TRYPTOPHANYL CIRCULAR DICHROISM IN A SPECIAL HEMOGLOBIN

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1. Introduction

The monomeric and dimeric hemoglobins found in the hemolymph of *Chironomus th. thummi* (Diptera) represent a set of model compounds whose comparison should provide insight into details of their molecular structure. The component CTT I deserves special attention because of some remarkable features of its CD spectrum: The ellipticity band at 540 nm is negative and the circular dichroism of the Soret region consists of two negative bands, one at 379 nm dominating at low pH, the other at 409 nm dominating at high pH. These properties reflecting heme–globin interaction will be dealt with in another paper [1]. This communication concentrates on the most striking peculiarity, a pronounced narrow positive band at 292 nm. A molecular interpretation of the band is attempted.

2. Materials and methods

Hemoglobins of *Chironomus th. thummi* were prepared from larvae and separated as met-hemoglobins using a modified method of Braun et al. [2]. Apo-hemoglobin was prepared by the method of Rossi-Fanelli et al. [3]. The first fraction of DEAE-cellulose chromatography, CTT I, was characterized by end group determination (Gly) and polyacrylamide gel electrophoresis. The isoelectric point of CTT I is above 8.3; this is about 2 pH units more alkaline than that found for the other fractions. Molecular weight determination by comparative gel filtration on Sephadex G 100 gave a value of 15,800 ± 1,150. Protein concentration was checked by dry weight in the case of globin and photometrically in the case of hemoglobin using a molar absorbance of ϵ_{540} = 13,100* for the cyano-met derivative. Tryptophan was reacted with NBS in 0.1 M Na acetate buffer pH 4. CD spectra were recorded with a Cary model 61 dichrograph calibrated with d-10-camphosulfonic acid. The spectral bandwidth was 10 Å. The helix content was calculated from the mean residue ellipticity at 222 nm on the basis of the data of Greenfield and Fasman [4].

Abbreviations:
CD : circular dichroism
CTT: hemoglobin of *Chironomus*
NBS: *N*-bromosuccinimide

3. Results and discussion

In the near UV, absorption can be due to disulfide bonds, the heme group [5], and aromatic amino acids. CTT I does not contain any cysteine or cystine and the 292 nm band persists in the globin after heme extraction (fig. 1), thus this band can be attributed to aromatic side chains. From the ellipticity at 222 nm we obtain a helix content of 80% in CTT I hemoglobin and 57% in CTT I globin. The finding that removal of the heme and the concomitant decrease of helix content leave the band substantially unchanged implies that the responsible residue(s) cannot have heme contact and must be located in a part of the molecule which is not affected by the structural change.

CTT I is a favorable case in which to identify specific

* The authors are indebted to Dr. H. Sick for the determination of this value.

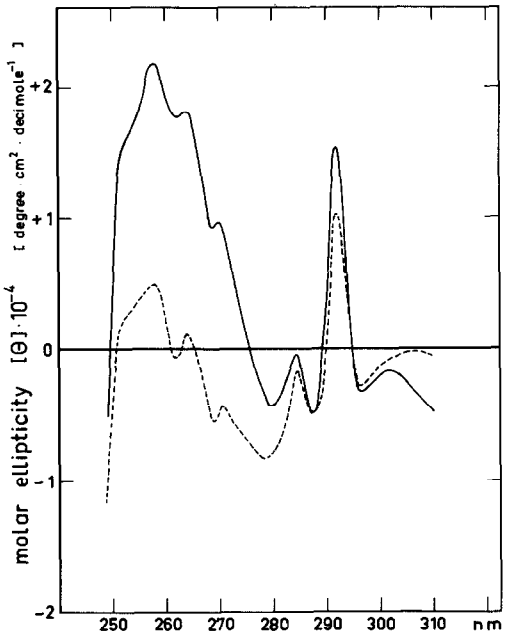

Fig. 1. CD spectra of CTT I hemoglobin (——) and CTT I globin (- - - -) in the near UV. Glycine/NaOH buffer (0.1 M, pH 6.8).

