IMMOBILIZATION OF ENZYMES BY COVALENT BINDING TO AMINE SUPPORTS VIA CYANOGEN BROMIDE ACTIVATION*

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SUMMARY : Enzymes can be readily linked to amine carrying supports activated by cyanogen bromide. High yields of binding and activity were obtained, and the bound enzymes exhibited greater stability than the free form toward denaturing effectors. In contrast with proteins bound to cyanogen bromide activated Sepharose, those bound to amine supports were not released from the matrix even in the presence of high concentrations of nucleophiles.

The most widely used method of immobilization of enzymes is their covalent binding to Sepharose via cyanogen bromide activation of the support (1-3). This simple and straightforward method generally results in a high efficiency of binding and the maintenance of most activity. However, it suffers from a drawback : the immobilized enzyme is leaky and in aqueous solution it is slowly released from the Sepharose (4-6). In the presence of strong nucleophiles the detachment is quite rapid, which is probably due to the solvolysis of the susceptible isourea bond linking the protein to the Sepharose (6-8). It has been expected that replacing the isourea bond by a guanidino group will lead to a more stable immobilized enzyme. For this purpose we investigated the preparation and properties of immobilized systems formed by coupling enzymes to amine carrying supports via cyanogen bromide activation.

MATERIALS AND METHODS

Bovine α-chymotrypsin was purchased from Worthington Biochemical Corporation, bovine trypsin and horse heart cytochrome C from Sigma, and subtilisin (Type Carlsberg) from Novo Industri, Copenhagen. Acetyl tyrosine

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ethyl ester, benzoyl arginine ethyl ester, Sepharose 4B and glass beads (G-700-50) were from Sigma. Polyacrylamide, Bio-Gel P-4, was from Bio Rad Laboratories. Aminoethyl cellulose and 3-aminopropyl triethoxysilane were from Aldrich. Cyanogen bromide, ethylene diamine, 1,8-diamino octane, hydroxylamine hydrochloride and hydrazine were the products of Fluka AG. Urea and and guanidinium chloride, ultra pure grade, were from Schwarz/Mann.

Binding of proteins to cyanogen bromide activated Sepharose was performed according to Axen and Ernback (9).

Binding of proteins to aminoethyl cellulose by glutaraldehyde was according to Glassmeyer and Ogle (10).

Binding of enzymes to amino derivatives of polyacrylamide : The hydrazide, aminoethyl and aminooctyl derivatives of polyacrylamide were prepared according to Inman and Dintzis (11). 1 gr of the polyacrylamide, substituted with diamine, was suspended in 15 ml of 0.1M sodium bicarbonate and then 500 mg of cyanogen bromide in 5 ml of bicarbonate was added. The pH was adjusted to 11 and maintained at this value for 6 minutes. Excess of the reagents was removed by filtration and the gel was resuspended in 20 ml 0.1N bicarbonate containing 20 mg of enzyme. The suspension was kept overnight in the cold and then thoroughly washed.

Binding to amine glass : Aminoalkylation of glass beads was performed according to Weetall (12). Protein coupling was carried out by cyanogen bromide activation of the amine glass by a procedure similar to that adopted for coupling them to the Bio-Gel amine derivatives.

Binding of protein to periodate oxidized Sepharose was according to Axen et al. (13).

The extent of protein coupling was estimated by subtracting the amount of free protein in the filtrate washings from that of total protein. The concentration of unbound protein in solution was determined by monitoring the absorbancy at 280 nm for chymotrypsin, trypsin and subtilisin, and at 410 nm for cytochrome C. The concentration of free enzyme in solution was also estimated from its activity towards its specific substrate.

Activity of free and matrix-bound enzymes was measured in the pH stat, using a Radiometer titrator in conjunction with a SBR 2c recorder. The total volume of the reaction mixture was 10 ml, the temperature was kept at 25°, and the pH was held constant by the addition of 0.02 N or 0.10 N NaOH. Pure nitrogen was bubbled through the vessel. In the case of chymotrypsin and subtilisin the reaction mixture contained 2 mM acetyl tyrosine ethyl ester at pH 8. Trypsin was assayed toward 1 mM benzoyl arginine ethyl ester at pH 8.

Denaturation : The degree of thermal denaturation was determined by heating the enzyme, free or bound to support, at the specified temperature for 15 minutes, and subsequently measuring the residual activity at 25°. Urea denaturation was carried out generally in 6 M reagent, pH 8. At different times samples were transferred to the pH stat reaction vessel and the residual activity determined.

Protein detachment from the support : A known amount of immobilized protein suspension was added to the solvolytic nucleophile solution, 1 M hydroxylamine or 1 M hydrazine, pH 9, or 1 M ammonia, pH 10. At different times the suspension was filtered and washed, and the filtrates checked for protein content and enzymatic activity. The solid was resuspended in the nucleophile solution.

