SEPARATION OF ERYTHROCYTE ENZYMES FROM HEMOGLOBIN BY CHROMATOGRAPHY ON BLUE-SEPHAROSE

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1. Introduction

The spectrophotometric determination of the oxygen consumption of biological samples with HbO_2 as oxygen donor and indicator [1,2] has the advantage over conventional methods of being accurate, sensitive and suitable for automation [3,4]. Since interferences due to the erythrocyte enzymes are usually negligible, highly purified hemoglobin preparations are not generally required [5–8]. However, when extending the spectrophotometric method to determinations such as quantitation of ethanol or simultaneous measurement of oxygen consumption and glycolysis of isolated cells, the presence of catalase and glycolytic enzymes in the HbO_2 preparations became a serious inconvenience.

Blue-Sepharose, in addition to specifically binding dinucleotide fold containing enzymes [9–12], also binds other proteins at pH-values below their isoelectric points. This property of blue-Sepharose, common to conventional ion-exchangers, combined with its specific affinity for dinucleotide-requiring enzymes, may be useful in the separation of HbO_2 from other erythrocyte proteins.

2. Materials and methods

2.1. *Chemicals*

All natural nucleotides, substrates and coupling enzymes were products of Boehringer Mannheim (a generous gift of Professor H. F. Schmidt). Sepharose 4B obtained from Pharmacia Uppsala was crosslinked according to [13], omitting $NaBH_4$ from the reaction medium. Cibacron blue 3G-A, a product of Ciba-Geigy Basel, was coupled to the crosslinked Sepharose as in [14].

Abbreviations: HbO_2, oxyhemoglobin; SDS, sodium dodecyl sulfate; blue-Sepharose, Cibacron blue 3G-A Sepharose 4B-CL

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2.2. *Recycling of the blue-Sepharose*

Blue-Sepharose was washed in the column with 2 vol. 6 M urea in 2 M NaCl, followed by 1 vol. bidistilled water. The gel was then removed from the column and dispersed by passing it through a metal mesh. Bidistilled water was added during this procedure. The gel was then stirred in excess water and allowed to sediment. The supernatant was siphoned out and this step was repeated 3 times. Blue-Sepharose was finally poured into the chromatographic column and equilibrated with 'starting buffer', i.e., 20 mM K-phosphate (pH 6.9). The maximum binding capacity of blue-Sepharose at pH 6.9 was ~20 mg HbO_2/ml.

2.3. *Preparation of the hemolysate*

Erythrocytes from human blood were washed 3 times with isotonic-buffered saline. The white cell layer was removed by suction after the final centrifugation. The sedimented blood cells were lysed by 30 s homogeneisation with an Ultraturrax apparatus (Janke and Kunkel, Breisgau), with 1 vol. bidistilled water and 0.1 vol. CCl_4. After 20 min centrifugation at 2000 × *g* in a swing-out rotor, the hemolysate (the upper layer) was recovered by syphonation. It was then filtered through a glass wool and HbO_2 content estimated from the absorption at 577 nm [15].

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2.4. *Analytical procedures*

The activities of adenylate kinase, nucleoside diphosphate kinase, pyruvate kinase, hexokinase, 3-phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase and glucose 6-phosphate dehydrogenase were measured in systems involving the interconversion of pyridine nucleotides. All enzymatic rates were determined at 25°C in 1 ml final volume photometrically (366 nm) or fluorimetrically (366 nm excitation filter, 470–3000 nm emission filter) by using an Eppendorf 1101 M-type photometer equipped with a W + W 4410-type recorder. Details concerning the composition of the reaction mixtures are given in [16]. Carbonic anhydrase activity was measured manometrically at +2°C as in [17], and catalase activity was determined spectrophotometrically at 240 nm [18]. SDS–polyacrylamide gel electrophoresis was done as in [19,20].

