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Immobilized hemoglobin in the purification of hemoglobin-based oxygen carriers

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ABSTRACT

Chemically modified hemoglobins can be used as oxygen carriers in cell-free fluids provided that they have a low oxygen affinity and are stable towards dissociation into subunits. The latter species are undesirable because they are filtered rapidly through the kidneys, have renal toxicity and are characterized by a high oxygen affinity. A most important step in the preparation of hemoglobin-based oxygen carriers is therefore their purification from any dissociable material. Hemoglobin immobilized as $\alpha\beta$ dimers on Sepharose lends itself naturally to this purpose as it is able to interact in a specific and reversible way with soluble $\alpha\beta$ dimers. Hemoglobin affinity columns are very effective in the purification of cross-linked and pseudo-cross-linked human and bovine hemoglobin. The applicability of the technique is enhanced by the ease with which $\alpha\beta$ dimers from different species cross-interact to yield hybrid $\alpha_2\beta_2$ tetramers. It is shown that hemoglobin affinity columns may provide analytical information on the cross-linking reaction itself.

INTRODUCTION

In the search for alternatives to blood for transfusion or perfusion purposes, the use of cell-free hemoglobin as an oxygen carrier has been proposed by several laboratories [1]. The hemoglobin molecule has to be modified chemically to stabilize the tetrameric state and hence impede the rapid renal clearance of the dissociated $\alpha\beta$ dimers. Further, the oxygen affinity of the molecule has to be rendered similar to that of whole blood as the physiological modulators such as 2,3-diphosphoglycerate are no longer segregated in the red cells.

Among the most promising modifications of hu-

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man hemoglobin studied to date are those in which an intramolecular cross-link has been introduced between the βEF5 lysines [2] or between the αG6 lysines [3] or, alternatively, a pseudo-cross-link between the βEF5 lysines [4]. Such modifications endow human hemoglobin with the proper stability and oxygen affinity. Bovine hemoglobin has also been modified in similar ways [5,6]. Interest in this readily available protein is growing as it can be employed when the use of human hemoglobin may be unnecessary and too costly (*e.g.*, for organ perfusion and organ storage). Moreover, the stroma-free bovine hemoglobin has unusual oxygen-binding properties; owing to its marked sensitivity to chloride ions, at physiological concentrations of chloride its oxygen affinity is lower than that of human blood [7].

Irrespective of the source of protein, a most important step in the preparation of hemoglobins to be used as oxygen carriers is represented by the purification of the cross-linked material from all dissociable forms of hemoglobin which are undesirable owing to their high oxygen affinity and very short intravascular retention time and renal toxicity. On a laboratory scale, purification is achieved by ion-exchange chromatography [5]. On an industrial level advantage is taken of the higher thermal stability of the cross-linked material [8].

We propose the use of subunit-affinity columns of immobilized hemoglobin to purify hemoglobin-based oxygen carriers. Human hemoglobin is known to be immobilized on cyanogen bromide (CNBr)-activated Sepharose as $\alpha\beta$ dimers which maintain the capacity to interact in a highly specific and reversible way with soluble $\alpha\beta$ dimers under conditions where the latter are in association–dissociation equilibrium with the $\alpha_2\beta_2$ tetramers [9,10]. The experiments presented here demonstrate that immobilized $\alpha\beta$ dimers extract very effectively any dissociable hemoglobin from preparations of cross-linked or pseudo-cross-linked protein. The versatility of the approach is enhanced by the cross-interaction between dimers from hemoglobins of different species which leads to the formation of hybrid $\alpha_2\beta_2$ tetramers.

EXPERIMENTAL

Hemoglobin

Human hemoglobin (HbA) was prepared from expired blood obtained from the Blood Bank of the University Hospital in Baltimore following a published procedure [4]. Bovine hemoglobin (HbBv) was prepared from bovine blood using the standard procedure of Fronticelli *et al.* [5].

HbA and HbBv were pseudo-cross-linked between the βEF5 lysines by reaction with mono-(3,5-dibromosalicyl)fumarate (FMDA) as described in refs. 4 and 6, respectively. The pseudo-cross-linked hemoglobins, HbA–FMDA and HbBv–FMDA, are predominantly non-dissociable tetramers stabilized by electrostatic and hydrophobic interactions.

