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EVIDENCE FOR THE INDUCTION OF A CONFORMATIONAL CHANGE OF TRYPSIN BY A SPECIFIC SUBSTRATE AT pH 8.0

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A method is described that permits the covalent attachment of enzymes to solid matrices in the presence of saturating levels of a specific substrate under conditions where the enzyme is optimally active. Bovine trypsin immobilized on glass and agarose in the presence of saturating levels of *N*-α-benzoyl-L-arginine ethyl ester differs in a number of respects from the enzyme bound in the absence of substrate. Trypsin bound in the presence of substrate (*ES* form) is attached to porous glass through three more side chains than the enzyme coupled in the absence of substrate (*E* form). The rate of inactivation by iodoacetamide for the *ES* form is much greater than that for the *E* form. The two enzyme forms differ also in their reactivity with TLCK and DFP, active site-directed inhibitors. The pH dependences of the *ES* and the *E* forms bound to agarose differ; the pH optima are 9.0 and 8.5 for the *ES* and *E* forms, respectively. The thermal denaturation of the agarose–trypsins occurs in two distinct steps. The inactivation rate of the *ES* form is significantly slower than the *E* form in both stages. Also, for the hydrolysis of both *N*-α-benzoyl-L-arginine ethyl ester and *N*-α-benzoyl-L-arginine amide, the K_m values for the *ES* form were lower than the K_m values for the *E* form. Our data suggest that a substrate-induced conformatonal change occurs with trypsin, and that this conformation is stabilized when the enzyme is bound by multiple attachments to a solid support. These findings support the "induced-fit" theory in general and lend support to the interpretation of the activation of trypsin by methyl guanidine that is part of a specific substrate (Inagami, T., and Hatano, H. [1969] J. Biol. Chem. 244, 1176).

INTRODUCTION

In this paper, we address the problem of enzyme specificity as manifested at saturating levels of substrate. There are at least two theories that explain this phenomenon. The nonproductive-binding hypothesis states that a poor substrate may bind strongly to a rigid enzyme, but in multiple modes, some of which do not lead to a catalysis (1–5). A specific substrate possesses the maximum number of specificity determinants and binds in one productive mode. According to the induced-fit theory, a specific substrate is capable of bringing about a conformational change in the enzyme that repositions catalytic groups to a more favorable arrangement (6–9). A poor substrate may bind well, but not in such a way as to induce the conformational change.

Test of the induced-fit theory requires the detection of a conformational change of the enzyme while the specific substrate is present. This is

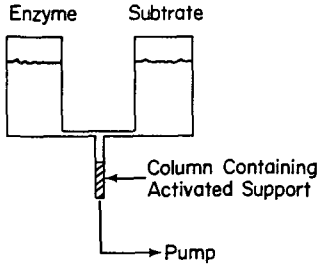


FIG. 1. Device for immobilization of an enzyme to a solid support in the presence of saturating levels of a specific substrate.

difficult, of course, because of the rapid transformation of the substrate to product. As a consequence, the experimental approaches taken in the past have been either (a) to study the enzyme conformation in the presence of a *specific* substrate under conditions where the enzyme is inactive or (b) to observe the enzyme under optimal conditions when a *nonspecific* substrate or inhibitor is present. In this report, we describe a method that appears to permit stabilization of a conformational state brought about by a specific substrate at the pH optimum of the enzyme. We used the apparatus shown in Fig. 1 for covalent binding of bovine trypsin to porous glass and agarose in the presence of saturating levels of a specific substrate.

The work of Inagami and coworkers suggested that trypsin would be an appropriate choice for this study (10,11). These investigators found that methyl guanidine brings about a 7-fold increase in the rate of the trypsin-catalyzed hydrolysis of Ac-Gly-OEt[1]. In addition, the rate of carboxamidomethylation of His-46[2] is enhanced by a factor of 6, when methyl guanidine is present. These results were interpreted as evidence in support of a substrate-induced conformational change. We have immobilized trypsin in the presence and absence of saturating levels of Bz-Arg-OEt. Chemical reactivity of the enzyme bound in the presence of substrate (*ES*) differs from that of the enzyme bound in the presence of substrate (*E*). Also, the pH dependences and temperature stabilities of the two enzyme forms differ.

EXPERIMENTAL PROCEDURE

Materials

Bovine trypsin (EC 3.4.4.4), 3×recrystallized was from Worthington Biochemical Corporation (Lot TRL 2DA). Sepharose 4B was obtained from Pharmacia Fine Chemical Company. The arylamine derivative of

[1]The abbreviations used are: Ac-Gly-OEt, acetylglycine ethyl ester; Bz-Arg-OEt, *N*-α-benzoyl-L-arginine ethyl ester; Bz-Arg-NH_2, *N*-α-benzoyl-L-arginine amide; TLCK, *N*-*p*-toluene sulfonyl-L-lysine chloromethyl ketone; DFP, diisopropylphosphorofluoridate.

