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AFFINOPHORESIS OF ANHYDROCHYMOTRYPSIN WITH AN AFFINOPHORE BASED ON POLY-L-LYSINE AS A MATRIX

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SUMMARY

Affinophoresis is an electrophoretic separation technique for biomolecules using an affinophore. The affinophore is a macromolecular polyelectrolyte bearing affinity ligands. It migrates rapidly in an electric field, and molecules that have affinity for the ligand are carried with it and separated from other molecules. An anionic affinophore for anhydrochymotrypsin was prepared by using poly-L-lysine (average molecular mass, 40 000) as the matrix. The amino groups of poly-L-lysine were succinylated. L-Tryptophan, an affinity ligand for anhydrochymotrypsin, was coupled through its α-amino group to one fifth of the carboxyl groups of the polymer by the use of water-soluble carbodiimide. The remaining carboxyl groups of the polymer were coupled with aminomethanesulphonic acid. Affinophoresis was carried out in 1% agarose gel plates, and the protein bands were detected with Coomassie brilliant blue R250. The migration of anhydrochymotrypsin toward the anode was greatly increased in the presence of the affinophore. The affinophoresis directly demonstrated that the conversion from serine-195 of chymotrypsin to a dehydroalanine residue in anhydrochymotrypsin results in an increase of over ten-fold in the affinity for the ligand, which resembles a product of the chymotrypsin-catalysed reaction.

INTRODUCTION

Affinophoresis [1] is a separation technique for biomolecules based on their specific affinity. An affinity ligand is attached to a soluble macromolecular polyelectrolyte and the resultant molecule is called an affinophore. In an electric field, the affinophore migrates rapidly due to its charges, and carries with it molecules that have specific affinity for the ligand.

Affinophoresis has been successfully applied to trypsin with either a cationic or an anionic affinophore bearing benzamidine, a competitive inhibitor of trypsin, as an affinity ligand. Both affinophores brought about considerable changes in the electrophoretic mobility of trypsin and the changes were large

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enough to separate trypsin from a mixture of proteins [1, 2].

Two types of ionic polymer have been used as matrices for affinophores, i.e. diethylaminoethyl-dextran for the cationic affinophore [1] and polyacrylyl-β-alanyl-β-alanine for the anionic affinophore [2]. Although both polymers are promising as general matrices for affinophores, the use of a commercially available polymer would be preferable from the viewpoint of easy application of this technique in a variety of fields. Here, the potential usefulness of poly-L-lysine, a commercially available polymer, as a general matrix for affinophores is reported.

Anhydrochymotrypsin (AnCht) is a catalytically inert derivative of bovine chymotrypsin (Cht) in which the active-site residue, serine-195, has been chemically converted to a dehydroalanine residue [3]. Ishii et al. [4] found that the conversion enhances the affinity of the protein for peptides containing an aromatic amino acid at the free carboxyl terminal, i.e. products of the chymotrypsin-catalysed reaction. Such a phenomenon, which was also observed in the case of trypsin [5], indicates the participation of the hydroxyl group of the active-site serine residue in the catalytic system, not only in cleaving the peptide bond of the substrate but also in the release of the product from the active site for effective catalytic turn-over.

In this work, a poly-L-lysine-based affinophore having a product-type ligand of Cht as an affinity ligand was prepared. Enhancement of the affinity for this product-type ligand as a result of the conversion of serine-195 of Cht to a dehydroalanine residue was directly demonstrated by affinophoresis.

EXPERIMENTAL

Poly-L-lysine hydrobromide [molecular weight (M_r), 30 000–70 000; average degree of polymerization, 190], α-chymotrypsin (from bovine pancreas, 3 × crystallized), chymotrypsinogen A (Chtg) (from bovine pancreas, 6 × crystallized) and phenylmethanesulphonyl fluoride were obtained from Sigma (St. Louis, MO, U.S.A.). Phenylmethanesulphonylchymotrypsin (PMS-Cht) [6] and anhydrochymotrypsin [7] were prepared as reported. AnCht was purified by high-performance affinity chromatography (HPAC) on a column of immobilized tryptophan on a hydrophilic vinyl-polymer gel, Toyopearl HW65S [8]. AnCht was also purified by affinity chromatography on an immobilized lima bean inhibitor [7].

