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SEPHADEX-BOUND HISTAMINE IN THE CATALYSIS OF ESTER HYDROLYSIS

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Ester hydrolysis by Sephadex-bound catalysts was studied in a flow-through system. Three different immobilized preparations were synthesized and used: histamine-, coimmobilized histamine-octylamine-, and octylamine-Sephadex; octylamine-Sephadex was used as a reference. Immobilization was carried out using water-soluble carbodiimide, which gave amide linkages between carboxymethyl Sephadex and the groups attached. It was found that the coimmobilized histamine-octylamine preparation was three times more efficient than immobilized histamine alone in the hydrolysis of the ester *p*-nitrophenylcaproate, whereas hardly any difference was found in the hydrolysis of the less hydrophobic substrate *p*-nitrophenylacetate. We attribute this enhancement of the hydrolysis of *p*-nitrophenylcaproate to local enrichment of the substrate on the histamine-octylamine matrix caused by the presence of hydrophobic octyl groups.

INTRODUCTION

There has been considerable interest for some time in the preparation of enzymelike artificial catalysts (1,2). The preparations obtained so far have been given various names, such as synzyme (3) or enzyme analogue (4). Yet, although some progress has been made, the entire area is still in its infancy, as is underlined by the fact that, to the authors' knowledge, catalytic action has only been reported with artificial and "activated" substrates. In the studies reported, soluble catalysts have been used preferentially, such as short peptides (5) or soluble polymers derived by the coupling of functional groups (e.g., imidazole) (1–3). In the present communication we wish to report on an alternative approach, i.e., the immobilization of catalytic groups on insoluble matrices such as Sephadex. This line of approach is likely to offer the same advantages, including their use in continuous flow systems, as found with immobilized enzymes (6).

MATERIALS AND METHODS

1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide HCl (EDC), *p*-nitrophenylacetate (PNPA) and *p*-nitrophenylcaproate (PNPC) were of

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analytical grade and obtained from Sigma Chemicals, St. Louis, Mo. Carboxymethyl-Sephadex C-25 was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. (Ring-2-C14)-histamine-dihydrochloride (59 mCi/mmol in 250 μl of 2% ethanol) was from The Radiochemical Centre, Amersham, U.K., Triton X-100 and 2,4,6-trinitrobenzenesulphonic acid (TNBS) were bought from BDH, Poole, U.K. Octylamine and the buffer materials were of analytical grade and obtained from Merck, Darmstadt, West Germany.

Immobilization

CM-Sephadex C-25 (6 g, dry weight) was treated, after swelling in water and filtration, with 48 g of EDC in 100 ml of water for 90 min at pH 5.5 and at 22°C; the pH was kept constant with 6 M HCl. Subsequently, the gel was filtered after washing quickly with 50 ml of cold distilled water, pH 5.5. Portions of the gel (3 g wet weight, which corresponds to about 1 g dry weight) were then quickly added to the following 10-ml solutions: (I), 1.5 g of histamine dihydrochloride and 15 μl of the above C14 labeled histamine-dihydrochloride (3.6 μCi) solution; (II), the same as (I) but containing in addition 3 g of octylamine; (III), 1 g of octylamine. After addition of the gels, the pH in each solution was brought to 8.2 with 6 M NaOH; the final volume in each sample was about 15 ml.

The coupling reaction was allowed to proceed for 30 h at room temperature on a rocking table. The gels were then washed with 1l each of the following solutions: 0.01 M HCl, 0.5 M NaCl, followed by aqueous acetone of decreasing water concentration from 80 to 20%, and finally with pure acetone. The gels were then freeze dried and weighed. No optimization of the coupling conditions was attempted, but the amount of EDC used could be reduced to 1 g per g of dry CM-Sephadex C-25. The resulting gels, although substituted to a lesser degree with histamine and octylamine, respectively, also catalyzed ester hydrolysis, but the "hydrophobic effects" observed were not as pronounced. Only the results obtained with the higher substituted preparations will be discussed under below.

Determination of the Degree of Substitution of the Gels

The freeze dried gels (20 mg of each) were allowed first to swell in acetone, then washed with acetone-water (50/50) and finally with water; after this, concentrated HCl was added to give a concentration of 6 M. The samples were heated at 60°C for 6 h in order to dissolve the gels. The hydrolysates were then neutralized with 5 M NaOH.

The hydrolysates from gels I (histamine only) and II (histamine and octylamine) were analyzed for their radioactive histamine content. One ml of each of the hydrolysates was added to 10 ml of scintillation liquid (toluene/Triton X-100, 3/1), and the radioactivity was measured. The hydrolysate from gel III (octylamine) was used as blank. The hydrolysate obtained from gel III (octylamine only) was tested for its content of primary amine groups using TNBS (7), and octylamine was used as reference.