[2]Trypsinogen numbering system (12).

porous glass (550 Å) was the product of Corning Glass Works. The Bz-Arg-OEt, Bz-Arg-NH_2, and DFP were purchased from Schwarz/Mann. The TLCK (lot 1106–0840) was a product of Sigma Chemical Company. Iodacetamide was from Eastman Kodak Company. Bromoacetone and methylguanidine were purchased from Schuchardt Company, Munich. Doubly distilled, deionized water was used throughout.

Methods

Enzyme Immobilization. The apparatus for immobilization of trypsin in the presence and absence of Bz-Arg-OEt is shown in Fig. 1. The buffer was 0.05 M Tris, pH 8, containing 25 mM $CaCl_2$. The reaction was carried out at 4°C. Typically, 1 g of either diazotized glass (13) or 1 ml cyanogen bromide–activated Sepharose 4B (14) was placed into a column (4.5 × 1.5 cm) and 1 cm of buffer layered on top. Enzyme solution (1 mg/ml, 200 ml) and Bz-Arg-OEt (10 mM, 200 ml) were pumped from the bottles as shown in Fig. 1; the flow rate was 5 ml/min. The *E* form of the enzyme was prepared analogously, with the Tris buffer substituted for the substrate solution. In both cases, the reaction time was 70–80 min.

For the preparation of the *ES* form of the enzyme, the following calculations may be performed to show that the enzyme is predominantly in the form of the *ES*-complex. The rate of substrate hydrolysis is given by $dP/dt = k_{cat}E_0$. A value of 3.2 s^{-1} for k_{cat} was calculated from the data of Gutfreund (15); E_0 is 2×10^{-5} M. For a contact time of 12 sec, ΔP is 0.84 mM. At the very bottom of the column, therefore the substrate concentration would be >4 mM, which is saturating. In the calculation of final substrate and enzyme concentrations, a dilution factor of 1/2 is used.

The amount of protein bound to porous glass was determined by amino acid analysis as previously described (16). The loss of lysine and tyrosine for glass-bound trypsin was also determined in these experiments using hydrolyzed native trypsin as a standard. Control experiments in which coupling runs were performed with substrate but no enzyme showed no binding of substrate to the support.

Rate Determinations. The hydrolysis of Bz-Arg-OEt was followed titrimetrically with a Radiometer pH-stat system. The standard overhead stirrer was used at high speed in all assays. The temperature of the reaction vessel was controlled at 25° ± 0.1. For a typical assay, 20 mg insoluble trypsin was suspended with a substrate solution of 1 mM Bz-Arg-OEt, 25 mM $CaCl_2$, and 0.5 mM Tris, pH 8.0. The standard base used as titrant was about 0.01 N NaOH. In the determination of the pH dependence, the following conditions were used: 10 mM Bz-Arg-OEt, 0.05 M Tris, 0.1 M KCl and 0.05 M $CaCl_2$. The titrant in this case was 0.05 N NaOH.

Corrections were made for nonenzymatic hydrolysis at the higher pH values (≥ 9.0).

Iodoacetamide inactivation. The reaction conditions used by Inagami and Hatano (11) for soluble trypsin were used for the immobilized *E* and *ES* forms. Autolysis of the bound trypsin derivatives need not be considered (Fig. 2). Buffers and the reaction vessel were purged with nitrogen that had been bubbled through Feiser's solution for removal of oxygen (17,18). The reaction vessel was covered with black tape to exclude light. Immobilized trypsin (20 mg) was suspended in 15 ml of a solution containing 0.1 M $CaCl_2$ and 0.4 M KCl. Solid iodoacetamide (1.39 g) was added, and the pH was maintained at 7.0 with the Radiometer pH-stat. The temperature was 25°C ± 0.1. At 2 h intervals, the bound enzyme was collected on a sintered glass filter, washed with 500 ml water, and assayed against Bz-Arg-OEt as described above. Fresh iodoacetamide was added to the recovered enzyme, and the reaction was continued. A control experiment without iodoacetamide showed no activity loss. The standard error in the rate determinations in the chemical modification studies is less than ±4%.

TLCK inactivation was performed at 25°C and pH 7.5 in 0.05 M Tris buffer containing 0.4 M KCl and 0.1 M $CaCl_2$. The TLCK concentration was 0.184 mg/ml. Remaining activity was determined at 50 min intervals. Fresh TLCK was added after each assay.