Preparation of affinophore

Solid succinic anhydride (100 mg) was added all at once to a solution of poly-L-lysine hydrobromide (100 mg) in 5 ml of 0.1 *M* sodium chloride. The pH of the solution was maintained between 8 and 10 with 6 *M* sodium hydroxide over 10 min. Unreacted amino groups were determined in a 10-μl aliquot of the solution by reaction with fluorescamine, and amounted to less than 1% of the original. The solution was dialyzed against 0.1 *M* sodium chloride (3 × 1 l). The dialysate (6.1 ml) contained 340 μmol of lysine residue as determined by amino acid analysis (see below). L-Tryptophan methylester hydrochloride (17.2 mg, 68 μmol) was added to the solution and the pH was adjusted to 4.75 with 1 *M* hydrochloric acid. 1-Ethyl-3-(3-dimethylamino-

propyl)carbodiimide hydrochloride (65 mg, 340 μmol) was added and the mixture was left for 15 min without any adjustment of pH. Almost all the L-tryptophan methylester was bound to the polymer [checked by UV absorption measurement after thin-layer chromatography (TLC) on silica gel, R_F = 0.87, chloroform–methanol (3:1, v/v)]. Aminomethanesulphonic acid (57 mg, 510 μmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (130 mg) were added to the solution at pH 5 and the pH was maintained between 4.5 and 5.0 for 1 h with 1 *M* hydrochloric acid and 1 *M* sodium hydroxide. The solution was dialysed against 0.1 *M* sodium chloride (3 × 1 l). Then 0.13 ml of 6 *M* sodium hydroxide was added to the dialysate (7.4 ml), and the mixture was left for 30 min at 24°C to hydrolyse the ester. After neutralization with 6 *M* hydrochloric acid, the solution was dialysed against water (3 × 1 l) and freeze-dried (112 mg). Stock solution was prepared at 1% (w/v) in water and stored in a refrigerator.

Determination of the concentration of the polymers

A polymer sample (0.1–0.5 mg) was hydrolysed in 0.5 ml of 6 *M* hydrochloric acid at 110°C for 16 h in an evacuated and sealed tube. In the case of succinyl-poly-L-lysine, hydrolysis was not complete under the above hydrolysis conditions and corrections were made for the recovery (83%) of lysine. A 1% (w/v) solution of the affinophore contained 28 m*M* lysine residues.

Affinophoresis

Affinophoresis was carried out in the same system as reported before [2]. An agarose gel plate (1%), 1 mm thick, was made on a Gel Bond film (12.5 × 8 cm; FMC, Marine Colloids Division, Rockland, ME, U.S.A.). The gel plate contained 0.1 *M* sodium phosphate buffer (pH 7.2) and the affinophore (0.01–0.1%). Samples (2 μl), which consisted of 4 μg of protein (8 μg in the case of crude AnCht) and 8 μg of the affinophore in 0.1 *M* sodium phosphate buffer (pH 7.2), were applied on the middle of the plate using Sample Application Foil (LKB, Product No. 2117-206, Bromma, Sweden). Electrophoresis was carried out on a flat-bed apparatus (ATTO, SJ-1073, Tokyo, Japan) under cooling with ice-cold water at a constant current of 100 mA per plate for 30 min in a cold room (4°C). Sodium phosphate buffer (0.1 *M*, pH 7.2) was used as an electrode solution. Proteins were stained with Coomassie brilliant blue R250 after drying of the gel plates as reported previously [2].

RESULTS

Preparation of poly-L-lysine-based affinophore (Fig. 1)

The coupling reaction of L-tryptophan methylester with succinyl-poly-L-lysine by the use of water-soluble carbodiimide proceeded well, like the coupling reaction of *p*-aminobenzamidine with polyacrylyl-β-alanyl-β-alanine [2]. L-Tryptophan methylester used in the reaction was coupled to the polymer in a short time (15 min), with a coupling yield of > 95% as determined by absorption measurement at 278 nm of the outer solution of dialysis. The ionic groups of the affinophore were converted from carboxyl into sulphonate by sulphomethylamidation. After the coupling reaction with amino-

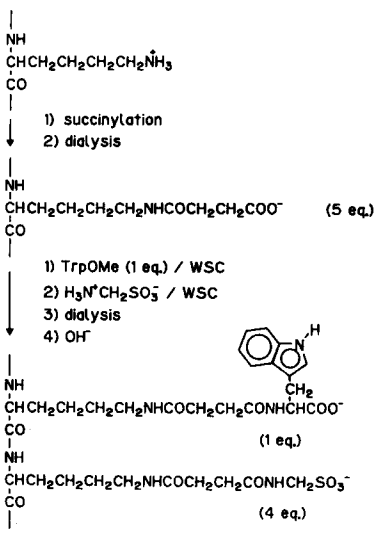

Fig. 1. Preparation of the affinophore for anhydrochymotrypsin. WSC = Water-soluble carbodiimide.

methanesulphonic acid, the amount of titratable carboxyl groups was equivalent to only 1—2% of the content of lysine in the affinophore solution; alkali treatment increased it to 24%, which is comparable to the amount of L-tryptophan methylester coupled to the polymer. These data support the progression of the reactions as represented in Fig. 1.

