ELSEVIER

Journal of Chromatography B, 664 (1995) 89–95

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Preparative and analytical applications of immobilized haemoglobin

E. Chiancone[a,*], M. Gattoni[a], C. Fronticelli[b], A. Termine[c], L. Silvestri[c], P. Sarti[d]

[a]*CNR Centre of Molecular Biology, Department of Biochemical Sciences, University "La Sapienza", 00185 Rome, Italy*
[b]*Department of Biochemistry, University of Maryland, Baltimore, MD, USA*
[c]*Istituto Dermopatico dell'Immacolata IRCCS, Rome, Italy*
[d]*Institute of Biological Chemistry, University of Cagliari, Cagliari, Italy*

Abstract

New applications of haemoglobin immobilized on Sepharose 4B are proposed for the removal of cell-free, potentially toxic haemoglobin from either natural biological specimens or artificial haemoglobin-containing systems (e.g., blood substitutes, haemosomes) to be employed for biomedical purposes. In a model study, an affinity column of immobilized haemoglobin was used to remove free haemoglobin from blood serum in which controlled haemolysis had been induced. The affinity column retains all the free haemoglobin, does not retain the haemoglobin–haptoglobin complex(es) and leaves the composition of the serum samples unaltered. When immobilized met-haemoglobin is used, haem is transferred readily to albumin, with which it forms a complex. This observation on the one hand shows that immobilized oxyhaemoglobin should be preferred for preparative purposes, and on the other opens the way to the characterization of the haem transfer reaction to albumin by means of immobilized met-haemoglobin. This reaction is difficult to study in solution owing to the overlap of the met-haemoglobin and methaemalbumin spectra.

1. Introduction

The vertebrate haemoglobin $\alpha_2\beta_2$ tetramer is known to undergo a reversible association–dissociation equilibrium into $\alpha\beta$ dimers which is influenced by a variety of factors, namely haem ligands, state of oxidation of the haem iron, allosteric effectors and ionic strength. The $\alpha_2\beta_2$ tetramer is stabilized by organic or inorganic phosphates and removal of the haem ligand; in deoxyhaemoglobin, for example, dissociation of the tetramer cannot be assessed directly. In contrast, dimer formation is favoured by oxidation of the haem iron, an increase in ionic strength or a departure from neutral pH [1].

Human haemoglobin is immobilized on hydrophilic matrices such as CNBr-activated Sepharose 4B as $\alpha\beta$ dimers that are linked to the matrix via either chain [2,3]. The immobilized dimers maintain the capacity of associating in a specific and reversible manner with soluble dimers and as a result part of the protein initially in solution polymerizes on the solid phase. The amount of matrix-bound tetramer thus formed is

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00405-6

a function of the same factors which control the association–dissociation equilibrium in solution, a phenomenon that can be exploited for both analytical and preparative purposes [2–4]. The most obvious analytical application is the use of immobilized subunits to study precisely their interaction with the soluble protein and to establish whether the coupling reaction has affected the association capacity. With human haemoglobin this property is slightly impaired, in particular in the presence of phosphate since this allosteric effector partly loses its stabilizing effect [3,4]. In spite of this difference, insoluble $\alpha\beta$ dimers have been used as a preparative tool, e.g. to separate in an easy way haemoglobin from myoglobin for the purpose of determining myoglobin in cardiac or skeletal muscle [5] or purifying cross-linked haemoglobin to be used as an oxygen carrier from any dissociable haemoglobin that passes rapidly through the kidneys [6].

The applications of immobilized haemoglobin proposed in this paper address a different problem, namely the removal of cell-free haemoglobin from biological specimens. In fact, free haemoglobin is known to be potentially toxic when in contact with cell membranes owing to its capacity to initiate radical-mediated membrane oxidation [7,8]. The results obtained with haemolysed serum as a model system suggested that immobilized haemoglobin, when oxidized, can be used advantageously to investigate the transfer of haem to albumin.

2. Experimental

Human haemoglobin (Hb) was prepared from outdated blood obtained from the Blood Bank of the Red Cross in Rome following the procedure described by Berger et al. [9]. Haemoglobin concentration was determined spectrophotometrically at 540 nm on the basis of the specific absorbance $A_{1\%,\,1\,cm} = 8.4$ for the oxygenated protein [9].

