Brief Report

Absorption Spectra of Inclusion Bodies in β-Thalassemia

By PHAEDON FESSAS, DIMITRIS LOUKOPOULOS AND BO THORELL

NORMOBLASTS and, to a much smaller extent, red blood cells of patients with homozygous β-thalassemia present large inclusion bodies, which have the staining and solubility properties of precipitated hemoglobin; they have been considered to represent the excess of α-chains.[1] This report presents the results of the examination of the absorption spectra of these inclusions, in situ, as well as in preparations from bulk isolation.

MATERIAL AND METHODS

For the bulk isolation of inclusion bodies, blood was obtained from four unrelated splenectomized patients with homozygous β-thalassemia, who had a high percentage of inclusion carrying cells; the parents of two of these patients had high-A_2 β-thalassemia trait, but the exact genetic constitution of the other two could not be established. Blood from healthy controls and from nonsplenectomized patients with homozygous β-thalassemia, presenting only few inclusion-carrying cells, served for comparison. The red cells were washed repeatedly with cold saline and finally packed by centrifugation at 2.200 g; 4 ml. of the packed red cells, of either patients and controls, were lysed with a 15 to 20-fold volume of a solution of 1 mM EDTA in distilled water, buffered to pH 7.2 with *tris*.[2,3] The hemoglobin content of the hemolysate was determined on a small aliquot, whereas the bulk of the erythrocyte ghosts was packed by centrifugation at 4 C. and 2.200 g for 1 hour. The supernatant was removed and the sediment was resuspended in the hemolysing solution and centrifuged, as above, as many times as necessary (up to 20 times) until the supernatant was perfectly colorless to the eye.

After the final centrifugation the volume of the packed ghosts was about ¼ of the initial packed red cell volume. This sediment was solubilized by making up to 4 ml. with either 10 per cent NaOH, according to the directions of Hunter for altered hemoglobin,[4] or with 0.1 N NaOH. An aliquot of the solubilized ghosts, 0.2 ml., was used for the determination of the residual hemoglobin by the benzidine method;[5] an alkali-denatured hemoglobin solution, prepared from a measured amount of hemoglobin, served for the spectrophotometric comparison. For the spectroscopic study, the solution of the ghosts was cleared by centrifugation, placed in a photometric cuvette of 10 mm. light path and overlayered with liquid paraffin. A small quantity of powdered $Na_2S_2O_4$ was added and the absorption spectrum was determined immediately with a Zeiss PMQ II spectrophotometer.

Microspectrophotometric analyses of single inclusions in situ were made on thin smears prepared from fingertip blood of two splenectomized homozygous β-thalassemics, the parents of which had high-A_2 β-thalassemia trait; the cells of one case were used also for the bulk isolation of inclusions. The smears, on quartz slides, were air dried and then treated with methanol for 2 minutes, which gives constant and stable proportions of intracellular hemi- and hemoglobin.[6] The microspectrophotometric technic was essentially

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Table 1.—*Microspectrophotometric Data on Single Thalassemic Inclusions in Situ*

	Absorbancy Soret	Absorbancy 280 mμ	Quotient Abs. 280/Abs. Soret
Normal erythrocyte on air-dried smear treated with methanol	0.200	0.400	2.0
Thalassemia, Case 1			
Erythrocytic inclusion	0.065	0.250	3.8
	0.130	0.290	2.2
	0.030	0.060	2.0
Erythroblastic inclusion	0.151	0.302	2.0
	0.150	0.300	2.0
	0.200	0.400	2.0
Thalassemia, Case 2			
Erythroblastic inclusion	0.030	0.200	6.7
	0.050	0.100	2.0
	0.055	(0.600)*	(10.9)*

Complete absorption spectra were run between 240 mμ and 450 mμ, thus covering the protein band at 280 mμ and the heme-absorption in the Soret region, which under the present conditions (6) range between 410 and 420 mμ. The reproducibility in measured absorbancy ($\log \frac{I_0}{I_1}$) is estimated to be better than ± .001. As an expression of protein/heme ratio the quotient between the absorbancies at the maxima 280 mμ and 415 ± 5 mμ has been calculated.