The affinophore bearing carboxyl groups was weakly stained by Coomassie blue dye. At a low concentration (< 0.02%, w/v) of the affinophore there was no problem in the detection of protein bands, but at higher concentrations a dark background resulted. Conversion from carboxylate to sulphonate made it unstainable by Coomassie blue dye, as already observed in the case of the affinophore bearing *p*-aminobenzamidine as an affinity ligand on a matrix of polyacrylyl-β-alanyl-β-alanine [2]. For this reason, the affinophore bearing sulphonate groups was used in the affinophoresis described below. This type of affinophore, however, inhibited the staining of protein and the sensitivity of the detection of protein was slightly impaired.

The affinophore (1%, w/v; 2 μl) was subjected to zone electrophoresis in an agarose gel plate under the same conditions as used in the affinophoresis described below. A section of the agarose gel was transferred onto a silica gel plate containing fluorescent indicator for TLC and migration of the affinophore was observed under UV light. The affinophore migrated to a position 6 cm anodal from the origin, as a definite band (100 mA/plate, 30 min).

Affinophoresis of AnCht and Cht

AnCht, Cht and inactive proteins related to Cht were subjected to affinophoresis (Fig. 2). All the proteins scarcely migrated under these conditions in the absence of the affinophore (plate A). Enhanced anodic migration upon affinophoresis was seen clearly for AnCht (plate B, Nos. 5 and 6) and, to a

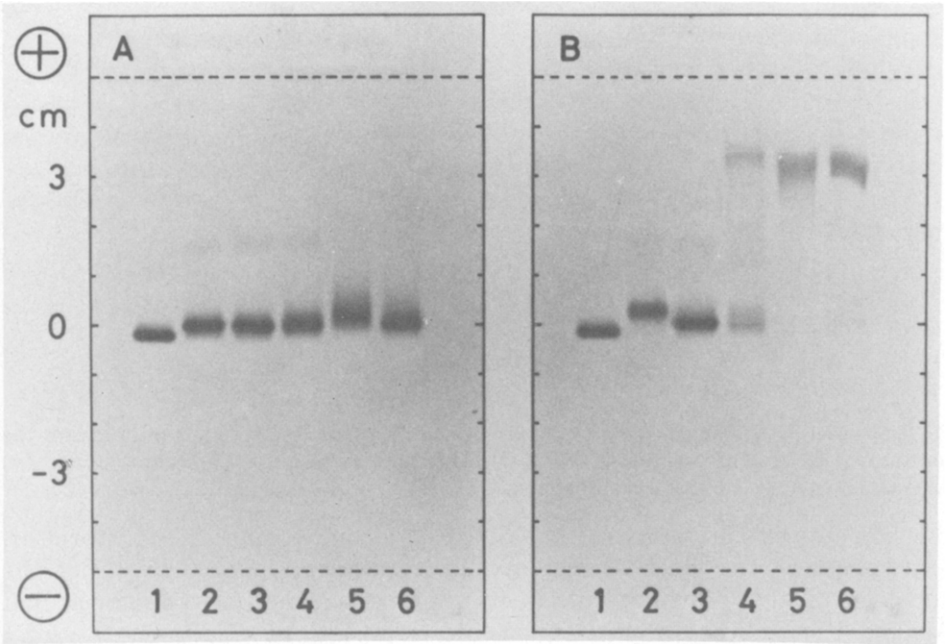

Fig. 2. Affinophoresis of anhydrochymotrypsin and related proteins. Electrophoresis of proteins (4 μg) was carried out in the absence (A) or presence (B) of the affinophore (0.01%, w/v). Other conditions were 100 mA per plate, 30 min, in 0.1 *M* sodium phosphate buffer (pH 7.2). 1 = Chymotrypsinogen A; 2 = α-chymotrypsin; 3 = PMS-chymotrypsin; 4 = crude anhydrochymotrypsin (8 μg); 5 = anhydrochymotrypsin purified on an immobilized L-tryptophan column; 6 = anhydrochymotrypsin purified on an immobilized-lima-bean inhibitor column.

much lesser extent, for Cht (plate B, No. 2). For Chtg and PMS-Cht, in which the substrate binding site is occupied by the phenyl ring of the covalently bound inhibitor, the affinophore had hardly any effect. This indicates that the remarkable effect of the affinophore in the case of AnCht is due to the specific affinity of the protein for the affinity ligand.