HbA covalently cross-linked between the two αG6 lysyl residues with a fumaryl residue (α–αXL HbA) [3] was a gift from the Department of Defense (LAIR, San Francisco, CA, USA). Following the chemical modification, the cross-linked material was purified by heating for 10 h at 60°C in the presence of reducing agents such as sulfites and dithionite. This treatment sterilizes the preparation and precipitates the residual less stable non-cross-linked material; α–αXL HbA is a tetramer which does not dissociate into dimers [3]. Typically the preparations have concentrations of 13 g/dl and contain 10–13% of iron(III) hemoglobin.

HbBv was cross-linked covalently with a fumaryl residue (β–βXL HbBv) by treatment of either the oxygenated or the deoxygenated derivative with bis (3,5-dibromosalicyl) fumarate as described in refs. 11 and 5, respectively. Reaction of the oxygenated protein leads to cross-linking between the two βEF5 lysyl side-chains, whereas reaction of the deoxygenated protein modifies both the α- and the β-subunits [5]. Unless stated otherwise, the protein cross-linked in the oxygenated state was purified by high-performance liquid chromatography on a Toyopearl 650M DEAE anion-exchange resin (Toso-Haas) using a gradient formed by 0.015 *M* Tris buffer (pH 8.0) and 0.015 *M* Tris buffer (pH 7.7) in 0.2 *M* sodium acetate; the main fraction was collected and concentrated by forced filtration. In contrast, the protein cross-linked in the deoxygenated state was not purified and thus contained both non-reacted and over-reacted protein, namely protein reacted at sites other than βEF5 and/or αG6. In all

instances, purified preparations of β–βXL HbBv are stable tetrameric hemoglobins.

Coupling procedure

Covalent coupling of hemoglobin was performed on Sepharose 4B activated by the CNBr method or on commercial CNBr-activated Sepharose 4B (from Pharmacia, Uppsala, Sweden). The concentration of immobilized protein was determined by means of spectrophotometric measurements on a Cary 14 spectrophotometer using a 2-mm cell; the effect of turbidity was minimized by the use of protein-free gel in the reference beam as described [10].

Determination of associating capacity of immobilized hemoglobin

The capacity of the various preparations of immobilized hemoglobin to interact with the soluble protein was assessed with a chromatographic column containing 5–6 ml of immobilized protein, thermostated at 8–10°C and eluted at a constant flow rate of 10–12 ml/h. The absorbance of the effluent was monitored at 541 nm with a Gilford apparatus equipped with flow-through cells. The experiments were of two kinds. For a semi-quantitative indication of the associating capacity, a small volume (1 ml) of protein solution in associating buffer [0.1 *M* phosphate buffer (pH 7.0)] was percolated through the column equilibrated with the same buffer. For a quantitative measure of the associating capacity the column was saturated, *i.e.*, sufficient protein solution was applied until a steady state was reached and the absorbance of the effluent was the same as that of the inflowing solution. In either type of experiment, interaction of the soluble protein with the immobilized protein results in an increase of the elution volume with respect to a non-interacting protein, *i.e.*, with respect to the void volume of the column, V_0 [12,13]. The column was freed from the retained protein with dissociating buffer, 1.98 *M* NaCl–0.01 *M* phosphate (pH 6.7) [9,10].

Gel electrophoresis

The fractions eluted from the hemoglobin affinity column were analyzed by polyacrylamide gel electrophoresis (PAGE) performed with the buffer system of Laemmli [14] at pH 8.6 in the presence of 0.1% sodium dodecyl sulfate (SDS); the stacking and running gels were 5 and 15% in acrylamide, respectively.

Intravascular retention time in the rat

The protocol described by Urbaitis *et al.* [15] was used. The rats received a bolus injection of 20 mg per 100 g body weight. Timed blood and urine samples were collected and analyzed for their hemoglobin content. Semi-logarithmic plots of hemoglobin concentration *vs.* time allowed the half-time of intravascular retention to be calculated. The urine samples were used to determine the fraction of hemoglobin eliminated through the kidneys.