Crystallized and lyophilized human serum albumin was purchased from Sigma (St. Louis, MO, USA). Albumin concentration was determined using the specific absorbance $A_{1\%,\,1\,cm} = 6.7$.

Controlled haemolysis of clotted blood samples, provided by the Istituto Dermopatico dell'Immacolata in Rome, was achieved as follows: the serum was separated from the blood clot by centrifugation, the clot was disrupted mechanically, centrifuged and the haemoglobin thus obtained was added to the serum. The haemoglobin concentration was determined spectrophotometrically. The serum analytes were determined at the Istituto Dermopatico dell'Immacolata using the reagents and kits provided by Boehringer (Mannheim, Germany) for the Monarch Chemistry Systems (Instrumentation Laboratory, Lexington, MA, USA) or the Hitachi Model 704 autoanalyser (Boehringer). In these kits, nitrogen determination was carried out with the urease method [10].

2.1. Immobilization procedure

Haemoglobin was coupled to commercial CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) in 0.1 *M* $NaHCO_3$ in the presence of ethanolamine in a ten-fold molar excess over haem. This procedure yields concentrations of immobilized protein of ca. 8 mg/ml [6]. The concentration of immobilized protein was determined spectrophotometrically on a Cary 14 apparatus using a 1-mm cell; the effect of turbidity was minimized by the use of protein-free gel in the reference beam [3]. Immobilized methaemoglobin was obtained by addition of 2–3 equiv. of potassium hexacyanoferrate (III) at neutral pH to the immobilized oxygenated protein; excess of hexacyanoferrate (III) and hexacyanoferrate (II) produced during the oxidation reaction were removed by washing the gel on a filter funnel. The binding capacity of haemoglobin affinity columns was assessed as described previously [4] by means of chromatographic columns containing 5–6 ml of immobilized protein and eluted at a constant flow-rate of 15 ml/h. A small volume (1 ml) of concentrated haemoglobin (about 10 mg/ml) in associating buffer (0.1 *M* phosphate at pH 7.0) was percolated through the column equilibrated with the

same buffer and the absorbance of the effluent was monitored at 540 nm with a Jasco Model 7800 apparatus equipped with flow-through cells. Interaction of the soluble protein with the immobilized protein results in an increase in the elution volume with respect to that of a non-interacting protein, i.e., to the void volume of the column. The haemoglobin affinity column was freed from the retained protein with dissociating buffer, 1.98 *M* NaCl = 0.01 *M* phosphate (pH 6.7) [2,4].

Polyacrylamide gel electrophoresis (PAGE) experiments were performed according to Davis [11] in the absence of sodium dodecyl sulphate (SDS) at pH 8.6; the running gel was 7.5% in acrylamide.

3. Results and discussion

Cell-free, potentially toxic haemoglobin may be present in natural biological specimens (e.g., bone marrow contaminated by damaged erythrocytes, blood used for regional perfusion hyperthermia that is haemolysed on extracorporeal circulation) or in artificial haemoglobin-containing systems such as haemosomes (liposomes contained haemoglobin) to be used for therapeutic purposes. The possible use of haemoglobin affinity columns for removing cell-free haemoglobin from specimens of this kind was investigated by employing as a model system blood samples in which controlled haemolysis has been induced (see Experimental).

Haemolysed serum, containing a known amount of haemoglobin, was applied to a met-haemoglobin affinity column of sufficient binding capacity and the absorbance of the eluate was monitored. Immobilized haemoglobin binds only part of the soluble protein, as indicated by the faint red-coloured effluent. The effluent contains the haemoglobin–haptoglobin (Hb–Hp) complex(es) and no free haemoglobin (Fig. 1). Further, its spectrum differs from that of the original haemolysed sample in that it is characterized by the presence of a significant amount of met-haemoglobin, e.g., the Soret maximum is at 408 nm (Fig. 2). The lack of recognition of the Hb–Hp complexes by immobilized haemoglobin accounts for non-proportionality between the amount of haemoglobin retained by the affinity column and the concentration of haemoglobin in the serum (Table 1). In this connection it is also relevant that the concentration of haptoglobin in the serum varies depending on the physiopathological condition of the patient. The lack of recognition of the Hb–Hp complex(es) by immobilized haemoglobin is perhaps not unex-

Fig. 1. SDS-PAGE patterns of four haemolysed sera before and after passage through a column containing immobilized human met-haemoglobin. The column was equilibrated with associating buffer (0.1 *M* phosphate, pH 7.0). For each serum, the lane on the left corresponds to the haemolysed serum and that on the right of the treated serum; first and last lanes, human haemoglobin. (A) Haem staining with benzidine; (B) protein staining with Coomassie Brilliant Blue. Hb = haemoglobin; Hb–Hp = haemoglobin–haptoglobin complexes; Alb = albumin.

