Journal of Solid-Phase Biochemistry, Vol. 2, No. 1, 1977

COVALENT IMMOBILIZATION OF ENZYMES ON IONOGENIC CARRIERS
Effect of Electrostatic Complex Formation Prior to Immobilization[1]

V. P. TORCHILIN, E. G. TISCHENKO, and V. N. SMIRNOV

National Research Center of Cardiology AMS USSR
Moscow, USSR

Accepted March 18, 1977

Using a water-soluble carbodiimide, α-chymotrypsin was immobilized on synthetic insoluble and water-soluble copolymers of acrylamide and acrylic acid. It was shown that under conditions which allow the formation of electrostatic complexes between the carrier and the enzyme prior to immobilization, it is possible to bind much larger amounts of the enzyme as compared to standard immobilization techniques. The enzyme thus immobilized almost preserves its original catalytic activity. It can be characterized by higher thermal stability than that obtained without preliminary complex formation. It was also demonstrated that by changing pH and ionic strength of the immobilization medium it is possible to switch from one type of interaction between the enzyme and the carrier to another. The results of studies on the kinetic behavior of the immobilized enzyme in reactions with the low molecular weight substrate *N*-acetyl-L-tyrosine ethyl ester (ATEE) and the high molecular weight protein inhibitor are also presented.

INTRODUCTION

In recent years the preparation and the study of immobilized enzymes have become an important aspect of biochemical investigations. The stabilization of enzymes by immobilization on insoluble solid carriers is in fact quite popular (1–3). Considerable success is also observed in the stabilization of enzymes with soluble polymer carriers, a procedure of importance when the product of immobilization is used for the treatment of insoluble or high molecular weight substrates (4,5).

At present the binding of high amounts of enzymes per unit weight of a carrier and the preservation of activity by the larger amount of bound protein comprise the most important problem. The fact that only a few

[1]Abbreviations: EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; ATEE, *N*-acetyl-L-tyrosine ethyl ester; CP-2, copolymer of acrylamide with 2% acrylic acid; CP-10, copolymer of acrylamide with 10% acrylic acid; CP-30, copolymer of acrylamide with 30% acrylic acid. Enzyme: α-chymotrypsin, E.C. 3.4.4.5.

preparations correspond to these requirements is probably explained by the absence of some universal recommendations.

When ionogenic carriers are used for immobilization, the electrostatic interaction plays the important role in the immobilization process. It was shown that opposite charges on the carrier and the enzyme facilitate immobilization (6). From this point of view the results of investigations on complexes of protein with other polyelectrolytes (7–10) are of great interest. The results of these and other studies (11) suggest that the processes of electrostatic complex formation can be successfully used as an intermediate step in the immobilization of enzymes on ionogenic carriers.

In this investigation we attempted to understand the differences between the properties of immobilized enzymes when immobilization was carried out on the same carriers with and without electrostatic complex formation. To answer this question the immobilization of α-chymotrypsin on copolymers of acrylamide and acrylic acid in the presence of water-soluble carbodiimide was used as a model reaction.

MATERIALS AND METHODS

Materials

Crystalline bovine α-chymotrypsin (E.C. 3.4.4.5.) was the product of Olain chemical reagents plant (USSR) and had a specific activity of 60% as determined by the technique described by Bender et al. (12). Acrylamide, acrylic acid, *N,N*-methylenebisacrylamide, ammonium persulfate, and ATEE were from Koch-Light (Great Britain); EDC was from Sigma Chemical Company (St. Louis, Missouri); soya bean trypsin protein inhibitor, methanol, and acetone were obtained from Reanal (Hungary); Sephadex G-75 was the product of Pharmacia (Sweden); and all other reagents were produced by Reachim (USSR).