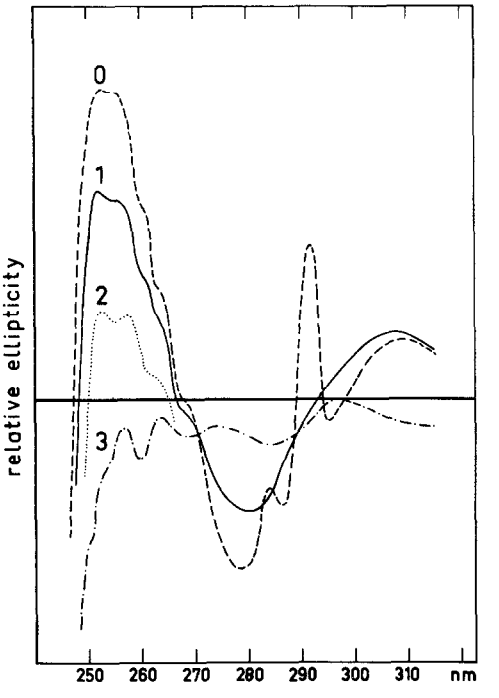

Fig. 2. Effect of *N*-bromosuccinimide on the CD spectrum of CTT I hemoglobin. Numbers indicating moles NBS per mole hemoglobin. Sodium acetate buffer (0.08 M, pH 4.0).

CD bands in the near UV as originating in tryptophan, from comparisons with spectra of Trp derivatives and the selective disappearance of the bands upon addition of NBS.

Comparative attribution: In table 1 the positions and signs of the CD extrema of hemoglobin, globin, and *N*-acetyl-L-tryptophan-amide (*N*-Ac-L-Trp-A) [6] are juxtaposed and tentatively related to certain vibrational transitions in the Platt notation [7]. This suggests that the prominent 292 nm band can be attributed to the O–O ^1L_b transition. Also the weaker band at 284 nm has the same (positive) sign expected for all ^1L_b transitions [6]. Strickland and coworkers [6] have grouped the CD spectra of Trp derivatives of low molecular weight into 4 classes: CTT I can be ascribed to either type III with resolved bands belonging both to ^1L_a and ^1L_b transitions, or to type IV with bands which do not coincide with the positions of the corresponding absorption bands.

The assignment of CD bands to Trp is further supported by comparison with spectra of monomeric [8] and polymeric Trp [9] and certain proteins such as lysozyme [10], cytochrome *c* [11, 12] and chymotrypsinogen A [6]. On the other hand, an alternative assignment to tyrosine is incompatible with the spectra of *N*-acetyl-L-tyrosine ethyl ester [13] ribonuclease A [13] and Trp-free insulin [14, 15].

Attribution by quenching with NBS: Tryptophan can be determined with NBS by following the decrease in absorbance at 280 nm on oxidation of the indole ring to oxindole. On the basis of the molar absorbance difference $\Delta\epsilon_{280} \doteq 4{,}200$ for free Trp, titration is completed with an average of 2–4 equivalents of NBS [16]. Hence, this method is stoichiometrically unsatisfactory and particularly problematic with heme proteins [17]. In our case the total absorbance difference indicates at least 3 Trp per molecule. Sequence work on CTT I has so far revealed only 2 Trp [18]. However, the 292 nm band is cancelled by only one equivalent of NBS (fig. 2). This demonstrates that it is really due to Trp which is oxidized preferentially under the conditions applied. But in this phase of the titration, the tryptophans are oxidized to only a small extent. There-

Table 1
Positions and signs of CD extrema of CTT I hemoglobin, CTT I globin, and *N*-acetyl-L-tryptophan-amide.

CTT I* hemoglobin		CTT I** globin		*N*-Ac-Trp-A type III** methanol, 77 °K, [6]		
Position (nm)	Sign	Position (nm)	Sign	Position (nm)	Sign	Designation [6, 7]
302	+					
296.5	–	296.5	–	297	–	O–O ^1L_a
292	+	292	+	290	+	O–O ^1L_b
287.5	–	287.5	–	287	(–)	
284.5	+	284.5	+	283	+	O+850 ^1L_b
279.5	–	278.5	–			
270	(+)	270.5	+			
269	((–))	269	–	269	–	^1L_a
264	+	264	+			
262	(–)	262	–			
258	+	258	+			
252.5	(+)	252.5	(+)			

* Taken from fig. 1.
** Taken from E.H. Strickland et al. [6].

