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Carrier membrane as a stationary phase for affinity chromatography and kinetic studies of membrane-bound enzymes

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ABSTRACT

The use of membrane supports as stationary phase, coupled with ligands of choice, allows all kinds of chromatography [Dj. Josić, K. Zeilinger, Y. Lim, M. Raps, W. Hofmann and W. Reutter, *J. Chromatogr.*, 484 (1989) 327] and offers a powerful alternative to both soft gel chromatography and high-performance liquid chromatography. In this work we present affinity membrane chromatography for purification of the enzyme carbonic anhydrase from haemolysates of human erythrocytes. Furthermore, the coupling of the enzymes to the membrane support allows kinetic investigations. As an example, kinetic experiments were carried out by means of carbonic anhydrase coupled to the membrane support using 4-nitrophenyl acetate and 2-chloro-4-nitrophenyl acetate as substrates.

INTRODUCTION

Affinity chromatography occupies a unique place in separation technology since it is the only technique that enables purification of almost any biomolecule on the basis of its biological function or individual chemical structure. Ligands containing primary amino groups can be coupled to polysaccharide matrices activated by cyanogen bromide [1]. The linkage induced by cyanogen bromide activation is probably of an ester type [2]. Owing to its esterase activity, carbonic anhydrase is able to cleave coupled sulphonamide groups, which are used as specific ligands in affinity chromatography but cause decreased reproducibility and yields [3].

The application of membranes for chromatographic separations and for enzyme immobilization has been discussed for several years [4,5]. Three different types of membrane have to be distinguished: hollow fibre membrane systems [5], resin beads enmeshed in hollow fibre membranes [6], and membranes containing polymerized beads, with internal structures identical with those of macroporous

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high-performance liquid chromatographic (HPLC) sorbents [7].

This paper describes the use of the third type of membrane, which is made of poly(glycidyl methacrylate–co-ethylene dimethacrylate). This support contains epoxy-activated groups, which allow the immobilization of molecules with amino groups, resulting in bonds that are stable towards hydrolysis [8].

Studies of enzyme kinetics have often provided important avenues for elucidating the mode of enzyme action. In general, kinetic investigations are carried out in cuvettes under static conditions. Immobilization of carbonic anhydrase on a carrier membrane turned out to be a promising system, which allowed kinetic studies under constant-flow conditions, thus reducing the disturbances caused by formation of product.

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade form Merck (Darmstadt, Germany) or Sigma (Munich, Germany).

Protein determination

Protein recovery was determined by measuring the protein concentration before and after separation according to the procedure of Lowry *et al.* [9].

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of 10–25 μl were mixed with 3–7 μl of 500 m*M* Tris–HCl buffer (pH 6.8) containing 10% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. Samples were heated at 95°C for 4 min, and 5–20 μg of protein were applied to each track. SDS-PAGE was performed according to the method of Laemmli [10] using the Bio-Rad mini system (Bio-Rad Labs, Munich, Germany).

Determination of CO_2 hydration activity

The assay was performed at 0°C, according to the method of Roughton and Booth [11] modified by Wilbur and Anderson [12]. Samples were made up to 1 ml with water, and 2 ml of diethylbarbituric acid (22 m*M*) with bromothymol blue (50 mg/l) were added. After addition of 2 ml of CO_2-saturated water, the time for a visible colour change at pH 6.3 was recorded. Activity was calculated with the formula $[(t_0 - t_s)/t_s] \times v \times 16.15 =$ U/ml (v = sample volume in μl; t_0 and t_s = recorded times for the uncatalysed and catalysed reactions).

Sample preparation for affinity chromatography

Human erythrocytes were washed with an equal volume of wash buffer (10 m*M* Tris HCl, pH 7.5, containing 0.15 *M* NaCl) and diluted with 1.5 volumes of a hypotonic solution (10 m*M* Tris–HCl) and then frozen at −20°C and thawed.

The homogenate was centrifuged at 100 000 *g* for 60 min and 10 ml were analysed by affinity chromatography.

Carrier membrane

Membranes were produced by radical copolymerization of (2,3-epoxypropyl)-methacrylate and ethyleneglycoldimethacrylate in the presence of porogene solvents. Ligands containing amino groups could be immobilized on the epoxy-activated groups of the membranes. Experiments were carried out in a membrane-carrying device (Fig. 1).