RESULTS AND DISCUSSION

Table 1 lists the extent of binding and relative activity of different insoluble enzymes, prepared by coupling enzymes to cyanogen bromide-activated amine supports and by previously reported procedures. It shows that coupling of enzymes to amine supports by the new method generally results in good yields

TABLE 1. Efficiency of binding.

Derivative	Means of enzyme binding	% of binding	% relative activity
Seph-CT[b]	CNBr	50	25-40
Seph-CT	KIO_4	100	6
Cell-$(CH_2)_2$NH-CT	GA	70	6
PA-NH$(CH_2)_8$NH-CT	EDC	10	5
PA-NH$(CH_2)_8$NH-CT	CNBr	40	30
PA-NH$(CH_2)_2$NH-CT	CNBr	25	8
PA-NHNH-CT	CNBr	25	8
PA-CT	$NaNO_2$	40	30
Glass-NH-CT	CNBr	65	35
Glass-NH-CT	GA	100	10
Glass-NH-Tr	CNBr	100	22
Glass-NH-Sub	CNBr	60	100
Glass-NH-Sub	GA	75	100
Seph-Sub	CNBr	90	40

[a]Relative to the activity of free enzyme.

[b]Abbreviations : Seph, Sepharose; Cell-$(CH_2)_2NH_2$, aminoethyl cellulose; PA-NH_2, polyacrylamide; GA, glutaraldehyde; EDC, 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide; CT, α chymotrypsin, Tr, trypsin; Sub, subtilisin; S, support.

and considerable preservation of activity, comparable to other enzyme-coupling efficient procedures. Thus coupling of chymotrypsin to the 8-aminoalkyl derivative of polyacrylamide via cyanogen bromide activation resulted in a better efficiency of binding and higher activity than coupling by soluble carbodiimide. Also chymotrypsin bound to aminoalkyl glass via cyanogen bromide activation was more active than that prepared by using glutaraldehyde as the coupling agent.

The stability of the derivatized enzymes toward thermal and urea denaturation was studied. Fig. 1 shows the thermal denaturation of several chymo-

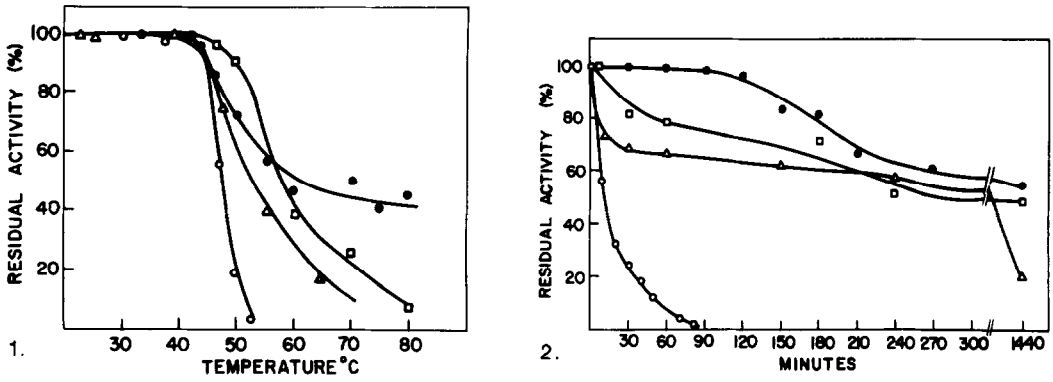

Fig. 1. Residual activity of free and insoluble chymotrypsin after heating for 15 min at different temperatures in 0.1 M phosphate buffer, pH 8. Activity was measured at 25°. o-native bovine α chymotrypsin; Δ - α-chymotrypsin bound to periodate oxidized Sepharose; □ -chymotrypsin bound to cyanogen bromide-activated Sepharose; ●-chymotrypsin bound to poly-(8-aminoocytyl)-acrylamide via cyanogen bromide activation.

Fig. 2. Irreversible denaturation of chymotrypsin derivatives in 6 M urea, 0.1 M phosphate buffer pH 8, 25°. Enzyme concentration 0.2 mg/ml. o-native enzyme; Δ-chymotrypsin bound to cyanogen bromide-activated Sepharose; □-chymotrypsin bound to aminopropyl glass via cyanogen bromide activation; ●-chymotrypsin bound to poly-(8-aminooctyl)-acrylamide via cyanogen bromide activation.

trypsin derivatives. The native enzyme undergoes complete irreversible denaturation on heating at 55° for 15 min, but the support-bound enzymes acquire increased stability and undergo denaturation at 20-30° higher. It is noteworthy that the enzyme coupled to the aminooctyl derivative of polyacrylamide preserves about half of its initial activity even after being heated to 80°. The greater stability of the bound enzymes is ascribed to diminished autolysis due to their fixation to the support. Greater heat stability was also observed with immobilized trypsin and subtilisin.

