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Journal of Chromatography B, 694 (1997) 39–48

JOURNAL OF CHROMATOGRAPHY B

New support for the affinity chromatography of hemoglobin

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Received 12 June 1996; revised 29 January 1997; accepted 6 February 1997

Abstract

A new support for affinity chromatography of hemoglobin was synthesised from EAH Sepharose-4B containing a hexamethylamine spacer. Benzenetetracarboxylic (BTC) or benzenehexacarboxylic (BHC) acids were covalently bound to the spacer arm. At pH close to the p*I* of the protein, the biospecificity of the support due to the interactions of the allosteric site of hemoglobin with immobilised polyanionic ligands was proved. When the allosteric site was blocked by covalently linked pyridoxalphosphate, the protein showed no more affinity for the support. Further investigations were done on the BHC support; the association constants between BHC support and the hemoglobin forms, oxyhemoglobin and deoxyhemoglobin, were determined. The deoxyhemoglobin affinity was ten times higher than that of oxyhemoglobin, both for fixed and for free ligand. The following values of binding constants K_{PX} and K_{PL} ($l\,mol^{-1}$) with fixed or free ligand respectively were found: for oxyhemoglobin, $K_{PX}=8.0\cdot10^{2}$, $K_{PL}=1.4\cdot10^{4}$; for deoxyhemoglobin, $K_{PX}=9.7\cdot10^{4}$, $K_{PL}=2.3\cdot10^{5}$. The BHC support capacity was about $4.7\cdot10^{-5}$ mol hemoglobin g^{-1} of dry gel corresponding to $1.5\cdot10^{-6}$ mol hemoglobin g^{-1} of hydrated gel or 0.1 g hemoglobin g^{-1} of hydrated gel.

Keywords: Hemoglobin; Benzenehexacarboxylic acid; Benzenetetracarboxylic acid; Oxyhemoglobin; Deoxyhemoglobin

1. Introduction

Among the possible oxygen-carrier blood substitutes, attention is focused on perfluorocarbons and a variety of hemoglobin preparations. The description of their properties is not the purpose of this paper as many specialised publications treat this topic [1–4]. Early methods for preparing hemoglobin solutions for use as a blood substitute involved hypotonic lysine of human red cells followed by crude separation of the soluble hemoglobin from the insoluble cell membranes. Another approach is the adaptation of recombinant DNA technology and the production of human hemoglobin in transgenic animals [5–8] that would enable the production of large quantities of human hemoglobin.

However, first and foremost, the product must be pure, free from contaminants that could induce toxicity. From recent research it has become clear that thorough purification of the hemoglobin solutions strongly lowers the risk of side effects. The criteria for safety evaluation of hemoglobin and perfluorocarbon based oxygen-carriers were elaborated and published [9] by the 'Workshop on Criteria for Red Cell Substitutes' held in Maryland in 1994 and sponsored by the Food and Drug Administration and different health institutions. Chromatographic purification methods are based mainly on weak ion-exchange supports, especially on the laboratory scale [10], or on affinity chromatography techniques.

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PII S0378-4347(97)00113-8

Affinity chromatography is based on the complementary relationship among the structure, the charge and the hydrophobicity between a protein and a stationary phase. The affinity supports are often designed to mirror the natural ligand to which the protein binds. The biospecific recognition between the two molecules is the result of complex multivalent interactions. Concerning hemoglobin affinity chromatography, different approaches were described.

Hemoglobin [11] is composed of four polypeptide chains called globins, two α and two β, each chain consisting respectively of 141 or 146 amino acids. The four globins are assembled together and form a central cavity containing the allosteric site. The allosteric site walls present positively charged amino acids which bind 2,3-diphosphoglycerate (2,3 DPG), the natural regulator of hemoglobin affinity for oxygen. The oxygenation of hemoglobin occurs with the rearrangement of the four units followed by the shrinking of the central cavity and the expulsion of 2,3 DPG. Anions other than 2,3 DPG can be fixed in the cavity and lead in the same way to the decrease of hemoglobin affinity for oxygen. Such anions are considered as effectors of hemoglobin. Hsia [12] proposed the chromatographic purification based on this principle. He immobilised polyanions (nucleotide triphosphate, diphosphoglycerate, inositolhexaphosphate and inositolhexasulphate) on crosslinked polysaccharides or on silica gel, linked to the chromatographic support by a hydrophobic spacer. Hemoglobin was thus retained on the gel, while impurities were eluted.