The freeze dried gels (100 mg) were also titrated in the presence of 1 M NaCl with 0.1 M NaOH. The amount of NaOH added to the first point of equilibrium was taken as a measure of remaining free carboxyl groups on the gels. The values obtained showed that approximately 10% of the carboxyl groups on the gels were substituted; all the gels were found to have nearly the same substitution level.

Assay of Immobilized Catalysts

Hydrolysis of the esters was measured at 402 nm following the appearance of *p*-nitrophenol. All measurements were carried out at 22°C under first-order reaction conditions, i.e., with a catalyst concentration (imidazole groups/ml reaction solution) far in excess of the ester concentration. After the substrates were dissolved in acetonitrile, ethanol and 0.07 M phosphate buffer, pH 7.8, were added so that the final composition of the solution was 1:4:95 of acetonitrile:ethanol:buffer, respectively.

The catalytic activity of the gels was assayed by passing substrate solution through a pasteur pipette (5 × 230 mm) of the appropriate gel (corresponding to 0.2 g of dry gel), and the resulting eluent was pumped to a 1-ml flow cuvette with a peristaltic pump, which was placed before the column. (It should be added that with some types of tubing, ester adsorption was noticed, which had to be compensated for to give a 0.060 mM substrate concentration entering the column; the product, *p*-nitrophenol, however, was found not to be adsorbed.)

Controls were carried out by stopping the pump to check upon possible leakage of catalytic groups. In no case was any increase in absorbance observed during these intervals, indicating strongly that the measured ester hydrolysis was caused by immobilized catalytic groups.

In a batch assay, 85 μmol of histamine (corresponding to the amount of histamine immobilized on 0.2 g of dry gel I) or 58 μmol of histamine (corresponding to the amount of histamine on 0.2 g of dry gel II) dissolved in the above buffer were added to *p*-nitrophenylcaproate present in the same buffer to a final volume of 20 ml. The amount of *p*-nitrophenol formed within 5 min. from an initial concentration of 0.060 mM *p*-nitrophenylcaproate was determined. Under these conditions autohydrolysis was negligible.

RESULTS AND DISCUSSION

In this study, catalysis of ester hydrolysis by the imidazole moiety of histamine immobilized on Sephadex was investigated. Although numerous studies have dealt with ester hydrolysis using artificial catalysts, e.g., imidazole, the immobilization of such catalytic groups has normally been to water soluble supports. We feel that the use of particulate matrices (e.g., Sephadex) offer a number of advantages; in particular, their easy application in flow systems.

Histamine was linked through amino groups to carboxyl groups of the gel by amide linkages formed on addition of carbodiimide. (Covalent binding of histamine to CNBr-activated Sephadex gave positively charged preparations, which had high blank activity, probably because of the formation of isourea groups; consequently, these derivatives could not be used.) In addition, histamine and octylamine were coimmobilized to give a more hydrophobic matrix, so as to compare the relative rate of catalysis of a more hydrophobic substrate, *p*-nitrophenylcaproate, with a less hydrophobic one, *p*-nitrophenylacetate.

In Fig. 1 the effect of different flow rates on the hydrolysis of the *p*-nitrophenyl esters by the gels is plotted. As can be seen, on increasing the flow rate, the degree of hydrolysis of both esters is reduced considerably. This behavior is expected and is analogous to what is known from studies with immobilized enzymes (8), where the residence time of the substrate in the column is the decisive factor, and not so much the fact that with increased flow rates, the unstirred layer surrounding the particles is diminished, thus decreasing the apparent K_m, which in principle could compensate for the shorter residence time existing using higher flow rates.

In Fig. 1 the degree of hydrolysis of *p*-nitrophenylcaproate by the more hydrophobic coimmobilized histamine-octylamine preparation is compared with a preparation containing histamine alone. As can be seen, the ester hydrolysis catalyzed by the former preparation is higher despite the fact that less histamine was bound (58 μmol) compared with the reference gel made up of only histamine (85 μmol). We attribute the enhancement of the catalysis by the histamine-octylamine gel to local enrichment of the substrate in the microenvironment of the catalytic groups, which is probably due to hydrophobic interactions between the substrate and the octyl groups on the gel (approximately 25 μmol as estimated from the above titration experiment). Hardly any difference was found in the hydrolysis of the less hydrophobic substrate *p*-nitrophenylacetate. This is in agreement with similar findings by other workers using water-soluble polyethyleneimine preparation (3).