Methods

Water-soluble copolymers of acrylamide and acrylic acid were obtained by radical copolymerization of monomers in the presence of ammonium persulfate in aqueous solution. To have copolymers with various acrylic acid units the composition of a monomeric mixture of acrylamide and acrylic acid was calculated using the constants of copolymerization $r_1 = 1.38$ and $r_2 = 0.36$, respectively (13). Copolymerization was carried out at 60°C during 4 h; total monomer concentration in the reaction mixture was 5% (wt/wt). The copolymers were precipitated by methanol and dried with acetone. The composition of copolymers was determined by the method of infrared spectroscopy by adsorbance of the carboxylic groups (1726 cm^{-1}). The

measurements were made on the UR-20 spectrophotometer (GDR). Insoluble copolymers of acrylamide and acrylic acid were produced by emulsion copolymerization of the monomeric mixtures in the presence of 3% (wt/wt) *N,N*-methylenebisacrylamide in silicon oil at 50°C during 4 h with ammonium persulfate as initiator. The granular product was separated from the oil and dried with ether.

Immobilization of α-chymotrypsin on the soluble and insoluble carriers was performed using the coupling agent EDC. For this purpose 40 mg of the carrier was dissolved or suspended in 10 ml of phosphate buffer, pH 7.1, and ionic strength 0.1 or 1.0 (ionic strength was adjusted by the addition of various amounts of NaCl). Twenty milligrams of the enzyme was added in 1 ml of the same buffer followed by the addition of 5 mg of EDC in 1 ml of the buffer. The immobilization reaction was carried out at 4°C for 6 h. When water-soluble preparations of immobilized enzyme were made the separation of bound and native α-chymotrypsin was performed by gel chromatography Sephadex G-75 column (60 × 2.5 cm) at 4°C. Elution was performed with phosphate buffer, pH 8.0, at the rate of 0.7 ml/min at various ionic strengths. Gel filtration was monitored spectrophotometrically by measuring the eluent absorbance at 280 nm or following the absorbance at 400 nm after titration of the enzyme active centers with *p*-nitrophenyltrimethylacetate and the formation of *p*-nitrophenol. The measurements were conducted on a Turner UV spectrophotometer (United States). When insoluble products were prepared nonbound protein was separated by washing the carrier granules successively with 200 ml of phosphate buffer, 200 ml of 1 M NaCl, 200 ml of 0.002 N HCl, and 200 ml of double distilled water.

The kinetic studies of immobilized and native α-chymotrypsin were carried out on a pH-stat TTT-1c (Radiometer, Denmark) using the specific substrate ATEE at pH 7, a cell temperature of 25°C, and a starting concentration of 10^{-2} M ATEE.

Temperature inactivation of native and immobilized enzymes was studied at $[E]_0 = 1.2 \times 10^{-6}$ M, 37°C, in phosphate buffer, pH 7.2, at various ionic strengths by periodic measurements of the primary rate of substrate hydrolysis under the conditions described.

The inhibition of α-chymotrypsin immobilized on copolymers by soya bean trypsin protein inhibitor was studied at $[E]_0 = 10^{-8}$ M, pH 7, and 25°C using ATEE as a substrate at a concentration of 10^{-2} M in 1 and 0.1 M KCl. Measurements were made with a pH-stat technique. K_i was calculated in Lineweaver–Burk coordinates.

The concentration of free amino groups in the immobilized enzyme preparations was determined by spectrophotometric titration as previously described (19).

RESULTS AND DISCUSSION

To immobilize α-chymotrypsin we synthesized copolymers of acrylamide and acrylic acid which contained 3% (wt/wt) (CP-3), 10% (CP-10), and 30% (CP-30) acrylic acid. The pH of the medium for immobilization was 7.1 since at this pH the carrier and enzyme macromolecules are oppositely charged (pK of the polyacid is about 4.5 and the isolectric point of the enzyme is about 9), but contain some nonionized COOH and NH_2 groups, which are necessary to bind enzyme and copolymer through activation with EDC. The immobilization of enzymes using EDC at slightly alkaline pH has also been described (14). Preliminary complex formation was carried out in the reaction medium with the ionic strength of 0.1, since high ionic strength prevents complex formation due to the shielding of macromolecular charges by low molecular weight electrolyte ions. The results of gel filtration chromatography of the reaction mixture are shown in Fig. 1. This figure demonstrates that when up to 500 mg of enzyme per gram of the carrier is present in the reaction mixture only bound enzyme is found upon completion of the reaction. After EDC treatment all enzyme is covalently bound to the carrier. This was proved by analysis of the reaction mixture by column chromatography at 1.0 ionic strength. Under these conditions no free enzymes was found. When EDC was omitted from the reaction mixture the increase in ionic strength during gel chromatography from 0.1 to 1.0 caused complete dissociation of the complex (Figs. 1b and 1c). The data presented in Fig. 1d show that when immobilization was carried out at ionic strength 1.0 only part of the enzyme was bound to the carrier and significant amounts of the enzyme were eluted as the native form. This is probably explained by the fact that during immobilization at high ionic strength, complex formation does not occur and only standard chemical reactions between activated carrier and the enzyme take place. (The increase in ionic strength does not change the reactivity of EDC since the prolongation of the reaction time from 6 to 42 h does not increase the amount of the enzyme immobilized.)

