EVIDENCE AGAINST THE BINDING OF NATIVE HEMOGLOBIN TO SPECTRIN OF HUMAN ERYTHROCYTES

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1. Introduction

Under suitable conditions, hemoglobin binds to red cell membranes prepared by hypotonic lysis of erythrocytes [1,2]. A report indicating that hemoglobin interacts with purified spectrin [3], has enforced the idea that hemoglobin could be a peripheral constituent of the erythrocyte membrane [1]. As our observations were in conflict with [3], we have reinvestigated this question.

The results presented here indicate that in vitro studies involving isolated spectrin are inadequate to explain hemoglobin retention by intact membranes. It is demonstrated on the other hand that in contrast to native hemoglobin, heme shows significant binding to spectrin in vitro. This observation may have some significance in vivo for the products formed by intra-erythrocyte degradation of hemoglobin and their effects on the properties of the red cell membrane.

2. Materials and methods

Spectrin was prepared by the method in [4] except that the final step in the purification of spectrin was performed on Sepharose 6B. Purity of spectrin was controlled for each preparation by dodecylsulfate–acrylamide gel electrophoresis. Human hemoglobin was prepared according to [5]. Aquomethemoglobin was obtained by oxidation of deoxyhemoglobin with a stoichiometric amount of ferricyanide, followed by dialysis against water. Spectrin concentration was calculated on a basis of mol. wt 460 000 and $E^{1\%}_{280}$ 8.8 [6]. For carboxy- and metcyanide hemoglobin we used $E^{mM}_{538.5}$ 14.85 and E^{mM}_{540} 11.5, respectively. CO-saturated buffers were prepared by bubbling high purity CO (Air Liquide). Hemin was from Koch-Light, Sepharose 6B and Sephadex from Pharmacia. Optical spectra were recorded on a Cary 118 spectrophotometer. Kinetic measurements were performed on a Durrum-Gibson stopped-flow apparatus interfaced to a minicomputer (Data General Nova 2). In fluorescence kinetics, excitation light was monitored at 280 nm and emission was recorded through a cut-off filter at 320 nm. The flash lamp had an output of 450 J with 45 μs width at half height.

3. Results

3.1. *Gel filtration*

The purpose of this work was to demonstrate whether or not spectrin could mediate the in vitro interaction of hemoglobin with the red cell membrane; therefore, experimental conditions which ensure retention of hemoglobin by stroma were chosen. Phosphate buffer, 10–50 mM, at pH 6.6, a value close to physiological conditions, which ensures good stability of hemoglobin and is just out of the range where polymerization of spectrin occurs [4]. In order to avoid the possible formation of some denatured materials, we repeated the experiments in [3] with the following modifications: the filtration on gels was performed in the cold room, and hemoglobin was used as the carbon monoxide derivative, which is one of its most stable forms. The pH was adjusted to 6.6 instead of 6.1; this difference does not change much the degree of interaction of hemoglobin with stroma [1].

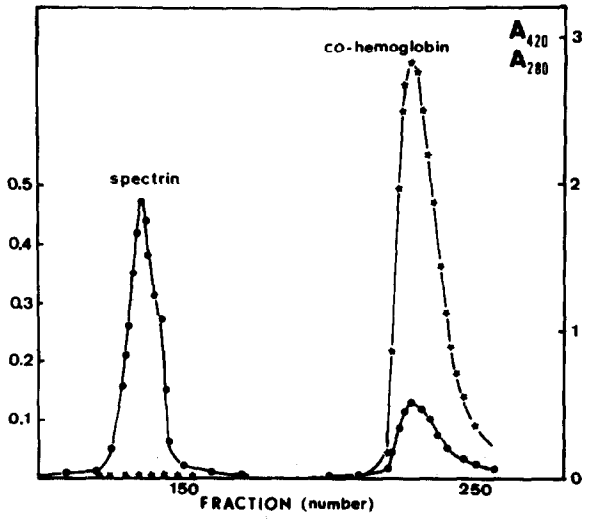

Fig.1. Elution profile of a mixture of spectrin and CO-hemoglobin on a Sephadex G-200 column. $A_{280\,nm}$ values (•). $A_{420\,nm}$ values (⋆). Ordinates have different scale for the spectrin and hemoglobin peaks. Sample solution containing 18 mg CO-hemoglobin and 16 mg spectrin was applied on a 3 × 120 cm column and eluted with 9 mM CO-saturated phosphate buffer, pH 6.6, 3°C. Fractions, 3.3 ml were collected.

As shown in fig.1, the elution pattern of a 1:1 mixture (by wt) of spectrin and CO-hemoglobin, obtained on a Sephadex G-200 column, shows a complete separation of the two proteins. However, contrary to [3] there is no retention of a heme compound in the spectrin fraction. The optical spectrum of the eluted hemoglobin shows no evidence of denaturation, even in the most dilute fractions.