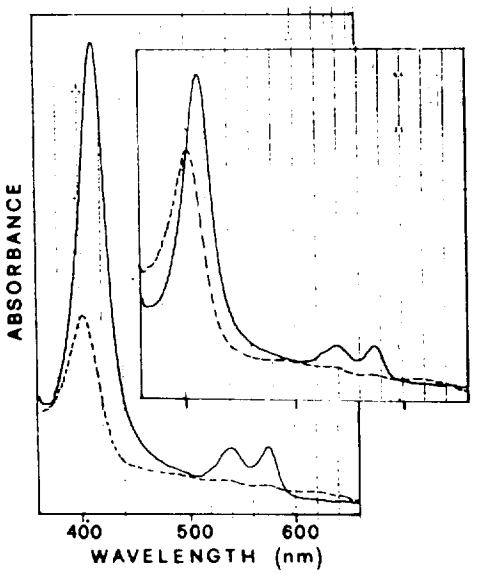

Fig. 2. Absorption spectra in the visible and Soret regions of two haemolysed sera and of the respective eluates from a column of immobilized met-haemoglobin. The column was equilibrated with associating buffer (0.1 *M* phosphate, pH 7.0). On passage through the column, the samples were diluted 4.6-fold and the haemolysed serum was diluted by the same factor for the sake of comparison. Dashed line, column eluate; solid line, haemolysed serum.

pected, as haptoglobin is known to bind haemoglobin as an $\alpha\beta$ dimer [12]. The haemoglobin affinity column, however, demonstrates in a clear and elegant manner that the same subunit interface which is cleaved on dissociation of the haemoglobin tetramer (the so-called $\alpha_1\beta_2$ interface) is involved in the interaction with haptoglobin. Moreover, it shows that the Hb–Hp complex(es) are more easily oxidized than free haemoglobin, in line with the lower stability of soluble $\alpha\beta$ dimers with respect to the $\alpha_2\beta_2$ tetramer [1].

Formation of the Hb–Hp complex(es) therefore diminishes the efficacy of haemoglobin affinity columns in removing the soluble protein from haemolysed serum. However, the use of immobilized haemoglobin could still be proposed provided that the biochemical composition and properties of the sample are not altered significantly by passage through the affinity column. To establish this, eight randomly chosen serum samples were divided into two aliquots; one aliquot was used as the reference ("original serum" in Table 1); the other was submitted to controlled haemolysis ("haemolysed serum" in Table 1) and was thereafter subdivided into two parts, one of which was passed through the affinity column ("treated serum" in Table 1). Table 1 shows that the activity of enzymes at high concentration in the erythrocytes, like lactate dehydrogenase (LDH) and glutamic–oxaloacetic transaminase (GOT), is increased significantly in the haemolysed and treated samples, whereas that of glutamic–pyruvic transaminase (GPT) and creatine kinase (CK) is increased only slightly.

The corresponding increase in the concentration of total proteins is more evident in the samples treated with the affinity column than in the haemolysed samples. This difference may be attributed to correction factors routinely introduced to compensate for the interference of contaminating haemoglobin which absorbs at 550 nm, the spectral region of the biuret reaction product. The values of total nitrogen and bilirubin are not changed significantly in the haemolysed and treated samples, while the concentration of glucose is on average lower. The latter finding in turn indicates that the glycolytic enzymes released from the erythrocytes are not retained on the affinity column.