An analogous effect is observed if the reaction of immobilization is performed at ionic strength 0.1 but at pH 9.4 when the carrier and the enzyme have the same charge (Fig. 1e).

Thus, our results demonstrate that electrostatic complex formation preceding the immobilization increases the amount of immobilized enzyme, probably due to facilitation of the reaction between active groups of a carrier and an enzyme which are in close proximity to each other in the complex formed. These data are in good agreement with the phenomenon of the increase in the sorption of alcohol dehydrogenase on alumina treated with albumin as compared to nontreated alumina (15). When albumin covers the

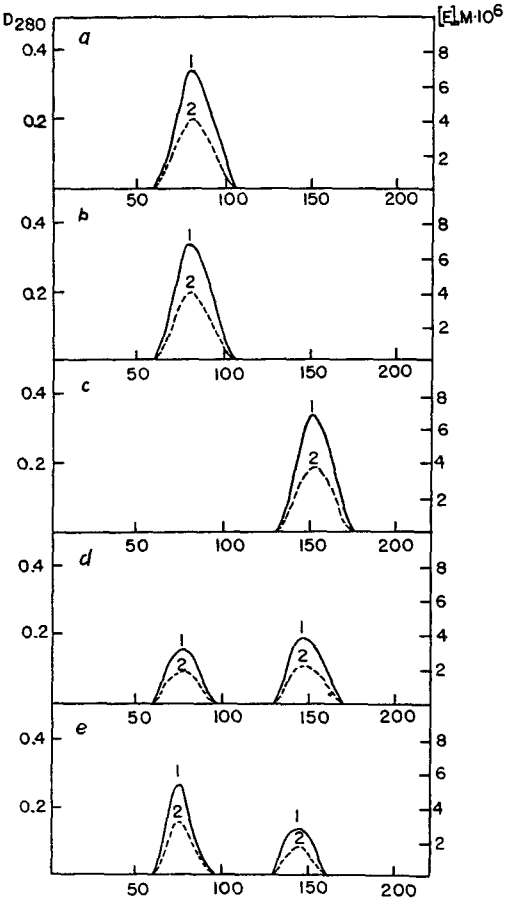

FIG. 1. Gel chromatography of α-chymotrypsin bound to CP-10 on Sephadex G-75 column. Curves: 1, absorbance at 280 nm; 2, concentration of the active enzyme in the eluent. (For details see the Methods section.) (a) Immobilization at pH 7.1, ionic strength 0.1, with EDC treatment. Eluent pH 8.0, ionic strength 0.1. (b) Same conditions as in (a), but ionic strength of the eluent 1.0. (c) Same conditions as in (a), but EDC treatment is omitted and ionic strength of eluent is 1.0. (d) Same conditions as in (a), but ionic strength of the immobilization medium is 1.0 and ionic strength of the eluent is 1.0. (e) Same conditions as in (a), but pH of the immobilization medium and the eluent is 9.4.

carrier and the enzyme has a slightly opposite charge, the amount of the protein adsorbed is always increased. This suggestion also explains the distortion of the interaction between protonized poly-4-vinylpyridine and serum albumin when the ionic strength was increased by the addition of NaCl (16). The shielding of the charges of macromolecules by low molecular weight electrolyte ions is what probably leads to the changes in macromolecular conformation, thus prohibiting the formation of polyelectrolyte complex between a protein and a synthetic polycation.