Another approach towards hemoglobin affinity chromatography is based on its easy glycosylation, in vivo or in vitro, by free glucose. A new support made from polyglucose chains linked by α-1,4-glucosidic bonds was used for selective hemoglobin retention and single step purification and concentration of erythrocyte hemoglobin [13]. The authors suggest that hemoglobin specifically recognises the polyglucose chains linked with α-1,4-glucosidic bonds since no affinity was observed for crosslinked dextran with α-1,6-glucosidic bonds.

A very important step in the preparation of hemoglobin based oxygen-carriers is its purification from any dissociable (toxic) material (hemoglobin has a tendency to dissociate into its subunits: α, β dimers). Chiancone et al. [14] immobilised hemoglobin as dimers on Sepharose. The bonded dimers are able to interact in a specific and reversible way with soluble dimers and thus eliminate them from the purified solution. Hemoglobin affinity columns were very effective in the purification of crosslinked and pseudo-crosslinked human and bovine hemoglobin.

Our affinity support is based on the natural affinity of hemoglobin for polyanions. In our previous work on hemoglobin oxygen carriers [15,16] we used the affinity of hemoglobin for polyanions to design and synthesise some conjugates of the protein with polymers substituted with benzenetetracarboxylic acid (BTC) or benzenehexacarboxylic acid (BHC). In this paper we present the synthesis of new supports based on Sepharose and containing BTH or BHC ligands linked by a hexamethylamine spacer, as well as the study of the hemoglobin interaction via its allosteric site with the fixed polyanions.

2. Experimental

2.1. Support

EAH Sepharose 4B containing hexamethylamine spacer was purchased from Pharmacia (Uppsala, Sweden). According to the commercial information the gel is formed of particles of 45–165 μm; the spacer arm concentration is 6–10 μmol ml^{-1} of hydrated gel and the pH range of utilisation is between 3 and 14.

2.2. Chemicals

Benzenetetracarboxylic (BTC), M_r=254.15 and benzenehexacarboxylic acid (BHC) M_r=342.17 were obtained from Aldrich (Steinheim, Germany), as well as the coupling agent 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDCI). The buffers used for chromatography were tris-(hydroxymethyl)aminoethane (Tris), pK_a 7.83, from Merck (Darmstadt, Germany), and 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bis–Tris) pK_a 6.5 from Aldrich.

Human hemoglobin (Hb) solutions were prepared by the Centre Régional de Transfusion Sanguine

(CRTS), Nancy, France, with hemoglobin concentrations 60–90 $g\,l^{-1}$. The exact hemoglobin concentration and the proportion of different hemoglobin forms (oxy-, deoxy- and methemoglobin) were determined by absorption measurements at four different absorption wave lengths [17,18]. We used Hb solution containing about 90% of oxyhemoglobin (oxyHb), 5% of methemoglobin (metHb) and 5% of deoxyhemoglobin (deoxyHb). Hemoglobin with its allosteric site blocked by covalently bound pyridoxalphosphate (Hb-PLP) was prepared at the Pharmaceutical Faculty in Nancy, France (Prof. Vigneron). The relative molecular mass of hemoglobin is 64 500. The isoelectric points (p*I*) of hemoglobin and Hb-PLP were measured by the electrofocusing method on acrylamide gel in CRTS laboratories. Thus the p*I* of Hb-PLP was found to be that of hemoglobin J. Broussais, i.e. 6.8 and the p*I* of unmodified hemoglobin was only very slightly higher, about 6.9. We can, therefore, consider that both molecules have very similar overall surface charges, the only difference being the accessibility to the allosteric site.

Bovine serum albumin from Serva (Heidelberg, Germany), has about the same relative molecular mass (66 400) as hemoglobin but a different structure and its p*I* is about 4.9.

Horse myoglobin from Sigma (St. Louis, MO, USA), has a structure very similar to that of one of four subunits of hemoglobin and the same p*I* (about 6.9). Its relative molecular mass is 16 700 and it has no allosteric site.

KNO_3 from Fluka (St. Queentin Fallavier, France), $M_r = 101.1$: was used to measure the hold up volume (V_o) since NO_3^- is not retained by an anionic column.

2.3. Chromatographic conditions

All chromatographic experiments were realised in a jacketed column 20 cm×9 mm I.D. from Pharmacia (Orsay, France), thermostated at 5°C. The eluent flow (about 22 $ml\,h^{-1}$) measured exactly at the column outlet, was provided by a peristaltic pump Minipuls 2 from Gilson (Villiers le Bel, France). We used UV absorbance detection at 276 nm in all experiments except where the competitor, free BHC, was added to the eluent, (strong BHC absorption in UV); in this case hemoglobin was detected at 415 nm.