A further and different use of immobilized haemoglobin was suggested by the electrophoresis patterns shown in Fig. 1, and more specifically by the observation that the albumin band is stained strongly by benzidine in the serum samples eluted from the met-haemoglobin affinity column. It is known that in vivo, when the level of plasma haemoglobin exceeds the haemoglobin binding capacity of haptoglobin, haem is transferred from human haemoglobin to albumin, thereby forming methaemalbumin [13]. It is also known that in vitro no haem transfer takes place with cyanomet-, oxy- or carbonmoxyhaemoglobin, whereas haem is transferred from methaemoglobin, haem dissociation being considerably faster from non-α-chains than from α-chains [13]. However, the study of the haem transfer reaction at physiological pH values is rendered

Table 1
Serum analytes after treatment of eight blood samples submitted to controlled haemolysis with a haemoglobin affinity column

No.	Serum analytes	Hb in serum		Original serum	Haemolysed serum	Treated serum
		mg/ml	% retained			
1	Proteins (total, g/dl)	2.10	63	6.9	6.8	7.2
	Nitrogen (total, mg/dl)			33	34	40
	Glucose (mg/dl)			84	83	82
	Bilirubin (total, mg/dl)			0.41	0.42	0.44
2	Proteins (total, g/dl)	2.44	56	7.5	7.3	8.0
	Nitrogen (total, mg/dl)			29	30	32
	Glucose (mg/dl)			150	125	140
	Bilirubin (total, mg/dl)			0.48	0.44	0.46
3	Proteins (total, g/dl)	3.57	80	7.4	7.3	8.4
	Nitrogen (total, mg/dl)			29	30	36
	Glucose (mg/dl)			76	62	60
	Bilirubin (total, mg/dl)			0.46	0.46	0.44
4	Proteins (total, g/dl)	3.67	69	7.5	7.3	8.0
	Nitrogen (total, mg/dl)			32	31	36
	Glucose (mg/dl)			100	72	72
	Bilirubin (total, mg/dl)			0.66	0.66	0.48
5	Proteins (total, g/dl)	1.35	nd	7.1	7.2	8.7
	Nitrogen (total, mg/dl)			38	40	33
	Glucose (mg/dl)			72	64	72
	Bilirubin (total, mg/dl)			0.61	0.57	0.62
	GOT (U/l)			24	27	21
	GPT (U/l)			21	29	45
	LDH (U/l)			219	459	903
	CK (U/l)			105	114	180
6	Proteins (total, g/dl)	1.48	nd	7.3	7.1	9.0
	Nitrogen (total, mg/dl)			39	41	30
	Glucose (mg/dl)			81	76	75
	Bilirubin (total, mg/dl)			0.73	0.69	0.59
	GOT (U/l)			29	30	51
	GPT (U/l)			23	30	36
	LDH (U/l)			219	255	927
	CK (U/l)			97	105	99
7	Proteins (total, g/dl)	5.78	nd	7.1	6.9	6.0
	Nitrogen (total, mg/dl)			15	12	13.8
	Glucose (mg/dl)			95	80	65
	Bilirubin (total, mg/dl)			0.52	0.47	0.23
	GOT (U/l)			18	41	46
	GPT (U/l)			7	7	9.2
	LDH (U/l)			441	1180	1210
	CK (U/l)			80	116	110
8	Proteins (total, g/dl)	7.1	nd	7.4	6.7	9.2
	Nitrogen (total, mg/dl)			43	41	44
	Glucose (mg/dl)			89	78	75
	Bilirubin (total, mg/dl)			1.17	1.12	0.93
	GOT (U/l)			18	49	48
	GPT (U/l)			12	12	14
	LDH (U/l)			306	1240	1420
	CK (U/l)			117	165	167

difficult by the similarity of the met-haemoglobin and methaemalbumin spectra [14]. The rapid transfer of haem to albumin during the passage of serum through the met-haemoglobin affinity column suggested that immobilized haemoglobin could be used advantageously to characterize the haem transfer reaction. Methaemalbumin can be separated readily from the immobilized haemoglobin (e.g. by means of a filter) and its spectrum measured without interference from haemoglobin. A first set of experiments showed that immobilized $\alpha\beta$ dimers, like the soluble protein, do not transfer haem to albumin when in the oxygenated, CO-bound or cyanomet state, but dissociate haem rapidly in the oxidized, met-form.