We have also studied the relation between the content of ionogenic groups in the carrier and the amount of immobilized enzyme (Table 1). If CP-2 under optimal conditions binds about 270 mg of the enzyme per gram of the carrier, the CP-10 and CP-30 are able to bind up to 500 mg of the enzyme per gram of the carrier. Although no differences in the amount of the enzyme bound are found for CP-2 with and without complexing, the amount of bound enzyme is decreased by 1.5- to 2-fold for CP-10 and CP-30 when complex formation does not occur (Table 1). These facts demonstrate that for successful complex formation some minimal number of charged groups in the carrier should exist. Under this limit the complex formation does not take place. The increase in the number of charged groups does not lead to any significant changes in the binding of the enzyme.

Several important facts have been found in the comparative study of the kinetic parameters of ATEE hydrolysis in the presence of immobilized α-chymotrypsin compared to native enzyme. These data are presented in Table 2. The most important observation is that immobilization of the

TABLE 1. **The Amount of α-Chymotrypsin Bound with Carrier versus Type of Carrier and Reaction Conditions**[a]

Carrier	Ionic strength of the immobilization medium	pH of immobilization medium	Amount of the enzyme immobilized (mg/g carrier)
CP-30	0.1	7.1	500
	1.0	7.1	400
	0.1	9.4	400
CP-10	0.1	7.1	500
	1.0	7.1	200
	0.1	9.4	325
CP-2	0.1	7.1	270
	1.0	7.1	250
Cross-linked CP-10	0.1	7.1	2.5
	1.0	7.1	1.2

[a]For conditions see the Methods section.

TABLE 2. **Parameters of ATEE Hydrolysis by Immobilized α-Chymotrypsin**[a]

Carrier	Ionic strength at immobilization	Ionic strength during hydrolysis	$K_m \times 10^3$	K_{cat} (sec^{-1})	Activity preserved (%)
CP-30	0.1	0.1	1.25±0.05	60±5	100
	0.1	1.0	1.15±0.05	140±5	—
CP-10	0.1	0.1	1.20±0.05	50±5	95
	1.0	0.1	1.25±0.05	70±5	75
	1.0	1.0	1.00±0.05	100±5	—
CP-2	1.0	0.1	1.30±0.05	60±5	80
Native	—	0.1	1.00±0.05	160±5	—
α-chymotrypsin		1.0	1.15±0.05	155±5	—

[a]For conditions see the Methods section.

enzyme does not change its activity (preservation of activity by 90–100%) if an intermediate stage of complex formation is involved. This is most likely explained by the suggestion that in the process of complex formation the active conformation of the enzyme is preserved and subsequent "sewing together" of the complex by EDC only stabilizes this conformation.

It is also found that the values of K_m for α-chymotrypsin immobilized with and without complex formation are practically similar and only insignificantly exceed K_m values for the native enzyme. At the same time K_{cat} values, which are similar for the enzyme immobilized both with and without complex formation, are two- to threefold lower than those for native enzyme. This is explained by a local decrease in pH of the medium in the vicinity of the active center of the enzyme due to high concentration of the COO^- carrier groups. Such an explanation seems quite logical since the increase in ionic strength of the reaction medium up to 1.0, which shields charges of the carrier molecules by oppositely charged ions of low molecular weight electrolyte, causes an increase in the K_{cat} of the immobilized enzyme by 2.5-fold (Table 2).

We have also studied the inhibition of immobilized enzyme by trypsin protein inhibitor from soya bean. It was found that for the enzyme immobilized on CP-2, CP-10, and CP-30 in all cases it is possible to observe 100% inhibition. At the same time the inhibition constant stays practically unchanged in the presence or absence of complex formation, and is about 2×10^{-9} mol/liter. Thus, it differs only slightly from the corresponding value for native α-chymotrypsin ($K_i = 1.4 \times 10^{-9}$ mol/liter). It is believed that the immobilization of α-chymotrypsin by the method described herein does not change its reaction ability toward macromolecular substrates.