Contamination of spectrin with heme compounds has been noticed [7,8]. Unfortunately, in all these studies the coloured fractions have not been spectro-

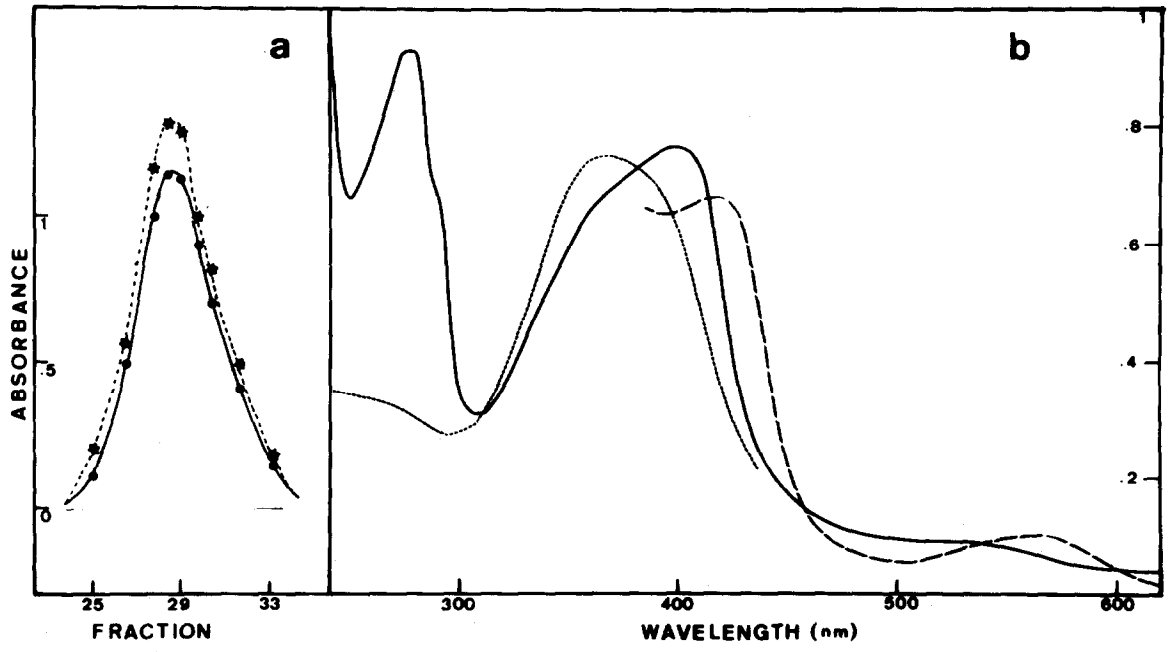

Fig.2. Elution profile of a mixture of spectrin and heme on a Sephadex G-25 column (a), and optical spectrum of the eluate (b). (a) Filtration of 16 mg spectrin mixed with a 40-fold molar excess of heme. $A_{280\,nm}$ values (•). $A_{410\,nm}$ values (⋆). Fractions, 3 ml, were collected from a 2.2 × 50 cm column equilibrated with 50 mM phosphate buffer, pH 6.6, 3°C. Fractions 27–32 had constant $A_{410/280\,nm}$ 1.15. (b) Optical spectrum of the spectrin–heme complex eluated from the gel (——); in the presence of dithionite as a reducing agent (– – –); optical spectrum of oxidized heme given for comparison (· · ·). Spectra were measured in the eluting buffer. Heme concentration was 1.9 μM in both cases.

photometrically characterized as native hemoglobin. One knows that at concentrations below a few nmol/ml, oxyhemoglobin becomes unstable. As the last step in the denaturation of hemoglobin is the release of the prosthetic group, we tried to estimate to what extent, if any, spectrin can interact with heme.

A mixture of spectrin with a 40-fold molar excess of heme was filtered through a Sephadex G-25 column. Part of the heme stays at the top of the column, and the other part is eluted together with spectrin (fig.2a). The optical spectrum of this fraction is shown in fig.2b. One can see that the heme which is bound to spectrin has a modified spectrum, as compared to free heme, with an increase in A_{400}. On the experiment shown in fig.2, spectrin is eluted from the column with a 10-fold molar excess of heme, as measured at 280 nm and in the visible at 557 nm after denaturation of spectrin and conversion of heme to an hemochrome derivative. From one experiment to the other, depending on the initial relative proportion of heme and spectrin, this ratio can vary. It indicates that the overall binding is not very strong. Preliminary titration experiments have shown that spectrin contains a heterogeneous population of sites which bind heme with different affinities. The optical spectrum of the chemically-reduced eluate of fig.2 shows no trace of hemochrome. These spectra alone do not identify the chemical nature of heme binding; however we cannot exclude the predominance of hydrophobic interaction between the heme pyrrole rings and some amino acid residues of spectrin.