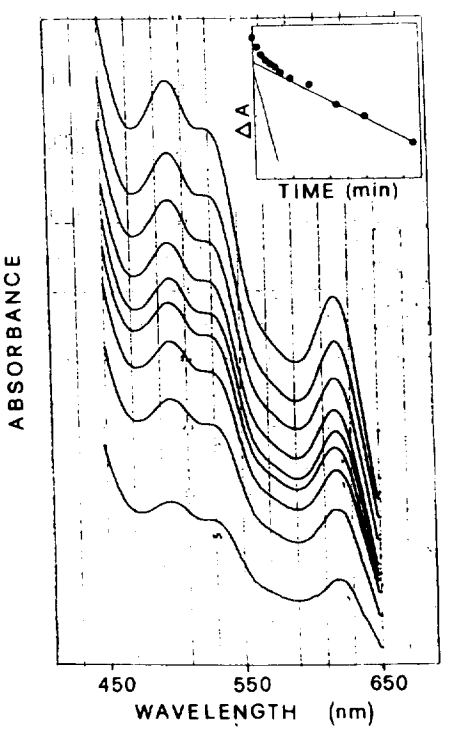

Fig. 3. Time dependence of the absorbance changes due to transfer of haem from immobilized met-haemoglobin to albumin at pH 7.5 and 25°C. Albumin at a concentration of $1.32 \cdot 10^{-4}$ *M* was mixed with $2 \cdot 10^{-4}$ *M* (haem) immobilized met-haemoglobin in 0.5 *M* Bis-Tris adjusted to pH 7.5 with HCl and containing 0.1 *M* NaCl; the liquid phase was separated from the insoluble material at times of 5, 10, 15, 20, 25, 30, 40, 60 and 90 min and the spectra were measured. The end of the reaction was taken as 1200 min, as the absorbance had increased by less than 10% with respect to that at 170 min. The inset shows the semi-logarithmic plot of the absorbance change as a function of time.

Fig. 3 shows the time course of methaemalbumin formation on exposure of albumin to immobilized met-haemoglobin at pH 7.5. The kinetics are characterized by the presence of two distinct phases of similar amplitude whose rates differ approximately tenfold ($k_f = 0.10$ min^{-1} and $k_s = 0.012$ min^{-1} under the experimental conditions used). The two rates are likely to reflect haem dissociation from the α- and β-chains [13]; the set-up used therefore lends itself to the analytical characterization of the haem transfer reaction from haemoglobin to albumin.

In conclusion, based on the data given in Table 1, haemoglobin affinity columns can be proposed as a preparative tool to remove free haemoglobin, although not the haemoglobin–haptoglobin complex(es), from biological samples. The oxygenated derivative should be preferred owing to the stability of the haem–globin linkage which is reflected in vitro in the lack of haem transfer to albumin.

Acknowledgements

This work was supported in part by the CNR target project on Biotechnology and Bioinstrumentation and by MURST grants 40% and 60% to E.C.

References

[1] E. Antonini and E. Chiancone, *Annu. Rev. Biophys. Bioeng.*, 6 (1977) 239.
[2] E. Antonini, M.R. Rossi Fanelli and E. Chiancone, in H. Sund and G. Blauer (Editors), *Protein–Ligand Interactions*, Walter de Gruyter, Berlin, 1975, p. 45.
[3] M.R. Rossi Fanelli, G. Amiconi and E. Antonini, *Eur. J. Biochem.*, 92 (1978) 253.
[4] E. Chiancone and M. Gattoni, *J. Mol. Recog.*, 3 (1990) 142.
[5] S. Schuder, J.B. Wittenberg, B. Haseltine and B.A. Wittenberg, *Anal. Biochem.*, 92 (1979) 473.
[6] E. Chiancone, C. Fronticelli, M. Gattoni, B.K. Urbaitis and E. Bucci, *J. Chromatogr.*, 604 (1992) 117.

[7] P. Sarti, N. Hogg, V.M. Darley-Usmar, M.T. Sanna and M.T. Wilson, *Biochim. Biophys. Acta*, 1208 (1994) 38.
[8] G. Papanga, C. Rice-Evans, R. Rule and D. Leake, *FEBS Lett.*, 303 (1992) 154.
[9] R.L. Berger, L. Carpenter, J. Everse and J.L. Kaplan, *Anal. Lett.*, 6 (1973) 125.
[10] L. Prencipe, S. Brenna and C.E. Sordi, *Clin. Chem.*, 29 (1983) 1315.
[11] B.J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
[12] A.C. Peacock, J.V. Pastewka, R.A. Reed and A.T. Ness, *Biochemistry*, 9 (1970) 2275.
[13] H.F. Bunn and J.H. Jandl, *J. Biol. Chem.*, 243 (1968) 465.
[14] R.E. Benesch and S. Kwong, *J. Biol. Chem.*, 265 (1990) 14881.