Nearly identical patterns were obtained for two AnCht preparations obtained by different purification procedures. Close observation of the plate, however, revealed some differences. AnCht purified by using an immobilized inhibitor column showed faint band(s) near the origin. This is consistent with the observation that this AnCht preparation contained a small amount of impurities resolvable by HPAC on an immobilized L-tryptophan column. On the other hand, AnCht purified by HPAC [8] appeared with some tailing. The reason for this is unknown.

Crude AnCht, which was obtained by alkali treatment of PMS-Cht, was also subjected to affinophoresis (plate B, No. 4). AnCht was completely separated based on its affinity for the ligand.

The dependency of the migrations of these proteins on the concentration of the affinophore in the agarose gel plate is summarized in Fig. 3. For AnCht, a saturation curve is obtained with half-saturation at a concentration of affinophore of $< 0.01\%$, which corresponds to the concentration of tryptophan

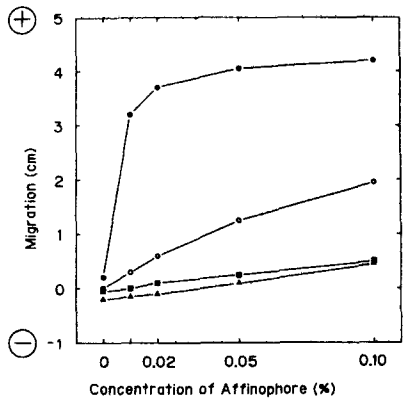

Fig. 3. Dependency of the migration of anhydrochymotrypsin and related proteins on the concentration of the affinophore. (•) Anhydrochymotrypsin; (○) α-chymotrypsin; (▪) PMS-chymotrypsin; (▲) chymotrypsinogen A.

residue on the affinophore of ca. 50 μM. On the other hand, for Cht, half-saturation is not attained even at the concentration of 0.1%, since the maximal migration of Cht expected in affinophoresis should be the same as that of AnCht. This indicates that over ten-fold enhancement of affinity toward a product-type ligand is caused by the conversion of serine-195 to a dehydroalanine residue. These observations are consistent with the reported dissociation constants of AnCht (14 μM) and Cht (0.4 mM) with acetyl-L-tryptophan, determined by quantitative affinity chromatography on immobilized AnCht and Cht columns (at pH 6.0 and 4°C) [4].

The increment in the anodic migrations of Chtg and PMS-Cht should be due to ionic interaction between the proteins and the affinophore.

DISCUSSION

Affinophoresis directly revealed the enhanced affinity of AnCht for a product-type ligand of Cht. Semi-quantitative information on the relative strength of the affinity for the ligand was obtained by performing the affinophoresis with various concentrations of the affinophore. In these experiments, six different samples could be analysed simultaneously within a short time (ca. 1 h). The capability of simultaneous analysis is one of the obvious advantages of affinophoresis.

Poly-L-lysine seems to be very useful as a matrix molecule for affinophores. Polymers of various degrees of polymerization are commercially available. The polymer can be easily derivatized in various ways by utilizing the nucleophilic reactivity of the side-chain amino groups. In addition, the amino group protrudes from the main chain of the polymer with a 4-CH_2 spacer, and this structural characteristic should reduce the possible steric hindrance by the main chain in the association with a target protein through an affinity ligand coupled to the amino group. It should also allow free positioning of the ionic group to avoid unfavourable ionic repulsion with a target protein. Succinylation should reinforce the characteristics of the polymer.

The sulphonate group has some advantages as an ionic group for anionic

affinophores in comparison with the carboxyl group, since an affinophore having sulphonate groups is not stained by Coomassie blue dye, and it should allow affinophoresis in a low pH region where ionization of the carboxyl groups is suppressed. The drawback to the use of sulphonate groups is in turn the difficulty of staining the protein itself after affinophoresis with this type of affinophore, as if the affinity of the dye for proteins had decreased. The difficulty of staining proteins presumably arises from ionic repulsion of the anionic dye by the anionic affinophore. Consequently, unless affinophoresis at low pH is to be carried out, introduction of sulphonate groups should be limited to a level that is sufficient to avoid staining problems.

Since the readily available polymer, poly-L-lysine, has been found to be a useful matrix for affinophores, affinophoresis should become more readily accessible for scientists in various fields.

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