3.2. *Fluorescence measurements*

Since heme is a potent inhibitor of protein fluorescence, we attempted to detect the binding of heme to spectrin fluorometrically. Upon mixing 1 μM spectrin with 1 μM heme there is a decrease of about 6% in the tryptophan fluorescence of spectrin. With 5 μM heme, there is a 12% quenching (fig.3). The kinetics of binding are fast and notably heterogeneous. When the same experiment was conducted with CO-hemoglobin instead of heme there was kinetically no trace of quenching although the stationary level of fluorescence observed was lower than that of spectrin alone. The possibility that the binding of spectrin to CO-hemoglobin could be so fast that the reaction would be completed in the dead time of the apparatus (2 ms) was considered. However the measured decrease

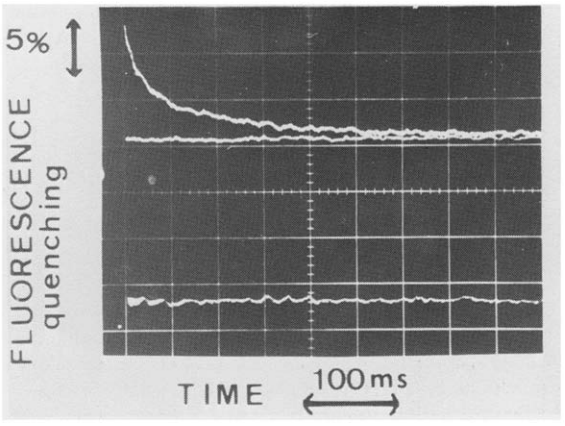

Fig.3. Kinetics of fluorescence quenching of spectrin by heme and oxyhemoglobin. The upper trace represents fluorescence quenching with heme, the lower trace corresponds to the same experiment made with oxyhemoglobin. Concentrations before mixing were: spectrin, 1.1 μM; heme, 5 μM; hemoglobin, 6 μM. Phosphate buffer, 50 mM, pH 6.6, 20°C.

in fluorescence corresponded only to that expected from the absorbance reduction in the intensity of exciting and emitting light in the optical cell, after mixing spectrin with hemoglobin.

3.3. *Functional studies on hemoglobin*

The detection of a possible interaction of spectrin with hemoglobin was tried by comparing some of the functional properties of hemoglobin measured in absence and in presence of spectrin. Kinetic measurements are very sentivite to small changes in molecular properties. Moreover, the computer facilities have allowed us to average up to 8 runs of the same experiment, further increasing the precision of the measurements. Kinetics for CO binding to deoxyhemoglobin, azide binding to aquomethemoglobin and oxygen dissociation from oxyhemoglobin were performed for equimolar (3 μM) solutions of hemoglobin and spectrin. There were no changes in the kinetics when the two proteins were allowed to interact (fig.4a–c). The possibility that spectrin binds preferentially to the hemoglobin tetramer or to the dissociated dimers, as it is the case for haptoglobin [9], has been considered by studying the kinetics of CO recombination after photodissociation of CO-hemoglobin alone and in presence of spectrin. In both cases (fig.4d) the relative proportion of the fast and slow phases, which

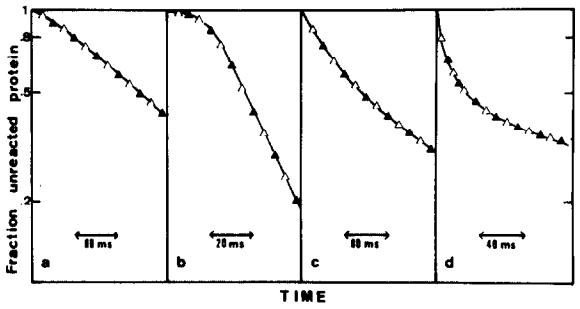

Fig.4. Kinetics of hemoglobin reactions with ligand in presence (▲) and absence (△) of spectrin. (a) CO binding: deoxyhemoglobin, 2.5 μM; CO, 46 μM; spectrin, 5 μM; 430 nm. (b) Deoxygenation: oxyhemoglobin, 2.5 μM; spectrin, 5 μM; 432 nm. (c) Azide binding: aquomethemoglobin, 5 μM; spectrin, 5 μM; azide, 50 mM; 405 nm. (d) CO recombination after photodissociation of CO-hemoglobin with a flash: CO-hemoglobin, 1.9 μM; spectrin, 4 μM; CO, 23 μM; 430 nm. Hemoglobin and spectrin were mixed for 30 min at 2°C before the experiments. The kinetics were measured in 50 mM phosphate buffer pH 6.6, 20°C. The indicated concentrations are those before mixing.

correspond, respectively, to the kinetics for the dimers and tetramers [10], were the same.

4. Discussion

The results presented in this work do not show evidence for an interaction between spectrin and hemoglobin in vitro. They are in conflict with a previous report: the experiment of fig.1 does not agree with the findings in [3]. The present results, obtained in experimental conditions favoring the best stability of hemoglobin, and the demonstration that spectrin has a strong affinity for heme (fig.2,3) suggest that in [3] some non-native form of hemoglobin is bound to spectrin.

The finding that spectrin binds heme possibly through hydrophobic interactions with the pyrrole rings is of some interest. It is highly probable that porphyrin and bilirubin do the same, as does serum albumin. In pathological cases of erythropoietic porphyria and Heinz body hemolytic anemia, these interactions could implicate a higher sensitivity of red cells to hemolysis through modifications of the spectrin matrix.

Acknowledgements

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