2.4. Synthesis of affinity support

The biospecific interaction between the immobilised ligand and its macromolecular counterpart must not be hampered by steric hindrance. We therefore used a matrix with a spacer, the commercially available EAH Sepharose 4B containing 1,6-diaminohexane spacer bound to the matrix and ending in a free amino group. We used the carbodiimide coupling procedure to fix the carboxylic ligand to the amino spacer (activation of carboxylic group by carbodiimide, followed by the condensation of activated carboxylate with Sepharose-linked amino group producing stable amide linkage).

The commercially available EAH Sepharose 4B (containing hexamethylamine spacer) was washed with a great amount of distilled water to get rid of conservation agent. Four syntheses were carried out under different conditions (with 20 or 50 ml of gel at pH 6 or 8) in order to find the optimal parameters. Slightly better results were obtained at pH 6. The following procedure was chosen for routine synthesis: 20 ml of gel was gently mixed with 15 ml of distilled water and the pH was adjusted to pH 6 by 0.01 *M* HCl. As both BTC and BHC are polyfunctional agents which can react with several amines, to avoid or to limit the possible crosslinking, the tenfold molar ratio of acid to amine was applied: 0.55 g of BHC or 0.40 g of BTC was dissolved in 1 *M* NaOH and pH was adjusted to 6. The acid solution was then slowly poured onto the gel under gentle mixing. The coupling agent EDCI (EDCI/acid molar ratio=1) was added in small portions and the reaction was carried out for 3 h. The substituted gel was then thoroughly washed with water until no traces of free polycarboxylic acid could be observed in UV spectrum. To remove the excess uncoupled ligand which might have remained ionically bound to the gel, several washings with pH 8.3 buffer, 1 *M* NaCl solution and distilled water were performed. The spacer non-reacted amine groups were blocked by acetylation procedure [19] to avoid ionic interactions with hemoglobin.

2.5. Analysis of the synthesised supports

The amount of BTC or BHC ligands linked to Sepharose was determined by UV absorption after hydrolysis of the bond. The ligands are supposed to be fixed by the amide bond to the amine spacer. The acid hydrolysis releases free BTC or BHC and their amount can be determined by spectrophotometric analysis. The complete ligand-support bond breaking depends on pH, temperature and hydrolysis duration. Firstly we optimised the conditions of pH from 0 to 4 and different temperatures up to 40°C, as well as different durations. It was found that the pH should be very acidic (pH≤1) and a hydrolysis time of 2 h at 40°C was necessary.

In order to establish whether some BHC or BTC molecules were fixed by ester bonds to OH groups of Sepharose, we also studied the hydrolysis with NaOH at pH 13 where only ester linkages were cut while HCl hydrolysed the ester and amide functions. These experiments verified that all ligands were attached by amide bonds to the spacer arm.

As the exact amount of gel (mass or volume) was difficult to determine because of the varying amount of water, we weighed the gel sample after 12 h freeze-drying and all results reported in the following text concern 1 g of freeze-dried gel.

Analysis procedure: a gel sample was washed, weighed and then hydrolysed at 40°C for 2 h in 1 *M* HCl. The absorbance of filtered solution containing released BTC or BHC ligands was measured at 240 nm. The reproducibility of synthesis was good. The mean value and standard deviation of BTC substitution obtained on three batches was $2.4\pm0.2\cdot10^{-4}$ mol BTC g^{-1} of support. Four syntheses of BHC support also gave reproducible results: $3.0\pm0.3\cdot10^{-4}$ mol BHC g^{-1}. In both cases 50–60% of spacer arms were substituted (Sepharose 4B has about $5\cdot10^{-4}$ mol NH_2 g^{-1} dried gel).

3. Results and discussion

3.1. Chromatographic behaviour of the new supports

The interactions of proteins with chromatographic support can be of different types: ionic, hydrophobic, Van der Waals, etc., or sometimes the result of multiple interactions can give a specific binding. Hemoglobin is composed of numerous amino acids providing the protein surface with positive or negative charges or hydrophobic areas which are the sites of possible interactions, according to pH conditions. We studied the interactions of hemoglobin with linked BTC or BHC ligands as a function of the pH of the eluent and we also examined the selectivity of the support i.e. its specificity toward the allosteric site of hemoglobin.

3.2. Specificity of interaction

The Hb samples contain about 90% oxyHb, 5% metHb and 5% deoxyHb. OxyHb has much lower affinity for anionic ligands than deoxyHb which is relevant to respiratory physiology [11,20] since the anionic ligand 2,3 DPG regulates the affinity of Hb for oxygen. We examined the behaviour of our supports with Hb samples and we obtained chromatograms containing a major peak of oxyHb and much smaller peaks of metHb and deoxyHb identified by prepared standards of metHb and deoxyHb. Thus we studied the interaction of the supports with the oxyHb component of Hb. In the following text, if not specified, the term Hb means oxyHb. In further studies concerning measurement of affinity constants we worked under special conditions in order to measure each binding constant (i.e. in nitrogen atmosphere in order to have hemoglobin converted to deoxyhemoglobin form).