RESULTS AND DISCUSSION

Effect of coupling conditions on the associating capacity of immobilized hemoglobin and formation of hybrid tetramers

Previous experiments on human hemoglobin immobilized on CNBr-activated Sepharose 4B have shown that the capacity of immobilized $\alpha\beta$ dimers to interact specifically with $\alpha_1\beta_1$ dimers in solution is influenced markedly by the coupling conditions [9,10]. Hence the associating capacity of the hemoglobin subunit affinity columns, expressed as milligrams of hemoglobin retained per milligram of immobilized protein, was assessed for bovine and human hemoglobin coupled to CNBr-activated Sepharose under the conditions detailed in Table I. In line with observations by Rossi Fanelli *et al.* [10], the highest capacity is displayed by the protein immobilized in the presence of ethanolamine, which facilitated single-point attachment of the protein to the matrix, thus leaving the subunit interfaces unaltered.

Thereafter the capacity of immobilized HbA and HbBv to cross-interact with soluble hemoglobin from the other species was determined. The results, included in Table I, show that immobilized hemoglobin interacts essentially with the same strength with either hemoglobin in solution. This finding reflects the ease with which hybrid tetramers are formed owing to the similarity of the relevant $\alpha\beta$ interfaces in the two proteins [9,10].

Purification of cross-linked hemoglobin from the non-cross-linked protein

After the characterization of their associating ca-

TABLE I

EFFECT OF COUPLING CONDITIONS ON THE ASSOCIATING CAPACITY OF IMMOBILIZED HEMOGLOBIN DIMERS

In all experiments, 1 ml of solution was applied.

Sample No.	Hb immobilized	Coupling conditions[a]	Hb per ml Sepharose (mg)		Hb applied		Hb retained per 100 mg Hb immobilized (mg)
			Added	Immobilized	Type	Concentration (mg/ml)	
1	HbBv	1	6.3	5.2	HbBv	10	8.3
2	HbA	1	6.0	6.0	HbA	9.2	7.8
					HbBv	18	8.1
3	HbA	2	4.6	3.7	HbA	9.7	8.6
					HbA	2.4	7.5
					HbBv	10.3	7.3
4	HbA	3	8.1	6.8	HbA	10.5	15.0
				6.5[b]	HbA	12.7	11.0
5	HbA	3	17.5	11.1	HbA	15.5	17.0

[a] Coupling conditions 1: commercial CNBr-activated Sepharose 4B, coupling carried out under standard Pharmacia conditions, namely 0.1 *M* $NaHCO_3$ (pH 8)–0.5 *M* NaCl for 2 h at room temperature, deactivation with 1 *M* ethanolamine. Coupling conditions 2: commercial CNBr-activated Sepharose 4B, coupling in 0.1 *M* phosphate buffer (pH 7.8) for 18 h in the cold. Coupling conditions 3: Sepharose 4B activated with 600 mg of CNBr per 12 ml of resin, coupling in 0.1 *M* $NaHCO_3$ (pH 8) containing 0.012 *M* ethanolamine for 1 h at room temperature, deactivation with 1 *M* ethanolamine.

[b] Experiment performed after keeping the immobilized hemoglobin for 4 months in the cold in the presence of sodium azide.

pacity, the different preparations of immobilized hemoglobin were employed to separate cross-linked from non-cross-linked hemoglobin. Only the latter protein is expected to interact with the hemoglobin affinity column as the cross-linked proteins, namely α–αXL HbA and β–βXL HbBv, which are stable tetramers, do not undergo reversible association–dissociation reactions into subunits and therefore elute in the void volume, V_0.