Immobilization of ligands

To prevent rapid hydrolysis of reactive epoxy groups, membranes were stored at −20°C in methanol. Before use, membranes were fixed in the device (Fig. 1) and then rinsed with water for 2 h at 4°C. Thereafter, membranes were equilibrated by washing with sodium phosphate buffer (0.2 *M*, pH 7.0) for 2 h. Coupling of the enzyme and of the substrate was achieved by dissolving 100 mg of carbonic anhydrase and 300 mg of *p*-aminomethylbenzol sulphonamide in 7 ml of

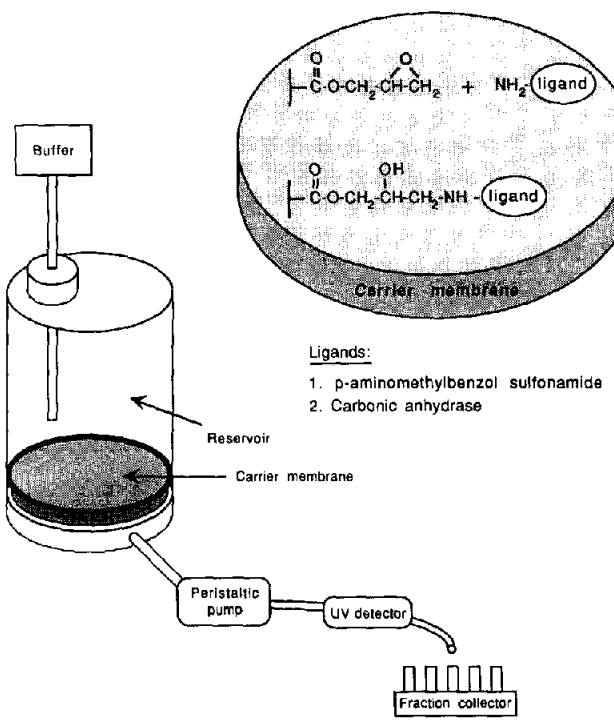


Fig. 1. Structure of the carrier membranes and its carrying device. Membranes consisted of a polymer matrix with an epoxy-activated surface. Ligands containing amino groups could be coupled by amine bonds. The membrane was tightly fixed in a vial. Buffer was loaded on the membrane and drawn by means of a peristaltic pump through the membrane and the UV detector towards the fraction collector.

the sodium phosphate buffer (0.2 *M*, pH 7.0) and recirculation overnight at room temperature. The remaining reactive groups were blocked by rinsing with Tris buffer (0.2 *M*) for 2 h. After immobilization of the ligand, membranes were stored at 4°C.

Kinetics and technique

4-Nitrophenyl acetate and 2-chloro-4-nitrophenyl acetate were used as substrates for determination of the esterase activity of carbonic anhydrase. Ester hydrolysis was followed at a wavelength of 405 nm with a Lambda 2 ™ spectrometer (Perkin Elmer, Überlingen, Germany). A typical procedure for a kinetic run in a conventional cuvette was to initiate the reaction by adding 0.1 ml of substrate solution to 2.9 ml of buffer (50 m*M* Tris–HCl, pH 7.6) with or without bovine carbonic anhydrase. Dynamic measurements with carbonic anhydrase immobilized on a carrier membrane were performed with the system shown in Fig. 1. Both substrates were made up in a stock solution of 50 m*M* substrate in dimethyl sulphoxide (DMSO). Just prior to use, one volume of stock solution was mixed with 49 volumes of buffer (50 m*M* Tris–HCl, pH 7.6) to produce a final substrate concentration of 1 m*M*. In order to obtain other substrate concentrations, different volumes of stock solution of substrate and buffer were mixed. About 4 ml of the substrate solution were drawn into the tube at the outlet of the membrane-carrying device, and *ca.* 20 ml were immediately put on the membrane. The former aliquot underwent spontaneous hydrolysis, and the latter, when drawn through the membrane, underwent enzyme-catalysed hydrolysis. During a run the absorbance was monitored continously.

RESULTS AND DISCUSSION

Affinity membrane chromatography

To separate native from denaturated enzyme molecules, carbonic anhydrase was applied to affinity membrane chromatography. Binding was achieved at pH 8.3 under low salt conditions and elution was accomplished by changing the pH to a value of 5.5 using flow-rates of 0.3 ml/min. The eluted fraction contained enzyme with a high specific activity, whereas the portion of the enzymes that passed through the membrane without binding showed a low specific activity, indicating its denaturation (Fig.2).

To purify carbonic anhydrase from a complex mixture, freshly prepared haemolysate was applied to affinity membrane chromatography. Elution was accomplished as described above. Enzymic activity was readily found in the eluate, indicating a good performance of affinity membrane chromatography (Fig. 3). The specific activity could be increased from 0.1 U/μg in the homogenate to 107 U/μg in the eluate fraction, giving a purification factor of *ca.* 1000. Recoveries of protein and enzymic activity ranged from 80 to 95%. With regard to recovery of mass and of enzymic activity, the efficiency of carrier membranes was similar to that of the high-performance affinity chromatography support [13].