The insoluble enzymes also exhibit increased stability toward denaturants. Fig. 2 shows the time course of irreversible denaturation of chymotrypsins in 6 M urea. Free enzyme is completely inactivated after 90 minutes, whereas the immobilized enzymes retain considerable activity even after 24 hours. The enzyme coupled to cyanogen bromide-activated Sepharose has 20% residual activity,

while those bound to amine supports via cyanogen bromide-activation still possess more than 50% of their initial activity. Similar results were obtained also with insoluble trypsin and subtilisin. After prolonged incubation of these enzymes (2 mg/ml) at pH 8 and 25°, the immobilized forms exhibited greater stability. Free chymotrypsin and subtilisin retained about 40% of their original activity after ten days, while the insoluble derivatives preserved all of their activity.

It has been reported that amine ligands and proteins bound to cyanogen bromide-activated Sepharose undergo detachment from the carrier. Thus Tesser et al. (4) demonstrated that a variety of amine ligands bound to Sepharose or cellulose via cyanogen bromide activation were detached from their support by solvolytic processes occurring above pH 5 in aqueous surroundings. At 4° and pH 8-9 the rate of ligand release was about 0.5% of the amount of bound ligand per hour. Dilute ammonia was found to release the ligand as the guanidinium derivative. Topper et al. (5,6,14) have noticed that insulin and other proteins were also detached from the Sepharose support in bicarbonate or ammonia solution.

It was therefore of interest to examine the stability of the linkage of proteins to amine supports in the presence of nucleophile solutions. For this purpose several proteins bound to cyanogen bromide-activated supports were incubated with 1 M hydroxylamine or 1 M hydrazine at pH 9 or with 1 M ammonia at pH 10, 25°, and the amount of enzyme released into the solution was estimated as a function of time. Fig. 3 shows the time course of protein detachment from cyanogen bromide activated Sepharose using hydroxylamine as a nucleophile. Within 24 hours most of the protein was released with a half life of about 3 hours. With ammonia at pH 10 or 1 M hydrazine pH 9, 25°, considerable release was found after two days for CT and cytochrome C bound to cyanogen bromide-activated Sepharose. In parallel experiments in which proteins bound to cyanogen bromide-activated amine glass were examined in the presence of 1 M hydroxylamine or hydrazine no release of protein into the solution was detected even after 3 days.

The linkage of protein to cyanogen bromide activated amine carriers is very much more stable to nucleophilic attack than the analogous linkage to

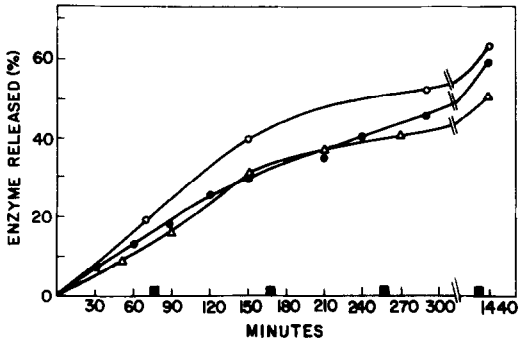

Fig. 3. Time course of protein detachment from supports in the presence of 1 M hydroxylamine pH 9, 25°. Δ-subtilisin type Carlsberg; ●-chymotrypsin; ○-horse heart cytochrome C, all bound to cyanogen bromide-activated Sepharose. ■-chymotrypsin bound to cyanogen bromide-activated aminoalkyl glass. Protein concentration - 0.5 mg/ml. Enzyme release was measured by the absorbancy of the supernatant after filtering the support.

Sepharose. This is expected in view of the greater stability of the C-N bond compared with the analogous C-O bond (e.g. amide versus ester bond). It is generally accepted that the bond between amine ligands and cyanogen bromide activated Sepharose is of the isourea type (6-8).

$$\text{Seph-OH} + \text{CNBr} \longrightarrow \text{Seph-O-C}{\equiv}\text{N} + \text{HBr}$$

$$\text{Seph-O-C}{\equiv}\text{N} + \text{RNH}_2 \longrightarrow \text{Seph-O-C(=NH)-NHR}$$

which is susceptible to nucleophilic attack and undergoes relatively facile solvolysis. By analogy it is assumed that with cyanogen bromide activation of amine support and protein binding a guanidine bond is formed which is very much more stable than the isosteric isourea bond.

$$\text{S-NH}_2 + \text{CNBr} \longrightarrow \text{S-NH-C}{\equiv}\text{N} + \text{HBr}$$

$$\text{S-NH-C}{\equiv}\text{N} + \text{RNH}_2 \longrightarrow \text{S-NH-C(=NH)-NHR}$$

The leakage problem can thus be overcome by the use of amine supports. Not only amines but also hydrazine and hydrazide groups can be activated by cyanogen bromide and then readily coupled to proteins, thereby providing a new and efficient method for the stable binding of proteins to solid supports.

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