The use of immobilized HbA is depicted in Fig. 1. In all experiments 1 ml of soluble hemoglobin was applied to the column. First the capacity for soluble HbA was calculated to be 4.2 mg (panel A). In the control experiment depicted in panel B, in which the amount of hemoglobin percolated was less than the column capacity (1.4 mg), all the protein was retained on the affinity column and eluted only after application of dissociating buffer. Thereafter α–αXL HbA purified by heat treatment at 60°C was applied to the column (panel C); most of the protein eluted in the void volume, but 5.4% was retained, indicating that this amount of dissociable hemoglobin had been left in solution after heat treatment. HbBv cross-linked in the oxygenated state (β–βXL HbBv) displayed a similar behaviour in that part of the protein was retained on the affinity column. On the other hand, β–βXL HbBv purified by ion-exchange chromatography contained no dissociable material, demonstrating the effectiveness of this purification procedure (panel D). Panel C also shows that on SDS-PAGE [14] the mobility of the retained protein corresponds to that of hemoglobin chains (lane c); thus, as expected, exclusively dissociable αβ dimers interact with the hemoglobin affinity column.

For large-scale purifications the column capacity, of course, has to be fully exploited in saturation experiments. Two major factors determine the extent to which a given subunit affinity column can be loaded under a set of experimental conditions, namely the amount of matrix-bound polymer formed per milligram of immobilized protein and the elution volume difference between the interacting protein and any other non-interacting protein present in solution. Both properties depend on the solution concentration of the interacting protein, but act against each other: when the concentration of soluble interacting protein is low, the difference in elution volume between the interacting protein and any other protein in solution is large; however,

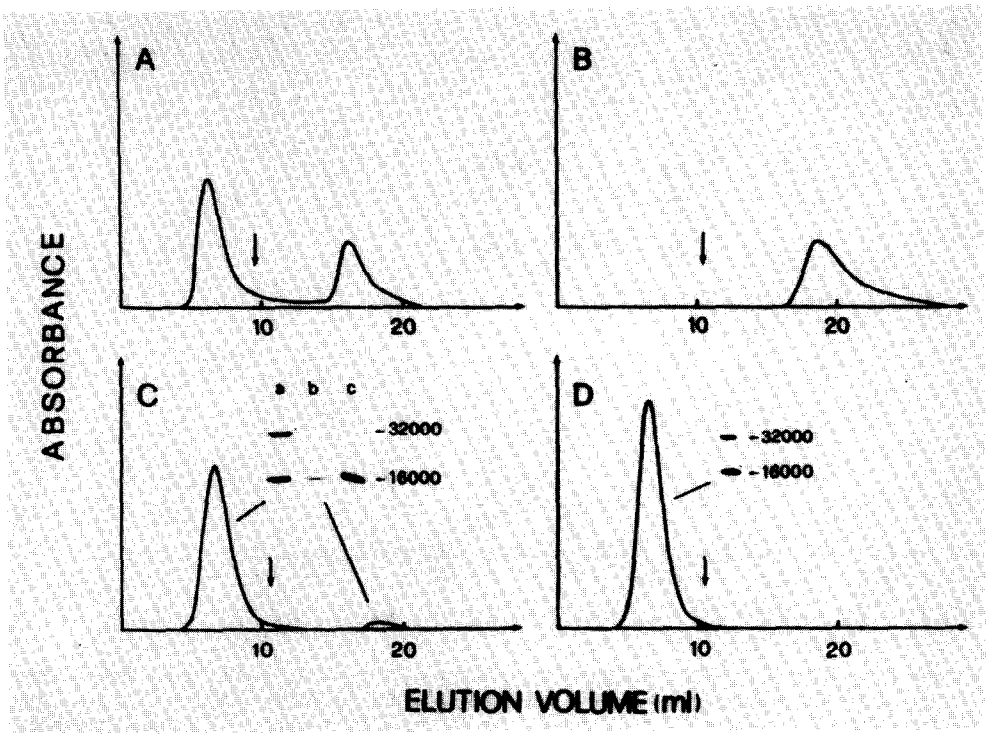

Fig. 1. Elution profiles of native and cross-linked hemoglobin solutions from a column containing Sepharose-bound HbA. The column (6 ml) was thermostated at 8°C and equilibrated with associating buffer, 0.1 *M* phosphate buffer (pH 7.0); 1-ml pulses of the various hemoglobin solutions in the same buffer were applied. Arrows indicate the application of dissociating buffer, 0.01 *M* phosphate buffer–1.98 *M* NaCl (pH 6.7). A = HbA at 12.7 mg/ml; B = HbA at 1.4 mg/ml; C = α–αXL HbA at 5.2 mg/ml; D = β–βXL HbBv, cross-linked in the oxygenated state and purified by ion-exchange chromatography, at 10.3 mg/ml. The inset of panel C shows the SDS-PAGE patterns of the fractions indicated on the elution profile (a and b) and of HbA (c). Lane a = α–αXL dimers and β-chain monomers; lanes b and c = α- and β-chain monomers. The inset of panel D shows the SDS-PAGE pattern of the indicated fraction containing β–βXL dimers and α-chain monomers.