The study of thermal inactivation of immobilized α-chymotrypsin (Fig. 2) demonstrated the differences in the thermal stability of the enzyme immobilized with and without electrostatic complex formation. Thermal stability was significantly higher for the enzyme which was immobilized via complex formation. This is probably explained by the fact that if complex formation occurs, larger numbers of bonds between the carrier macromolecules and the enzyme can be realized. This suggestion is supported by the noticeable decrease in the number of free amino groups for the enzyme immobilized via complexing in comparison with immobilization in the absence of complexation (9.5 and 11.5, respectively). These values were

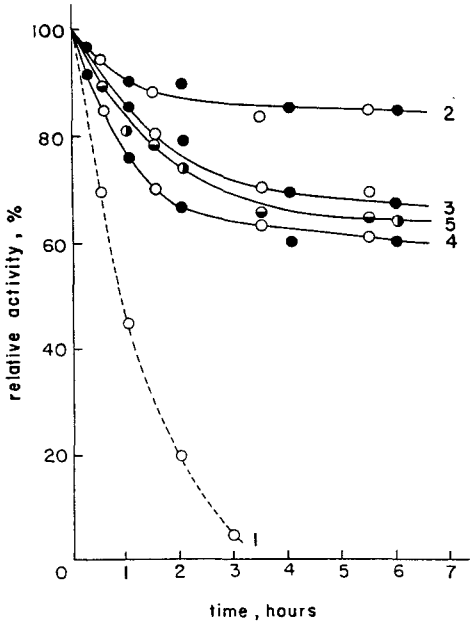

FIG. 2. Thermal inactivation (37°C, pH 7.1) of immobilized α-chymotrypsin preparations (curves 2–5) and native enzyme (curve 1) at similar active centers, concentration 1.2×10^{-6} M. Curve 2: α-chymotrypsin immobilized on CP-10 (○) and CP-30 (●) at ionic strength 0.1 and pH 7.1. Curve 3: α-chymotrypsin immobilized on CP-10 (○) and CP-30 (●) at ionic strength 0.1 and pH 9.4. Curve 4: α-chymotrypsin mmobilized on CP-10 (○) and CP-30 (●) at ionic strength 1.0 and pH 7.1. Curve 5: α-chymotrypsin immobilized on CP-2 at pH 7.1 and ionic strength 0.1 (◑) and 1.0 (◒).

determined by spectrophotometric titration. It follows from theoretical considerations previously discussed (17, 18) that thermal stability of the immobilized enzyme in this case should increase, which was in fact observed in our experiments.

It is essential to emphasize that at the same concentrations of carrier and enzyme for the preparations obtained with and without electrostatic complex formation, there is thermal stabilization (Fig. 2) but no decrease in the rate of autolysis. Autolysis should proceed at the same rate in both cases.

It is interesting to note that thermal stability of the preparations based on CP-2 in both cases was practically the same. This again shows that when the ionogenic groups in the carrier are too few, electrostatic complex formation does not occur even under favorable conditions.

It is quite likely that the important factor in stabilization of the enzyme is electrostatic repulsion of similar charges on the macromolecules of a carrier which results in its rigidity (negative charges on the macromolecule of the carrier are always in excess and are not completely neutralized by opposite charges of the enzyme molecule). That the rigidity of carrier macromolecules plays the important role in the thermal stabilization of the enzyme is proved by the following: When the ionic strength of the medium is increased, shielding of similar charges of the carrier from each other occurs. This results in the tendency to form statistical coils. The thermal stability of the immobilized enzyme is then no longer significantly different from that of native enzyme at the same ionic strength. Thus our data also demonstrate the importance of the rigidity of macromolecules of the carrier in increasing thermal stability of the water-soluble immobilized enzyme.

Electrostatic complex formation plays an important role in the immobilization of α-chymotrypsin on insoluble carriers, i.e., cross-linked copolymers of acrylic acid. It follows from the results shown in Table 1 and Fig. 3 that immobilization at low ionic strength (when electrostatic complex formation between the immobilized protein and the carrier is possible) causes significant increases in the amount of bound protein (1.2–2.5 mg enzyme/g carrier). In this case the presence of prereactional electrostatic complex formation causes significant increases not only in the amount of enzyme bound but also in its thermal stability. The incubation of the enzyme immobilized without complex formation at 37°C for 6 h leads to a drop in activity of 30%. The complex formation step prior to immobilization decreases the drop in activity to only 5%.

In conclusion, it is suggested that in immobilization of enzymes and other biologically active compounds on ionogenic carriers by covalent binding, electrostatic complex formation between the protein and the carrier can be successfully used. This allows the binding of larger amounts of active enzyme and a significant increase in the stability of the products.