3. Results and discussion

The fractionation procedure was tested for column volumes of 15, 150 and 1500 ml. The data reported here belong to an experiment in which a 150 ml (2.5 X 30 cm) column was used. Two column volumes of hemolysate at 10 mg HbO_2/ml in 20 mM K-phosphate buffer (pH 6.9) were loaded onto the column by hydrostatic pressure at 5 ml/min and 14 ml fractions were collected throughout (fig.1). At pH 6.9, hemoglobin and nucleotide-dependent enzymes bind to blue-Sepharose, whereas catalase and carbonic anhydrase (pI = 5.8 and 5.3, respectively [21,22]), emerge with the buffer. The column was then washed with 1.2 vol. 'starting buffer'. At this point the effluent was colourless. Virtually pure HbO_2 was eluted from the column with ~1.5 vol. of 'low ionic strength pH 8.0 buffer', consisting of 100 mM triethanolamine-acetate (table 1). The other erythrocyte proteins (mainly pyridine-linked dehydrogenases and phosphotransferases) could be further eluted with 'high ionic strength pH 8.0 buffer', consisting of 100 mM triethanolamine-acetate and 2 M NaCl. The pooled HbO_2 fractions were ~50 mg/ml. Glycerol was added to 50% final conc. The HbO_2 could be stored for several months at −12°C or at +4°C and used directly for the measurement of oxygen consumption by dilution with the appropriate buffer. In cases where glycerol was undesirable, it was removed by dialysis against buffer for several hours.

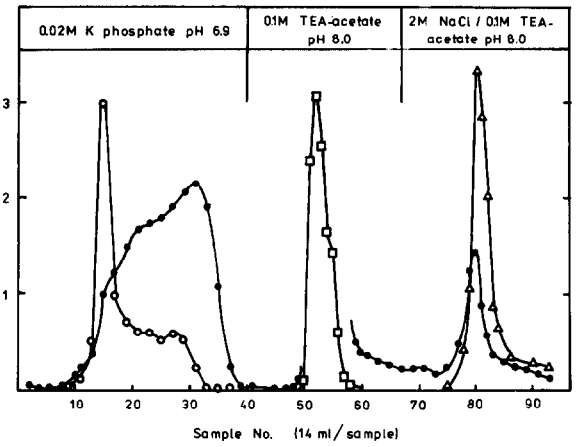

Fig.1. Separation of hemoglobin, catalase and lactate dehydrogenase from human erythrocytes (2.9 mg protein) by chromatography on blue-Sepharose. One interval along the vertical axis corresponds to 1 A_{280} unit (•), 5000 units of catalase/ml (○), 20 mg HbO_2/ml (□), and 0.5 units of lactate dehydrogenase/ml, determined fluorimetrically as NADH formation (△).

The SDS gel electrophoresis of purified HbO_2 showed a single band of protein at 16 000 M_r. In the starting hemolysate, at the same protein concentration, several protein bands in addition to the HbO_2 band were clearly visible. Fractions 10–20 in fig.1

Table 1
The activity of several erythrocyte enzymes in the human hemolysate and purified HbO_2 preparation[a]

Enzyme	Spec. act. (μmol . min^{-1} . mg protein^{-1})	
	Hemolysate	HbO_2
Lactate dehydrogenase	0.0314	0
Glucose 6-phosphate dehydrogenase	0.0047	0
Glyceraldehyde 3-phosphate dehydrogenase	0.0168	0
3-Phosphoglycerate kinase	0.0631	0
Adenylate kinase	0.0492	0
Nucleoside diphosphate kinase	0.0922	0
Hexokinase	0.0015	0
Pyruvate kinase	0.0038	0
Catalase[b]	200	0.32
Carbonic anhydrase	3.18	0.08

[a] The results are mean values of 3 separate expt.
[b] Enzymatic units are expressed as in [18]

(eluted before HbO_2) showed more bands, dominated by one at 31 000 M_r (carbonic anhydrase) and another at 60 000 M_r (the catalase subunit). These 2 proteins could be completely resolved by gel filtration on Sephacryl S200 SF, after concentration by ammonium sulfate precipitation. The fractions eluted with 2 M NaCl at pH 8.0 gave a multitude of bands (fig.2).