*The ultraviolet absorption curve displayed a high light-scatter component.

similar to the description in reference 6; some additional information is given in table 1. Typical inclusions, measuring over 1 μ in diameter and thus large enough to cover the photocell aperture of the microspectrophotometer, were selected by phase-microscopy.

Results

The red cells from normals and from nonsplenectomized thalassemia patients yielded pure white ghosts, the hemoglobin content of which was always less than 0.01 per cent of the initial preparation and insufficient for spectroscopic analysis. The splenectomized cases of β-thalassemia yielded a somewhat larger sediment, which was dirty white at the top, brownish in the middle and greenish-brown at the bottom layer; about 0.5 per cent of the hemoglobin of the initial preparation had been retained in this material. Examination of the centrifugate by phase contrast and by various staining procedures showed that it consisted of aggregates of inclusions, together with strands of amorphous material and "siderotic" granules (fig. 1). The inclusions had retained their morphologic and staining characteristics, as seen in intact cells,[1] whereas the amorphous substance stained like nuclear material.

The addition of NaOH to the centrifugate produced a brownish solution, which upon reduction, developed a clear pink color. The spectrum was typical of denaturated globin-hemochrome (alkaline hemochromogen),[4,7] with a narrow first band at 555 mμ, a weaker and broader second band at 525 mμ and a pronounced Soret band at 422 mμ (fig. 2). The same spectrum was obtained

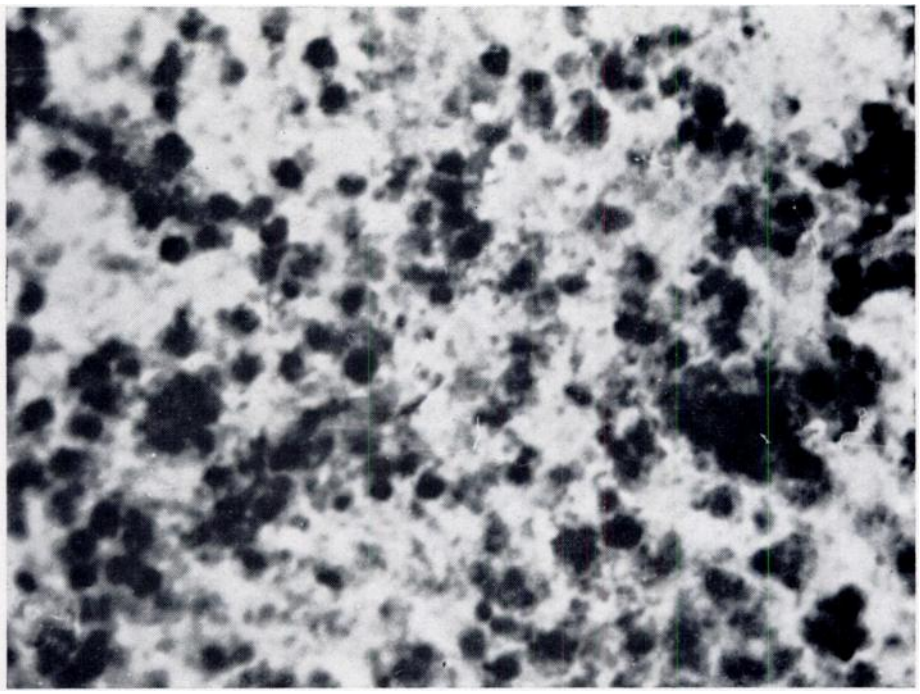

Fig. 1.—Lysed red blood cells from a splenectomized case of homozygous β-thalassemia after thorough washing of the ghosts and centrifugation, stained vitally with methyl violet. Intact individual inclusion bodies and clumps thereof can be recognized. Total magnification x 2,000.

on the samples of all four splenectomized homozygous thalassemics, using either the strong or the weak NaOH solution.