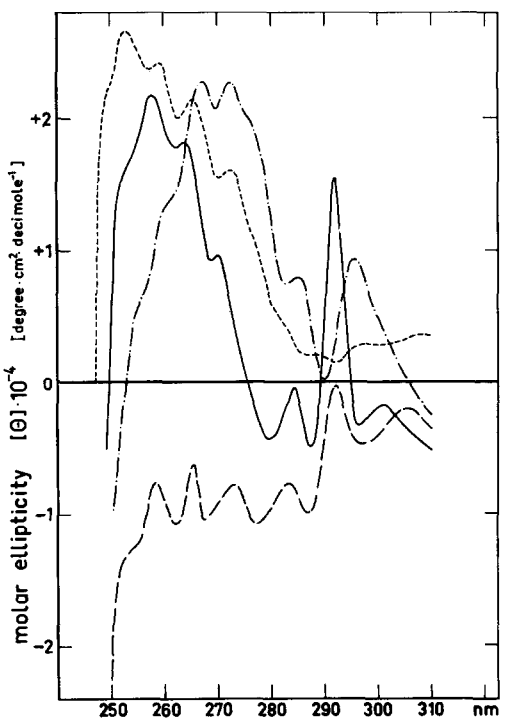

Fig. 3. Near UV circular dichroism of horse hemoglobin (- - - -), sperm whale myoglobin (—.—), CTT I hemoglobin (——), and CTT III hemoglobin (— —); oxidized form. Phosphate buffer (0.1 M, pH 6.8).

fore, the quenching of the band must be due either to selective and stoichiometric oxidation of one specific Trp residue or to a conformational change in the environment of the tryptophan(s) responsible for the signal. On the one hand we do not find stoichiometry in the photometric titration nor has it been reported elsewhere; on the other hand the helix content which is surprisingly constant down to pH 4, i.e. more than 5 pH units away from the isoelectric point, drops significantly upon addition of small amounts of NBS. As a consequence the bands below 284 nm, probably attributable to tyrosine and phenylalanine, are also affected (fig. 2).

Structural considerations: From amino acid sequences so far worked out, the individual hemoglobins of *Chironomus* contain Trp in different positions of the chain. Among them only CTT I contains Trp at A12 [19], and only CTT I produces the extraordinary circular dichroism described. The second Trp of CTT I which has been found with certainty is at or close to H7 [18]. The only Trp residue of CTT III is located in this position [20], and CTT III does not show a similarly pronounced band (fig. 3). These arguments are in favour of Trp A12 being the signal-giving residue. Since A12 is invariantly occupied by Trp in all vertebrate hemoglobins and myoglobins, the question arises why these proteins do not exhibit a

similarly accentuated band (fig. 3). It is unlikely that the CTT I band is merely a cumulative effect of a higher Trp content. Carp oxyhemoglobin, for instance, which contains 4 Trp in the β-chains [21] and 2 Trp in the α-chain [22], does not exhibit a comparable signal [23]. What additional factor could effect the observed sharpening and intensification of the band? Comparison of the atomic structure of CTT III [24] and sperm whale myoglobin [25] suggests an explanation that at least seems plausible: In CTT III the amino acids B1–B4 are deleted. This retrenchment of the first turn of helix B forces helix A to bend considerably towards helix B, the break in the direction of the helix A axis occurring at A13. Therefore residue A12, Phe in CTT III and even more Trp in CTT I, must be in a nearly rigid position. For instance, the restriction of conformational mobility of *N*-Ac-L-Trp-A at 77°K results in an 18-fold increase in rotational strength as compared to 298 °K [6]. This possible interpretation is being further investigated by comparative cleavage and chemical modification experiments. Fluorescence and differential denaturation studies are also in progress.

A concluding speculation may be permitted: Can the appearance of Phe in CTT III be considered to be a genetic substitution correcting the inadequate steric situation which results from the collision of the partially deleted helix B and the bulky Trp at A12?

Acknowledgements

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