Similarly, the coimmobilized histamine-octylamine preparation gives a higher degree of hydrolysis with the more hydrophobic caproate ester,

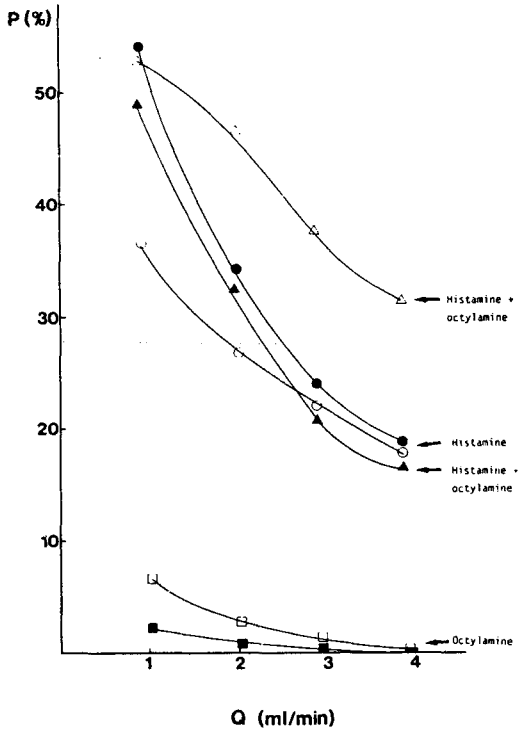

FIG. 1. Relationship between the degree of hydrolysis, P, of p-nitrophenylcaproate, PNPC, (open symbols) and p-nitrophenylacetate, PNPA, (solid symbols) and flow rate, Q, (ml/min). The substrate was pumped through a column containing 0.200 g (dry weight equivalent) of the appropriate immobilized preparation. Substrate concentration was 0.060 mM.

compared with the acetate ester. In contrast, the less hydrophobic preparation with only histamine immobilized showed a higher conversion of p-nitrophenylacetate than the corresponding caproate ester (at low flow rates). This effect could be due to diffusional restrictions, which may be more pronounced for the caproate ester at low flow rates. From Fig. 1 it can also be seen that a reference gel containing only bound octylamine (65 μmol) causes negligible background activity with either ester at high flow rates. With the more hydrophobic substrate, p-nitrophenylcaproate, and at low flow rates, some slight hydrolysis occurs; however, no ester hydrolysis takes place when nonsubstituted carboxymethyl (CM) Sephadex is used. The histamine gel preparations are true catalysts since, during the number of

assays run, many more moles of substrate are hydrolyzed by the gels than there are moles of catalytic groups present on the gel. Furthermore, the efficiency of the preparations did not decrease on repeated use.

The catalytic activity of soluble histamine was compared with that of the immobilized preparations based on the rate of formation of *p*-nitrophenol from *p*-nitrophenylcaproate. In the batch assay, the same amount of soluble histamine as coupled to the gels and the same substrate concentration was used; furthermore, an assay volume corresponding to the volume passing over the immobilized preparation during a given time and a given flow rate was used (see above). It was found that the rate of formation of *p*-nitrophenol, catalyzed by soluble histamine, was eight times less than that for the coimmobilized histamine-octylamine gel and three times less than that for the immobilized histamine gel. These values for the rates of hydrolysis observed with the gel preparations, at a flow rate 4 ml/min, were taken from Fig. 1.

The reason for the higher efficiency of both immobilized histamine preparations could possibly be caused by cooperative effects taking place on the matrix between the imidazole moiety and other functional groups present, such as carboxyl, hydroxyl, and amide groups. It appears unlikely that the immobilized form of histamine itself should be more efficient than the unmodified histamine used in the batch assay. As already mentioned, the reason for the 2.7-fold higher efficiency observed with the histamine-octylamine preparation compared with histamine immobilized alone is probably due to a higher local ester concentration on the matrix, caused by the hydrophobic octyl groups interacting with the substrate.

In conclusion, this report describes a highly convenient technique for the immobilization of an imidazole containing compound, histamine, to a well-characterized particulate support, Sephadex. In somewhat related recent reports, which appeared while this work was in progress, either protected histidyl groups coupled to cross-linked polyethyleneimine (9) or sodium imidazolate coupled to polystyrene (10) were used. With such insoluble preparations, (*a*) the assay is simplified as it becomes independent of any heterogeneity such as turbidity in the system, (*b*) possible leakage of catalytic groups can be easily detected, (*c*) they are readily reusable, and (*d*) they allow convenient separation of substrate and product.

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