The interactions of hemoglobin with the support containing BTC or BHC ligands were studied in the pH range 6.6–7.7 using Bis–Tris or Tris buffers as eluent, varying the pH values by 0.1 unit. After the chromatographic measurements at given pH, the column was washed with Tris at pH 8 in order to eliminate all possible remaining hemoglobin. The eluent pH values were adjusted at 20°C.

Our attention was focused on three zones: pH<p*I* where the protein has a positive global charge, pH> p*I* where hemoglobin is negatively charged and pH= p*I* where the protein has neutral overall surface charge. The chromatograms obtained for hemoglobin containing 95% of oxyHb, 5% metHb and 5% deoxyHb are represented in Fig. 1. For better legibility only chromatograms at certain pH values

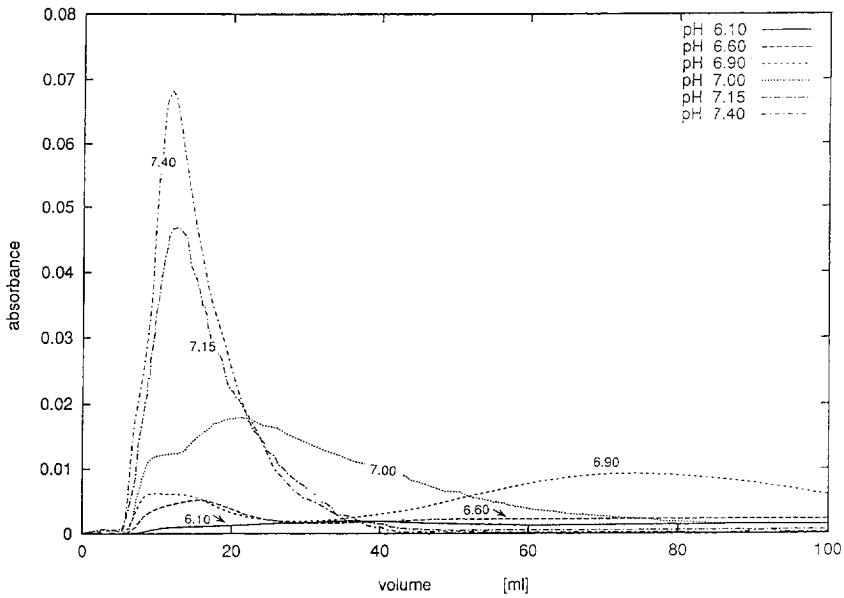

Fig. 1. Chromatograms of hemoglobin (oxyhemoglobin) on a BHC support at different eluent pH. Flow-rate: 22 ml h^{-1}. Column contained 5.5 g of hydrated gel corresponding to $8 \cdot 10^{-6}$ mol of active BHC ligands. Detection at 415 nm.

are shown, knowing that intermediate chromatograms have the same tendency. At pH<7 two more or less separated peaks can be observed, the major peak due to oxyHb is preceded by small metHb peak (metHb is formed by the oxidation of oxyHb). The deoxyHb peak sometimes slightly observable at great elution volumes could not be seen in the figure.

In the presence of both kinds of supports (BHC and BTC ligands) the same behaviour was observed:

*at pH = p*I* = 6.9

At this pH we observed very strong retention of Hb, (elution volume of about 75 ml) which was supposed to be due to the specific interaction with the Hb allosteric site. Other proteins, albumin, myoglobin or hemoglobin having its allosteric site blocked by covalently bound pyridoxalphosphate (Hb-PLP), were not retained and were eluted in the hold up (V_o) volume (measured by KNO_3) and which corresponded to 7–8 ml.

Albumin was chosen as a reference substance because it cannot interact with our supports in any way: it has about the same relative molecular mass as Hb but it is negatively charged at the studied pH range (pH 6–8), thus theoretically it cannot be retained by negatively charged ligands. The albumin elution in V_o volume proved also that no size exclusion phenomena were present. Myoglobin has the same p*I* as Hb but it has no allosteric site as it is formed of only one globin. It can interact only by possible partial surface charges. The absence of interaction at this pH suggests that no surface partial charges interact with the column ligands.