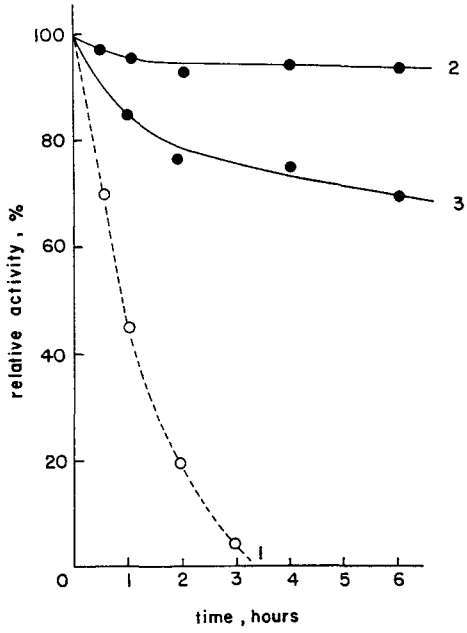

FIG. 3. Thermal inactivation (37°C, pH 7.1) of the native α-chymotrypsin (curve 1) and the enzyme immobilized on the cross-linked water-insoluble CP-10 (curves 2–3) at similar active centers, concentration 1.2×10^{-6} M. Curve 2: α-chymotrypsin immobilized at pH 7.1 and ionic strength 0.1. Curve 3: α-chymotrypsin immobilized at pH 7.1 and ionic strength 1.0.

REFERENCES

1. GOLDMAN, R., GOLDSTEIN, L., and KATCHALSKI, E. (1971) *In*: Biochemical Aspects of Reactions on Solid Supports, STARK, G. R., ed., Academic Press, New York, pp. 1–78.
2. MELROSE, G. J. H. (1971) Rev. Pure Appl. Chem. 21 : 83.
3. ZABORSKY, O. R. (1973) Immobilized Enzymes, WEAST, R. C., ed., CRC Press, Cleveland.
4. PECHT, M. (1972) Biochem. Biophys. Res. Commun. 46 : 2054.
5. FOSTER, R. L. (1975) Experientia 31 : 772.
6. KAY, G., and LILLY, M. D. (1970) Biochim. Biophys. Acta 198 : 276.
7. KINT, J. A. (1973) FEBS Lett. 36 : 53.
8. DAY, L. A. (1973) Biochemistry 12 : 5329.
9. ANDERSON, A. J. (1963) Biochem. J. 88 : 460.
10. POLDERMAN, A. (1975) Biopolymers 14 : 2181.

11. STRELTSOVA, Z. A., BRAUDO, E. E., and TOLSTOGUSOV, V. B. (1975) Bioorgan. Khim. 1 : 267.
12. BENDER, M. L., BEGUE-CANTON, M. L., BLEAKELEY, R. L., BRUBACHER, L. J., FEDER, J., GUNTER, C. R., KEZDY, F. J., KILLHEFER, J. V., MARSHALL, T. H., MILLER, C. G., ROLSKE, R. W., and STOOPS, J. K. (1964) J. Am. Chem. Soc. 88 : 5890.
13. HAM, D., ed. (1971) Copolymerization, Khimia, Moscow, p. 488.
14. JOHANSSON, A. C., and MOSBACH, K. (1974) Biochim. Biophys. Acta 370 : 339.
15. AZAWA, M., COUGHLIN, R. W., and CHARLES, M. (1975) Biotechnol. Bioeng. 17 : 1369.
16. MISTAFAEV, M. I., TSAREVA, E. A., and EVDAKOV, V. I. (1975) Vysokomol. Soed. 17 : 2226.
17. MARTINEK, K., KLIBANOV, A. M., TCHERNYSHIOVA, A. V., and BERESIN, I. V. (1975) Dokl. AN USSR 223 : 233.
18. MARTINEK, K., GOLDMACHER, V. S., KLIBANOV, A. M., TORCHILIN, V. P., SMIRNOV, V. N., CHASOV, E. I., and BEREZIN, I. V. (1976) Dokl. AN USSR 228 : 1468.
19. FIELDS, R. (1971) Biochem. J. 124 : 581.