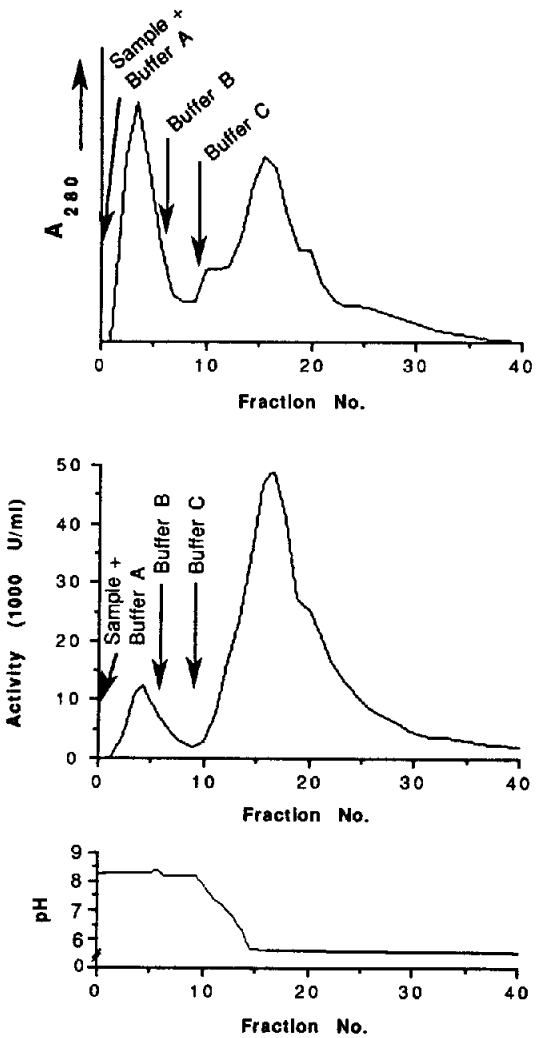


Fig. 2. Separation of denatured from native bovine carbonic anhydrase by affinity membrane chromatography. The membrane was equilibrated with buffer A (0.1 *M* Tris–HCl, pH 8.3) and then 4 mg of bovine carbonic anhydrase dissolved in 4 ml of buffer A were applied. After washing with buffer B (buffer A containing 1 *M* NaCl) elution was accomplished with buffer C [0.1 *M* sodium acetate (pH 5.5) containing 1 *M* NaCl].

Kinetic investigations

Immobilization of proteins on activated carrier membranes showed various binding capacities. Determination of the protein concentration of the binding buffer before and after immobilization elicited a binding capacity value of 6 mg of carbonic anhydrase per g of membrane, whereas bovine serum albumin showed a value of 60 mg of protein per g of membrane. These results are similar to immo-

