituted hemoglobin, 16.5 at 598 nm [8].

Absorption spectra were measured with a Cary 219 spectrophotometer; CD spectra were recorded in a Cary 60 spectropolarimeter equipped with a 6002 attachment. The molar ellipticities (deg.cm^2/dmol) are expressed on a heme basis.

3. Results and discussion

Figure 1a–c reports the CD spectra in the Soret region of the deoxy-, oxy and carbonmonoxy-derivatives of *Arenicola marina* and *Nereis diversicolor* erythrocruorins (see also table 1); the spectra of earthworm erythrocruorin are included for comparison. In all the derivatives the Soret Cotton effects are



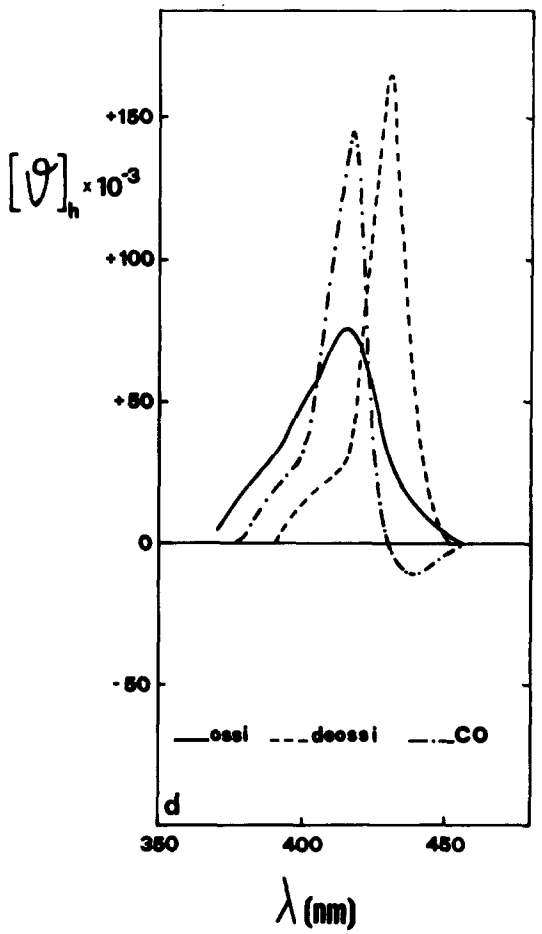


Fig.1. CD spectra in the Soret region of (a) earthworm, (b) *Arenicola marina*, (c) *Nereis diversicolor* erythrocruorin and (d) human hemoglobin. Oxy- (——), deoxy- (– – – –), carbonmonoxy- (—·—·—) derivatives in I = 0.1 M phosphate buffer (pH 7.0).

Table 1
CD data of *Arenicola* and *Nereis* erythrocruorins, *Spirographis* chlorocruorin and chloroheme containing hemoglobin (phosphate buffer (pH 7.0) = 0.1)

Derivative / Protein	Oxy				Deoxy				CO			
	λ (nm)	[θ]h	λ (nm)	[θ]h	λ (nm)	[θ]h	λ (nm)	[θ]h	λ (nm)	[θ]h	λ (nm)	[θ]h
Earthworm erythrocruorin	410	+34.2	430	− 9.5	420	−66	437	+ 79.5	417	+ 77.7	430	−26.4
Arenicola erythrocruorin	410	+42			418	−42.2	435	+108	418	+127	435	−14.7
Nereis erythrocruorin	410	+44			418	−47	435	+ 95	417	+119	430	−13.0
Spirographis chlorocruorin	424	+30	442	−27.5	439	+40	452	− 16.5	422	+126	440	−90
Chloroheme containing hemoglobin	440	+55.5			445	+69			433	+ 60.5	460	−15

complex and there is no coincidence between the dichroic and the absorption maxima. This behaviour is at variance with that of mammalian hemoglobins and myoglobins, which exhibit only a large positive band located at the wavelength of the Soret absorption band (fig.1d). The small additional negative band at higher wavelength in carbonmonoxy-hemoglobin, together with the splitting of the Q_0 and Q_1 bands, has been taken as evidence of a lowering of the heme symmetry, due to the non-linear iron–carbon monoxide bond above the heme plane [12].

In all erythrocruorins, the spectra of the deoxygenated derivative show two large Cotton effects of opposite sign; moreover upon addition of a heme ligand (oxygen, carbon monoxide) the bands are reversed in sign and shifted to lower wavelengths.