the amount of protein retained is low owing to a mass action effect [10,12,13]. Hence, unless the cross-linking procedure is standardized to yield similar concentrations of dissociable protein in the mixture, it is best to check every preparation of cross-linked hemoglobin on a small scale before performing a large-scale purification.

A column saturation experiment involving immobilized HbA to purify HbBv cross-linked in the deoxygenated state is given in Fig. 2. This specific example also illustrates that hemoglobin affinity columns may yield information on the cross-linking reaction itself. The occurrence of modifications at sites other than lysines αG6 and βEF5 was indicated by SDS-PAGE experiments which showed a substantial amount of tetramers, corresponding to overreacted hemoglobin, in addition to the expected dimeric and monomeric species (compare with panels and C and D in Fig. 1). The component retained on the column, analysed by SDS-PAGE, contained not only monomeric species derived from unmodified, dissociable αβ dimers, but also dimeric species deriving from undissociable $\alpha_1\beta_1$ dimers. Such dimers, which are absent in the purified cross-linked material [5], are still able to interact with the immobilized protein.

Purification of pseudo-cross-linked hemoglobin

HbA–FMDA and HbBv–FMDA are stabilized tetramers at neutral and slightly acidic pH values on the basis of sedimentation velocity experiments [4,6]. Consistent with this observation, a major fraction disappears from plasma in the rat at significantly slower rates (half-time ≈ 190 min) than na-

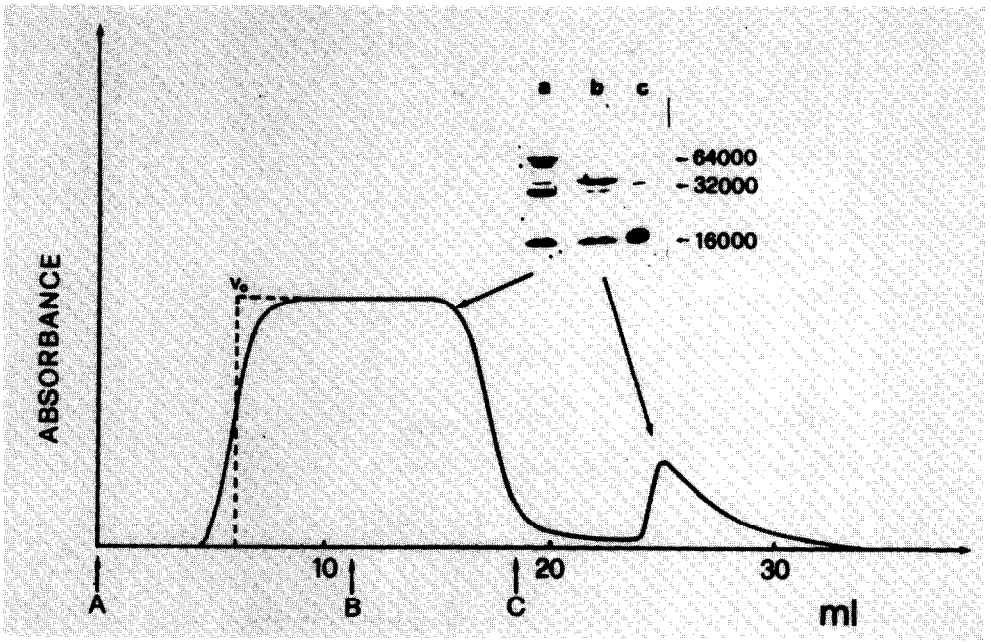

Fig. 2. Purification of dissociable hemoglobin from HbBv cross-linked in the deoxygenated state by means of immobilized HbA. Experimental conditions as in Fig. 1. Arrows indicate application of (A) cross-linked bovine hemoglobin at 5 mg/ml, (B) associating buffer and (C) dissociating buffer. The inset shows the SDS-PAGE pattern of the starting solution (lane a, any fraction in the plateau region), of the retained protein (lane b) and of HbA (c). The high-molecular-mass bands correspond to cross-linked tetramers and dimers in lane a and to $\alpha_1\beta_1$XL dimers in lane b; the 16 000 M_r band corresponds in all three lanes to α- and β-chains.