The purification of HbO_2 by chromatography on blue-Sepharose has several obvious advantages:

(i) The procedure is fast (2–4 h) and has a high yield (~70%). In this way the oxidation of Fe^{2+} is avoided. The absorption spectrum of purified HbO_2 is identical to that of the fresh hemolysate;

(ii) The pyridine-linked dehydrogenases and phosphotransferases, which represent the most abundant erythrocyte enzymes, are almost entirely removed. At most 0.2% catalase and 3% carbonic anhydrase could be detected in the purified sample as compared to the initial hemolysate;

(iii) The procedure may be used as a preliminary step in the purification of erythrocyte dehydrogenases and kinases, the behaviour of blue-Sepharose being analogous to that of DEAE-cellulose, used for the elimination of hemoglobin [23]. Under the experimental conditions described, several erythrocyte enzymes such as nucleoside diphosphate kinase, adenylate kinase, lactate dehydrogenase and glucose 6-phosphate dehydrogenase could be recovered by >80% in proportion to the initial hemolysate (table 1), whereas pyruvate kinase which is more sensitive to denaturation, was recovered in proportion to 30% only. Since HbO_2 does not bind to blue-Sepharose at pH 8.0, it is thus possible to purify such enzymes by a factor of 200–1000, using large volumes of hemolysate loaded at pH 8.0 onto small columns. Enzymes may be eluted by salt, coenzymes or a combination of both (J. L. et al., in preparation);

(iv) The convenience of using blue-Sepharose for obtaining large amounts of purified HbO_2 also relies on the qualities of blue-Sepharose, namely stability, mechanic resistance and easy regeneration.

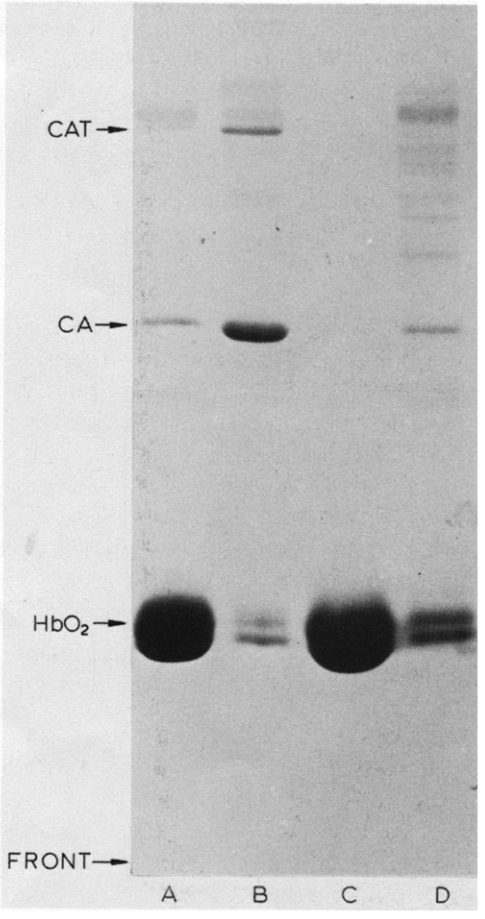

Fig.2. Polyacrylamide gel electrophoretic pattern of the fractions obtained by chromatography on blue-Sepharose of human hemolysate. Electrophoresis was run for 4.5 h on a gel containing 15% acrylamide, 0.4% bisacrylamide, and 0.1% SDS. Arrows indicate the positions of catalase (CAT), carbonic anhydrase (CA) and HbO_2: (A) hemolysate (33 μg protein); (B) pooled fractions (10–20, fig.1) containing catalase and carbonic anhydrase (15 μg protein); (C) purified HbO_2 (33 μg protein); (D) pooled fractions eluted with 'high ionic strength pH 8.0 buffer' (25 μg protein).

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