All microspectrophotometric records of single, intracellular inclusions displayed a distinct Soret band as in the normal, hemoglobin-containing erythrocyte, although of a variable magnitude, mainly depending on the size of the inclusion. The protein maximum at 280 mμ was less pronounced due to the superimposed light scatter (fig. 2). Mostly, however, a maximum or a plateau was present which allowed the calculation of the absorbancy ratio between protein and heme. This ratio was in most instances close to that of normal intraerythrocytic hemoglobin (table 1). In some cases, however, the quotient was significantly higher, which indicates an excess of protein relative to heme within the inclusions.

Moreover, the ultraviolet spectra from the single inclusions showed no evidence of absorption due to nucleic acids or nucleotides.

Discussion

Both methods of study of the spectral properties of the thalassemic inclusions leave no doubt as concerns the presence of a hemoglobin-like component in these structures. However, the exact form of the pigment—or pigments—involved, cannot be directly inferred from the results, as the exact position of

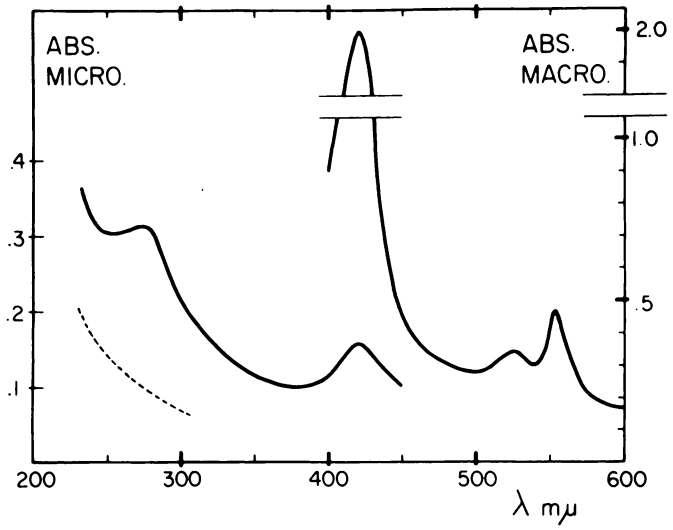

Fig. 2.—Absorption spectrum from a single erythroblastic inclusion in situ (left-hand curve) and absorption spectrum from inclusions, isolated in bulk (right-hand curve). Note the two different ordinate divisions ($\log\frac{I_0}{I_1}$). The maxima from left to right are: 280, 418, 422, 525 and 555 mμ. The dotted curve indicates the light "scatter" function $k_1 \cdot \frac{1}{\lambda 4} + k_2$, which type of function is superimposed on the "real" absorption of the inclusion in situ.

the Soret-band obtained by microspectrophotometry from the single inclusions in situ, is influenced by the method of preparation.[6] Judging from the color of a large part of the isolated inclusions, it is obvious that the denaturation of the hemoglobin has already advanced considerably; the greenish shade speaks for a choleglobin-hemochrome (or -hemichrome) type of transformation. This change, or further degradation and subsequent loss of the heme moieties could be one reason for the sometime higher protein: heme ratio, calculated from the microspectra. The presence of nonheme proteins in some of the inclusions with a high protein: heme ratio is also a possibility, which cannot be excluded at the moment.

The complete absence of ultraviolet absorption due to nucleotide or nucleic acids, as revealed by the microspectrophotometric results, agrees with the fact that the inclusions present no basophilia, after regular staining, or pyroninophilia. Apparently neither ribosomes, nor template RNA precipitates together with this instable hemoglobin fraction; these hemoglobin particles are probably released from the ribosomal attachments prior to their precipitation. The observation of free α-chain remaining in solution in the hemolysates of severe β-chain deficiencies[8] supports the latter conclusion.

The present study offers direct proof for the hemoglobinic nature of the β-thalassemia inclusion bodies; it does not permit, however, the characterization of this material as consisting of α-chains. Further studies to this effect are in progress.

Summary

The spectroscopic properties of isolated or single erythroblastic and erythrocytic inclusions of homozygous β-thalassemia prove that these structures contain a hemoglobin-like component.

Summario in Interlingua

Le proprietates spectroscopic de isolate e de singule inclusiones erythroblastic e erythrocytic in homozygotic thalassemia β indica que iste structuras contine un componente hemoglobinoide.

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