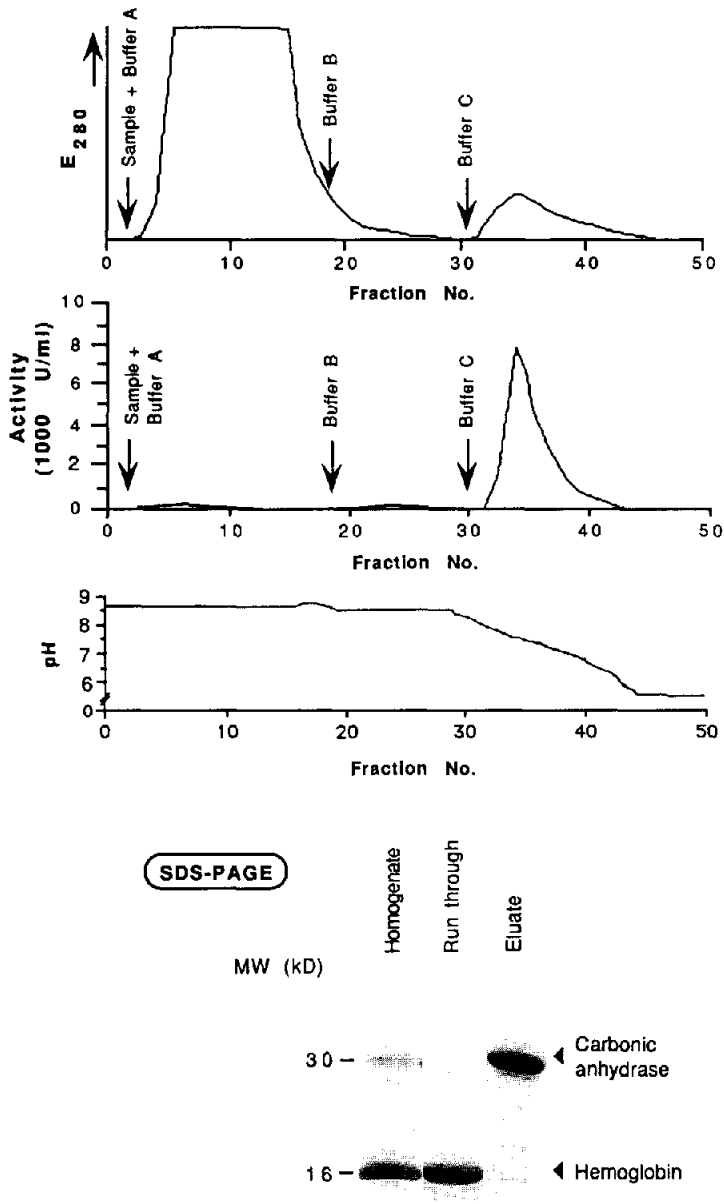


Fig. 3. Purification of carbonic anhydrase from haemolysate of human erythrocytes. Equilibration was achieved with buffer A (0.1 *M* Tris–HCl, pH 8.3). A 10-ml volume of haemolysate of human erythrocytes, containing 17 mg of protein, was loaded on the column. After washing with buffer B (buffer A containing 1 *M* NaCl), elution was accomplished with buffer C [0.1 *M* sodium acetate (pH 5.5) with 1 *M* NaCl]. Isolation of proteins was controlled by SDS-PAGE.

bilization on a succinylimide-activated silica support, where the binding capacities of different proteins ranged from 5 to 50 mg protein per g of support [14].

Immobilization of carbonic anhydrase on a carrier membrane provided an opportunity to carry out kinetic experiments under dynamic conditions, in contrast to the usually encountered steady-state conditions present in cuvettes. 4-Nitrophenyl acetate and 2-chloro-4-nitrophenyl acetate were used as substrates, which offered some distinct advantages over the measurement of reversible hydration of CO_2. The esters are easily handled, hydrolysis is irreversible at convenient rates, and precise spectrophotometric determination can be performed at constant pH. Constant spectrophotometric determination showed a gradual increase in absorbance, caused by spontaneous hydrolysis (Fig. 4). Thereafter, the substrate solution passing through the membrane showed increased absorbance caused by enzyme-catalysed hydrolysis. Measurements of the absorbance in the substrate pool at different times before passage through the membrane showed only a gradual increase. This is a strong indication that hydrolysis occurred only during passage through the membrane bearing the immobilized enzyme.

Enzymic activity was calculated from Lambert Beer's law. Increased flow-rates caused increased activity (Fig. 5), probably owing to accelerated removal of the product decreasing its local concentration.

These results agree with the investigations by Unarska *et al.* [15]. They compared the rates of association between γ-globulin and protein A, immobilized on either agarose beads or microporous nylon membranes. When the γ-globulin solution was forced through the pores of the membrane, the rate of association with protein A was 200 to 300 times faster than in the agarose bead affinity system. However, when the membrane with immobilized protein A was simply immersed in ligate solution, so that the fluid inside the pores remained static, this

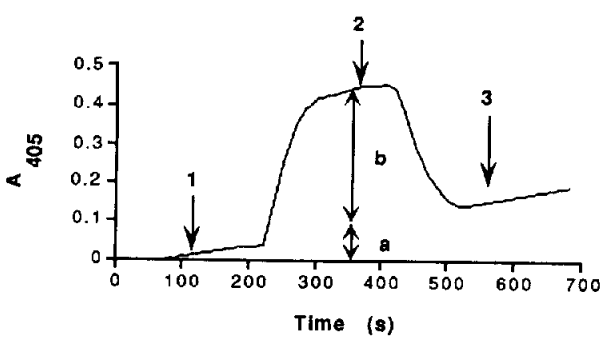
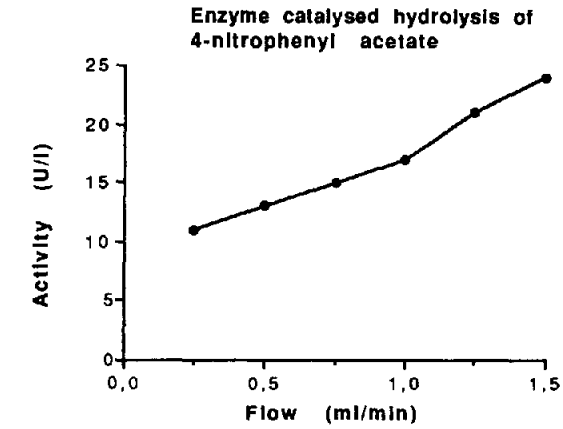