Hb-PLP has its allosteric site covalently blocked by PLP. It is obvious that the blocked allosteric site cannot interact with the polyanionic ligands and the absence of Hb-PLP interaction with linked BTC or BHC ligands at pH 6.9 and its elution in volume V_o, of about 7 ml (Fig. 2) confirms the specific interaction of Hb via its allosteric site.

*pH > p*I*

If the eluent pH increased above p*I*, the Hb retention rapidly decreased and the elution volumes were of about 20 ml or less (Fig. 1). The Hb overall surface charge became increasingly negative with rising pH and consequently the Hb specific interaction was progressively cancelled by the increasing repulsion between the ligands and the Hb surface and thus the elution became faster and faster. The Hb-

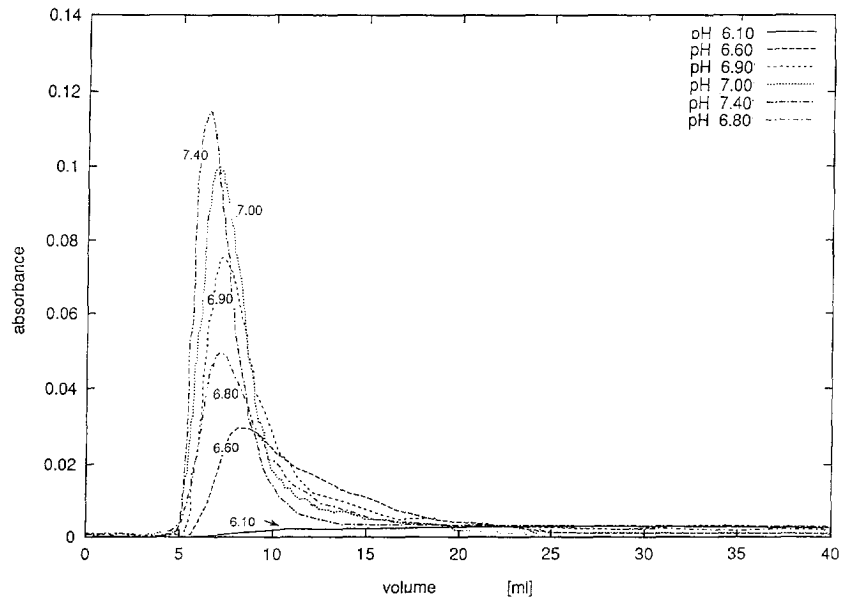

Fig. 2. Chromatograms of pyridoxylhemoglobin on BHC support at different eluent pH. Flow-rate: 22 ml h^{-1}. Column contained 5.5 g of hydrated gel corresponding to $8 \cdot 10^{-6}$ mol of active BHC ligands. Detection at 415 nm.

PLP elution did not change and remained in volume V_o at about 7 ml (Fig. 2).

*pH < p*I*

At pH<p*I* a strong oxyHb retention was observed but a new phenomenon was noticed: Hb-PLP also started mildly interacting with the column as the pH was moved away from the p*I* value (see pH 6.6 curve in Fig. 2 with elution volume slightly raising to about 8 ml). At pH 6.1, Hb-PLP as well as Hb were strongly retained as can be observed on curves pH 6.1 in Figs. 1 and 2. In the range of pH<p*I*, both proteins (Hb and Hb-PLP) take a positive charge leading to ionic interactions with column ligands, increasingly stronger as pH decreases. At pH 6.1, Hb interacted with COO^- column ligands obviously by non-specific electrostatic attraction due to its surface charges because all forms of hemoglobin were retained by the column: a small peak corresponding to metHb, a major peak corresponding to oxyHb (Fig. 1, pH 6.1 curve), as well as Hb-PLP (Fig. 2, pH 6.1 curve). It seems that as pH decreases below 6.9 the specific interaction of Hb with BTC or BHC ligands via allosteric site existing at pH=p*I*, started to be overlapped or replaced by non-specific ionic interactions at pH 6.6 and the latter became evident when pH reached 6.1. At pH close to p*I*, Hb interacts with BHC or BTC column ligands in a specific way via its allosteric site. Therefore, our support can be considered as an affinity chromatography support.

Further extensive investigations were done on a BHC ligand column and we present the obtained results below.

3.3. Capacity of the support

The capacity of BHC support was determined at pH 6.9 from the equilibrium isotherm using the frontal elution method [21]. Assuming that one Hb molecule is bound to one active BHC ligand, the number of Hb molecules retained by the column at saturation, Q_s, gives the number of active ligands, Q_x, or the capacity of the column. The amount of hemoglobin molecules, Q_s, which were retained by the chromatographic support was measured from the difference between the average retention volume V and V_o of the elution front: $Q_s = c(V - V_o)$, c being the solute concentration in mobile phase used for the saturation experiment. The number of active BHC

ligands, Q_x, is different from the number of linked ligands, because due to steric hindrance all the ligands cannot interact with Hb.