The spectra of the oxygenated derivative show the greatest variability in the various proteins; thus, in earthworm erythrocruorin two bands of opposite sign are visible, while in *Nereis* and *Arenicola* erythrocruorins there is a main positive band of different complexity. These findings are in keeping with observations on earthworm, *Eunice* [13] and *Amphitrite* [14] erythrocruorins. In earthworm erythrocruorin, which has been studied in greater detail, the CD spectrum of the oxygenated form has been shown to be the more sensitive one to local alterations in the heme environment; thus, the occurrence and the amplitude of the negative band at higher wavelength (~430 nm) reflects subtle changes in conformation which are not necessarily connected with changes in the subunit assembly [4].

Figure 2 reports the CD spectra of oxy-, deoxy- and carbonmonoxy-chlorocruorin and of the corresponding derivatives of chloroheme reconstituted hemoglobin. In general the dichroic bands are of lower intensity than in the protoheme containing proteins. In hemoglobin, substitution of proto- with chloroheme does not change the features of the Soret CD spectra. All the derivatives analysed show one main positive band; furthermore, as in HbA, the carbonmonoxy-derivative has a small negative peak at higher wavelength. The chlorocruorin derivative studies show a splitting of the Soret band into two dichroic components of opposite sign. Thus there is a similar change in the Soret CD spectra in going from hemoglobins to the high molecular weight hemeproteins irrespective of the type of heme. In contrast

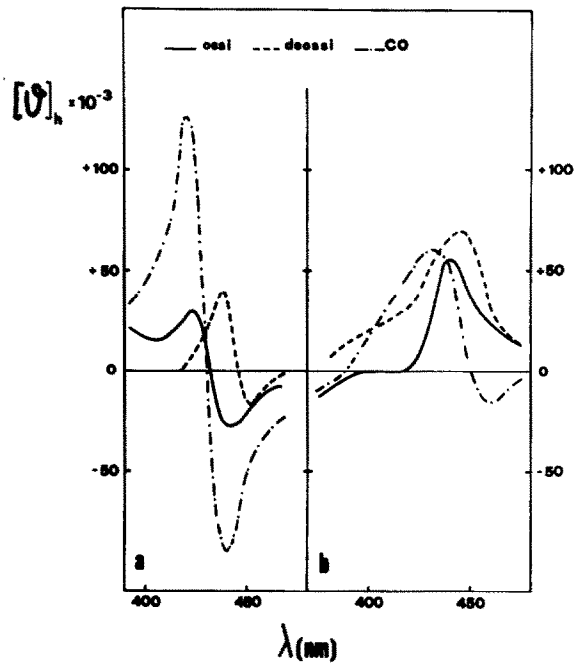


Fig.2. Circular dichroism spectra in the Soret region of (a) *Spirographis spallanzanii* chlorocruorin and (b) chloroheme-reconstituted hemoglobin. Oxy- (——), deoxy- (– – – – –), carbonmonoxy- (—·—·—) derivatives in I = 0.1 M phosphate buffer (pH 7.0).

to erythrocruorins the CD bands of chlorocruorin do not change in sign upon ligand binding.

The splitting of the Soret CD bands into two components and their changes in magnitude have been predicted theoretically [3]; they depend on the orientation of the two perpendicular heme transition moments. The orientation is in turn affected, according to [3], by the interaction of the heme group with nearby aromatic side chains situated within a distance of 1.2 nm.

More recently interaction of the heme with other groups, such as peptide bonds, was also shown to give a significant contribution [15]. The similarities in the erythrocruorins spectra indicate a similar effect of the environment on the orientation of the heme transition moments; the orientation itself is very different from that occurring in human hemoglobin, where, according to [3], the two transitions are perpendicularly oriented between 60° and 75° from the pyrrole nitrogens. Moreover a significant reorientation of the heme

transition moments occurs upon ligand binding and manifests itself as a reversal of the Soret Cotton bands. In chlorocruorin the nature of the heme environment is similar to that of oxy- and carbonmonoxy-erythrocruorin, and seems to be insensitive to the presence of a heme ligand. Thus the heme transition moments are similarly oriented in the liganded and unliganded forms. Another characteristic feature of the chlorocruorin spectra is the large ellipticity value of the negative peak in carbonmonoxy-chlorocruorin, which points to a high degree of asymmetry in this derivative.

In conclusion the following points can be made in the framework of the phenomenological approach outlined above:

(i) The immediate heme environment of erythro- and chlorocruorins differs from that of hemoglobins;

(ii) In spite of the differences in the detailed appearance of the CD spectra, their common features suggest a similarity in the conformation of the polypeptide chains around the heme as well as in the nature of the amino acid residues in its vicinity.

Hence, the CD spectra bring out the conservative character of the heme surrounding in erythro- and chlorocruorins regardless of the differences in minimum molecular weight and in the number of chains.

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