Fig. 4. Test assay for enzyme kinetics of bovine carbonic anhydrase immobilized on a carrier membrane. 4-Nitrophenyl acetate and 2-chloro-4-nitrophenyl acetate were used as substrates. (1) Substrate solution drawn into the tube behind the membrane showed gradually increasing absorbance caused by spontaneous hydrolysis. (2) Substrate passing through the membrane showed greatly increased absorbance caused by enzyme-catalysed hydrolysis. (3) Substrate solution kept as a pool over the membrane, but bypassing the membrane, revealed a decreased absorbance closer to the level of spontaneous hydrolysis.



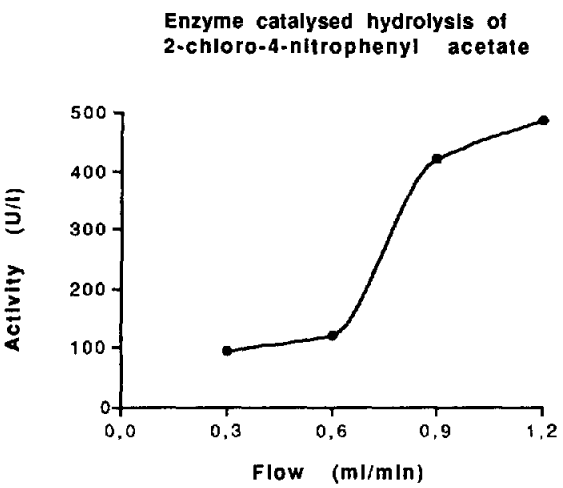


Fig. 5. Relationship between flow and activity of carbonic anhydrase immobilized on a carrier membrane. 4-Nitrophenyl acetate and 2-chloro-4-nitrophenyl acetate were used as substrates. Substrate solution was pumped through the membrane at different flow-rates. The enzymic activity for both substrates increased at greater flow-rates.

increase was not obtained. Unarska *et al.* [15] concluded that the increased rate of association between γ-glubulin and immobilized protein A is due to faster diffusion in the membranes.

Use of different substrate concentrations allowed the determination of kinetic data from Lineweaver–Burk plots. Under conventional measurements in cuvettes, bovine carbonic anhydrase revealed a Michaelis constant (K_M) of 11 mM and a specific activity of 19 U/μg for the hydrolysis of 4-nitrophenyl acetate, and a K_M of 2 mM and a specific activity of 122 U/μg for 2-chloro-4-nitrophenyl acetate. In contrast, membrane-coupled bovine carbonic anhydrase showed a K_M

TABLE I

KINETIC DATA OF IMMOBLIZED CARBONIC ANHYDRASE IN COMPARISON WITH SOLUBLE ENZYME

Enzyme	V_{max} (U/l)	k_{cat} (s^{-1})	K_M (mM)	Specific activity (U/μg)
4-Nitrophenyl acetate				
Soluble enzyme	36	9	11	19
Immobilized enzyme	27	0.2	7	12
2-Chloro-4-nitrophenyl acetate				
Soluble enzyme	215	55	2	122
Immobilized enzyme	200	2	1	83

of 7 mM and a specific activity of 12 U/μg for hydrolysis of 4-nitrophenyl acetate, and a K_M of 1 mM and a specific activity of 83 U/μg for 2-chloro-4-nitrophenyl acetate. The magnitudes of K_M for immobilized and soluble carbonic anhydrase are similar, which indicates similar rates of dissociation of the enzyme–substrate complex. In contrast, the esterase activity of immobilized carbonic anhydrase showed a lower turnover number than the soluble enzyme, which indicates a slightly decreased rate of conversion of enzyme–substrate complex into product (Table I). Comparison of specific activities of soluble and immobilized enzymes showed a ratio of 64 and 68%, respectively. In spite of immobilization of enzyme to a solid support, enzymic activity and affinity to ester substrates indicate the integrity of the biological conformation of carbonic anhydrase.

The results presented here are pilot experiments preparing for future applications of carrier membranes as a support for the immobilization of various ligands and enzymes. Carrier membranes turned out to be an efficient support for affinity chromatography. Kinetic data for immobilized carbonic anhydrase indicate the integrity of the original enzyme conformation. Recent results have shown that other enzymes, such as trypsin, can be immobilized on membranes without loss of enzymic activity.

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