The column contained 9.55 g (corresponding to 8.9 ml) of hydrated gel (or 0.296 g of dried gel). Hemoglobin was added to the reservoir of the eluent ($c = 2.18$ g l^{-1}, flow-rate = 19.8 ml h^{-1}) and the eluent volume necessary for the column saturation was measured. The saturation volume ($V - V_o$) was 416 ml. This corresponded to $1.4 \cdot 10^{-5}$ mol of retained Hb. The amount of retained Hb was also checked by a desorption method: the saturated column was washed with Tris buffer (pH 7.5) and the quantity of desorbed Hb was determined in the collected eluent by spectrophotometry. The result was the same as that evaluated in the adsorption procedure.

Another similar experiment with a hemoglobin concentration of 9 g l^{-1} confirmed this value.

The support capacity can be expressed in different ways:

$4.7 \cdot 10^{-5}$ mol Hb (or of active BHC ligands) per g of dry gel or

$1.47 \cdot 10^{-6}$ mol Hb g^{-1} of hydrated gel or 0.1 g Hb g^{-1} of hydrated gel or 3 g Hb g^{-1} of dry gel.

3.4. Determination of affinity constant of hemoglobin for BHC ligand

Binding parameters for non-covalent interactions of molecules can be measured from the retardation of these substances on affinity matrices containing immobilised ligand-interactants. In our work, the zonal approach in the presence of a competing ligand (free BHC) added to the eluent was used. The elution volume of the protein zone and the peak width decreased as the concentration of soluble competing ligand increased. The method is based on Chaiken theoretical considerations [22] for monovalent binding system:

The symbols used in the text are as follows:

X: immobilised ligand (BHC fixed on the column)

P: protein (hemoglobin)

L: competing ligands (free BHC added to the eluent)

Q_x is the quantity of the immobilised active (accessible) ligands on the column or column capacity

The solute P interacts with BHC ligands fixed on the column, X, or with free BHC molecules present in the eluent, L. Some conditions must be fulfilled: There is no interaction between ligands X and L. Only very small amount of P is introduced on the column compared with the amount of fixed ligand (P<X) and the amount of competing ligand is sufficiently high so that L ≫ PL. Both reactions are thermodynamic equilibria with two association constants K_{PX} and K_{PL}.

$$P + X \Leftrightarrow PX \qquad K_{PX} = \frac{[PX]}{[P][X]} \tag{1}$$

$$P + L \Leftrightarrow PL \qquad K_{PL} = \frac{[PL]}{[P][L]} \tag{2}$$

The retention volume V_z of protein at infinite dilution (only a very little amount of protein injected in order to be in the linear region of isotherm) is equal to the slope of the Langmuir equilibrium isotherm at the origin [22,23]:

$$\frac{K_{PX} Q_X}{1 + [L] K_{AL}} = V_z - V_o \tag{3}$$

where V_z is the elution volume of hemoglobin and V_o is the hold up volume. The elution volume is linearly dependent on the amount of immobilised ligand Q_x at a given concentration [L] of the free ligand.

If expressed as a function of the concentration of the free competitor in the eluent [L]:

$$\frac{1}{V_z - V_o} = \frac{1}{K_{PX} Q_X} + \frac{K_{PL}[L]}{K_{PX} Q_X} \tag{4}$$

where the elution volume is inversely proportional to the concentration [L] at known Q_x. The intercept gives the K_{PX} binding constant and the ratio of the slope to the intercept gives the K_{PL} constant.

The binding constants' measurements were performed at pH 6.9 at a column temperature of 5°C. As hemoglobin solution consists of different forms oxyHb, deoxyHb and metHb, only freshly prepared solutions of each component were used for the measurements and their purity was checked by the above mentioned spectrophotometric method.

OxyHb: the solution was oxygenated in order to have more than 95% oxyHb. The column outlet composition was controlled and the oxyHb form was

eluted quantitatively. Thus it was sure that the measured major peak corresponded to oxyHb.

DeoxyHb: The hemoglobin solution and eluent were deoxygenated during one night by flushing nitrogen. The deoxyHb composition was checked by spectrophotometry under nitrogen conditions in order to avoid reoxygenation. It was found that the solution contained about 70% deoxyHb, 25% metHb and 5% oxyHb. The samples were also injected on the column under nitrogen atmosphere. The effluent analysis confirmed that the major peak corresponded to deoxyHb.

MetHb was prepared by reaction with $K_3[Fe(SCN)_6]$.