tive HbA and HbBv (half-time 40 min). However, a fraction is excreted very quickly (half-time $\approx$ 20 min); its relative amount, although erratic, is always higher in HbBv–FMDA [6].

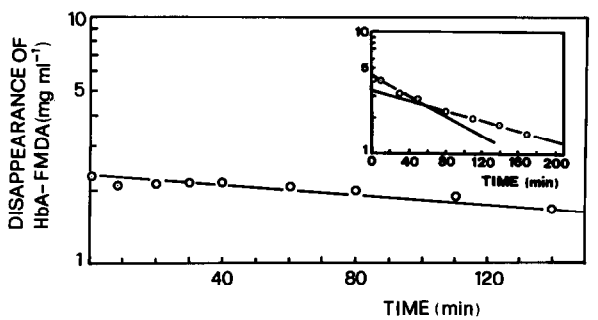

Fig. 3. Time course of disappearance from plasma of purified pseudo-cross-linked HbA–FMDA in the rat. The inset shows the biphasic curve obtained from the same material before purification on the hemoglobin affinity column. In the inset the fast component has a half-time of retention of 20 min and the slow component the same half-time as the purified material, 190 ± 10 min. The experimental protocol is described under Experimental.

In order to check whether the minor component filtered quickly by the kidney corresponds to dissociable hemoglobin, HbBv–FMDA was applied to a column of immobilized HbA in a pulse experiment. Surprisingly, most of the material (about 75%) behaved as dissociated hemoglobin and was retained on the column. In order to verify the possible existence of a slow dissociation equilibrium in the small fraction containing tetrameric protein which eluted in the void volume, this component was applied again to the hemoglobin affinity column on the following day. No protein was retained, pointing to the absence of a slow equilibrium.

A similar behaviour was observed with HbA–FMDA. In this instance the stability towards dissociation of the HbA–FMDA tetramer purified by means of the hemoglobin affinity column was checked *in vivo* by measuring the intravascular retention in the rat. The slope of the intravascular retention, shown in Fig. 3, was monophasic with a half-time (190 ± 10 min) similar to that measured for intramolecularly cross-linked hemoglobins [5].

This finding indicates that the purified species is stable also *in vivo*. There is, however, an inconsistency between the experiments *in vivo*, where the major fraction is retained by the kidney as a non-dissociable tetramer, and *in vitro*, where the major fraction interacts with the affinity column as a dissociable tetramer. Any explanation will reflect the difficulty of comparing biological and biochemical data. It is possible that the capillaries in the kidney favour or are neutral with regard to tetramer formation, whereas the column favours the dissociation of pseudo-cross-linked species. More probably the reason lies in the presence of isomeric species that are not resolved by the standard purification of the pseudo-cross-linked material and are recognized differently by the glomeruli in the kidney and by the immobilized hemoglobin in the column.

CONCLUSIONS

The experiments presented here demonstrate that hemoglobin affinity columns can be used effectively to purify modified hemoglobins to be used as oxygen carriers. The versatility of the technique is enhanced by the cross-interaction between dimers from different hemoglobin species, which reflects the ready formation of hybrid hemoglobin tetramers in solution; hence, for example, a column of bovine hemoglobin can be used to purify the human protein.

In addition, hemoglobin affinity columns may provide analytical information. They can be used to establish whether other purification techniques are effective in removing all the non-cross-linked material. More importantly, they can furnish information on the modification reaction itself, as exemplified by the case of bovine hemoglobin cross-linked in the deoxygenated state in which formation of intradimer cross-links in $\alpha_1\beta_1$ dimers was demonstrated for the first time.

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