The column contained $Q_x = 1.4 \cdot 10^{-5}$ mol of BHC ligands determined as mentioned above.

In all experiments 60 μl of hemoglobin solutions (10 g l^{-1}) were injected which corresponded to 10^{-8} mol of protein. It was previously verified that these conditions corresponded to the linear part of the isotherm (the different concentrations of Hb were injected in the range of 5–50 g l^{-1} and the elution volumes remained constant).

3.4.1. OxyHb affinity constants

Six different free ligand concentrations in the range 0.1–1.2 m*M* corresponding to 0.03–0.36 g l^{-1} of free BHC were used. At each concentration at least two injections were done and a very good reproducibility was observed. Therefore, the association constants K_{PX} and K_{PL} were determined from linear regression of 12 measurements. Fig. 3 presents the experimental values, where most of the points represent two superimposed nearly identical retention volumes, ($Q_x = 1.4 \cdot 10^{-5}$ mol). The association constants determined from Eq. (4) are given in Table 1.

Table 1
Hemoglobin association constants with fixed or free benzenehexacarboxylic ligands, K_{PX} and K_{PL} respectively, at pH 6.9

Compound	K_{PX} (l mol^{-1})	K_{PL} (l mol^{-1})
Oxyhemoglobin	$8.0 \cdot 10^2$	$1.4 \cdot 10^4$
Deoxyhemoglobin	$9.7 \cdot 10^4$	$2.3 \cdot 10^5$
Methemoglobin	None	None

3.4.2. DeoxyHb affinity constants

Four different free ligand concentrations in the range 0.15–0.6 m*M* were used and at each concentration 2–3 injections were done. The linear regression was done on 9 measurements. The results are shown in Fig. 4, where several points represent two superimposed nearly identical values ($Q_x = 1.4 \cdot 10^{-5}$ mol). The calculated deoxyHb affinity constants can be found in Table 1.

Concerning metHb, the elution volume of metHb was so close to the V_0 volume that it was not possible to determine the affinity constant.

As can be seen from Table 1, the calculated values of association constants for deoxyHb are within the range of usual magnitude of affinity constants (10^4–10^8 l mol^{-1}). The deoxyHb interaction with anionic BHC ligand was 10 times higher than that of oxyHb, whatever the state of ligand, free or fixed.

This phenomenon is not surprising because the above is relevant to the respiratory mechanism of hemoglobin and the role of the allosteric site. The concept of allosteric site is based on conformational

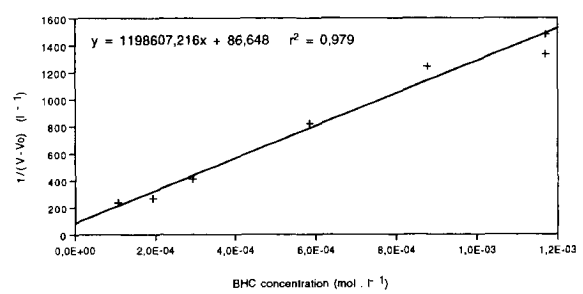

Fig. 3. Determination of oxyhemoglobin affinity constants at pH 6.9 with free and fixed BHC ligands, K_{PL} and K_{PX}, respectively. Quantity of active immobilised BHC ligands on the column $Q_x = 1.4 \cdot 10^{-5}$ mol. $K_{PL} = 1.4 \cdot 10^4$ l mol^{-1}; $K_{PX} = 8.0 \cdot 10^2$ l mol^{-1}.

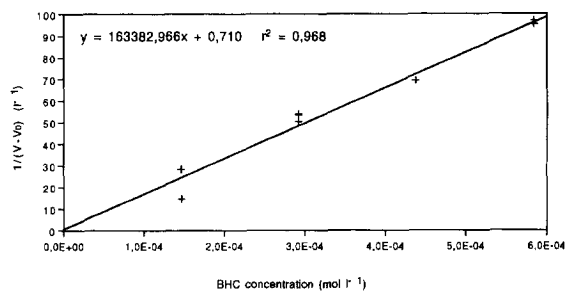

Fig. 4. Determination of deoxyhemoglobin affinity constants at pH 6.9 with free and fixed BHC ligands, K_{PL} and K_{PX}, respectively: Quantity of active immobilised BHC ligands on the column $Q_x = 1.4 \cdot 10^{-5}$ mol. $K_{PL} = 2.3 \cdot 10^5$ l mol^{-1}; $K_{PX} = 9.7 \cdot 10^4$ l mol^{-1}.

changes in protein molecule affecting its biological activity by the fixation of regulating ligand-effector. DeoxyHb has a quaternary structure, the so-called 'deoxy' or T (tense) structure, which differs significantly from that of hemoglobins such as oxy, carboxy and other hemoglobins along with methemoglobin, all of which have nearly identical quaternary structure, termed 'oxy' or R (relaxed) structure [20]. The T structure has a high affinity for effectors via its allosteric site and a low affinity for oxygen, contrary to the R structure with a low affinity for the effector. The natural effector 2,3 DPG binds to Hb which favours the oxygen transport within body. On the other hand, the oxygenated form of hemoglobin, oxyHb has a low affinity for 2,3 DPG. For example, the interaction constants published in [24] for oxyHb-DPG and deoxyHb-DPG complexes are respectively 210 and 7700 $1\,mol^{-1}$. Our support is supposed to interact (under conditions of pH close to p*I*) with Hb in the similar way as 2,3 DPG, via its allosteric site, and the interaction with deoxyHb is stronger than with oxyHb.

In both cases, oxyHb and deoxyHb, the interaction is stronger with free BHC ligands than with those linked to Sepharose, $K_{PL} > K_{PX}$. This can be due to the better steric accessibility of protein to the free ligand and also due to the higher number of available COO^- groups in the non-linked BHC molecule.

It was observed that metHb (a minor component due to the autooxidation of oxyHb) did not interact with BHC support at pH 6.9 while interacting at pH 6.1. This also confirms that the nature of interactions at pH close to p*I* and at pH<p*I* are fundamentally different. Those involved at pH<p*I* must be to the positive overall surface charge, whatever the structure (R or T), while those at pH close to p*I* must be due to the allosteric site interactions as it was confirmed by Hb-PLP experiments.

Bucci [25] measured the association constants of deoxyHb and benzenepentacarboxylate in a 0.05 *M* NaCl solution at 20°C and pH varying from 7.5–9 by a potentiometric titration method. The value of the affinity constant extrapolated in the range of pH 6.5–7, was 10^7. The measurements were done under very different conditions from ours, using a different method and with a slightly different ligand. Therefore it is difficult to compare the absolute value obtained by this method with that calculated in our experiments. Nevertheless, the exponential decrease of the interaction with rising pH observed by Bucci, can be compared with our results.

4. Conclusion

New chromatographic affinity supports were synthesised from Sepharose containing BHC or BTC molecules fixed on a hexamethylamine spacer arm. Both ligands exhibited a specific affinity for hemoglobin at a pH close to the p*I* (where total protein surface overall charge is zero). If hemoglobin had its allosteric cavity blocked by covalently linked pyridoxylphosphate, no retention was observed, which confirms the biospecificity of the interaction via the allosteric site of hemoglobin. The linear decrease of the elution volume of hemoglobin with competitor concentration further supports the hypotheses of the specific binding recognition of hemoglobin for fixed ligand.

As the eluent pH increased above the p*I*, the affinity of hemoglobin for BHC or BTC supports decreased; the specific interaction was progressively cancelled by the increasingly strong repulsion forces between negative column ligands and negative total charge of the protein surface. At pH 6.6, hemoglobin, as well as pyridoxylated hemoglobin, started to be mildly retained by the column by non-specific ionic surface forces. At pH 6.1, all hemoglobin forms (methemoglobin, oxyhemoglobin, deoxyhemoglobin and pyridoxylated hemoglobin) were strongly retained by the column. This confirms that the interactions at pH close to p*I* and at pH<p*I* are fundamentally different.

It can be concluded that no major non-specific factors are operating in the interaction of hemoglobin with the support at pH close to 6.9. Thus the adsorption for purification purposes can be done at this pH with desorption with an eluent at pH 7.5.

The described support, used at pH close to the p*I* of hemoglobin, can be considered as affinity support having biospecific interactions with hemoglobin allosteric site. The obtained values of binding constants of deoxyhemoglobin with free or fixed BHC ligands ($10^5\ 1\,mol^{-1}$) are within the range of usual affinity values. They are not very high but sufficient for affinity application. The observed oxyhemoglobin

affinity for anionic ligand 10–100 times lower than that of deoxyhemoglobin is relevant to the respiratory mechanism of hemoglobin. For the purification procedure it would be better, prior to the chromatographic step, to convert hemoglobin to deoxyhemoglobin form by nitrogen bubbling. After the desorption of hemoglobin from the column, the oxy-form can be easily recovered by oxygen bubbling.

Acknowledgments

The authors wish to thank P. Menu from the Pharmaceutical Faculty of Nancy and the Centre Régional de Transfusion Sanguine de Nancy for their help in supplying us with hemoglobin solutions and the students S. Caillet, V. Quessada and C. Emmerman for their contribution to the experiments.

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