Bromoacetone inactivation was performed with a procedure based on that of Beeley and Neurath (19). The reaction was done at 40°C, and the pH was maintained automatically at 7.0 with 1 N NaOH. The reaction vessel

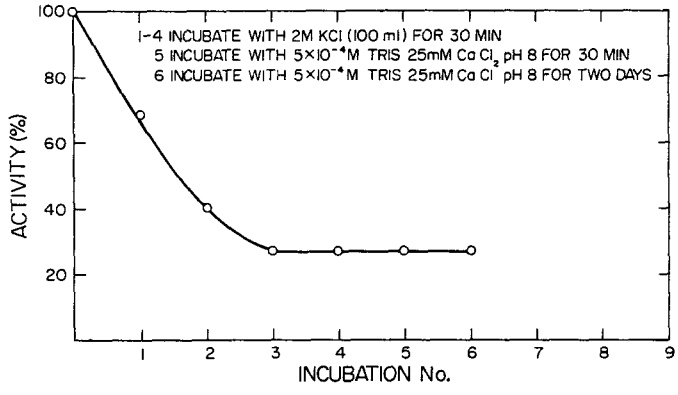


FIG. 2. A representative plot showing removal of adsorbed enzyme from a preparation of glass-bound trypsin. The final preparation shows no loss in activity after 2 days at pH 8.

was wrapped to exclude light. The reaction mixture contained 0.55 M $CaCl_2$, 0.375 M KCl, and bromoacetone at 0.29 M. A fresh bromoacetone solution was used after each assay.

DFP inactivation of the *E* and *ES* forms of trypsin was carried out in 0.5 M Tris at pH 7 containing 0.1 M $CaCl_2$ and 0.4 M KCl at 25°C. The DFP concentration was 25 μM. The bound enzyme was washed and assayed at timed intervals.

pH-dependence studies were performed with *E* and *ES* forms of trypsin bound to Sepharose 4B. The hydrolysis of Bz-Arg-OEt was followed titrimetrically, as described earlier. The Bz-Arg-OEt concentration was 0.01 M; the buffer was 0.05 M Tris containing 0.1 M KCl and 0.05 M $CaCl_2$. The relatively high Tris concentration was used to prevent a large difference in pH between the gel and the bulk solution (20,21).

Thermal denaturation of the Sepharose-bound trypsin derivatives was performed by incubation of the bound enzyme at 70°C for varying time periods. Damp Sepharose–trypsin (10 mg) was suspended in 18 ml 0.05 M Tris, pH 8.0, containing 0.025 M $CaCl_2$. The enzyme was stirred for the desired time period, after which the reaction mixture was cooled to 25°C. At this point, 2 ml 0.01 M Bz-Arg-OEt was added, and the rate of hydrolysis followed as usual.

The Michaelis constant for the hydrolysis of Bz-Arg-OEt by agarose-bound trypsin derivatives was determined with the enzyme in a fixed bed at pH 8.0, 0.05 M Tris, 25 mM $CaCl_2$, and 25°C ± 0.1. A ΔP was calculated from the change in absorbance at 254 nm on passage of the Bz-Arg-OEt solution through the column. The residence time was calculated from the measured flow rate and the void volume, which was determined by a published method (22). To obtain initial velocities, we used the method of Keyes and Semersky (23). At a given initial substrate concentration, ΔP was determined at 7 flow rates in the range of 60–280 ml/h. A rate free from external diffusional limitation was obtained by extrapolating to zero a plot of ΔP vs residence time or the reciprocal of the flow rate. A double-reciprocal plot was constructed, and K_m was calculated by the method of Wilkinson (24).

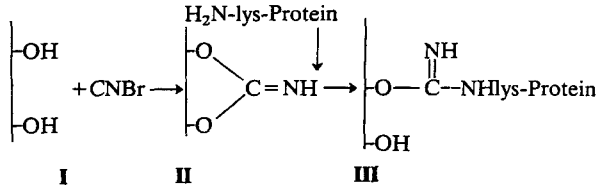
The hydrolysis of Bz-Arg-NH_2 was followed by the ninhydrin method (25). The enzymatic reactions were carried out at 25°C ± 0.1 in 0.05 M Na-Veronal, pH 8.0, which contained 25 mM $CaCl_2$. At timed intervals, aliquots were removed with a syringe fitted with a nylon net. The same enzyme sample was used throughout the K_m determination. After each run, the bound enzyme was washed in a sintered glass filter with 500 ml distilled water. Initial velocities were determined using substrate concentrations ranging from 0.5 to 20.0 mM.

RESULTS

Trypsin Immobilization

Trypsin was covalently linked to porous glass and agarose in the presence (*ES*) and absence (*E*) of the specific substrate Bz-Arg-OEt. Adsorbed protein was removed by stirring the bound enzyme with 2 M KCl. Figure 2 depicts a typical profile for a preparation of glass-bound trypsin. The final preparation can be stirred in buffer at pH 8.0 for 2 days with no loss in activity. The porous glass bound more enzyme when the substrate was absent: 5.5% (wt/wt) for *E* and 3.7% (wt/wt) for *ES*. In the case of agarose, the relative amounts of trypsin bound were reversed: 2.8% (wt/wt) for *E* and 4.5% (wt/wt) for *ES*. Glass-bound trypsin retained 10–15% of the original activity; Sepharose–trypsin retained 20–30%.

The amino acid analysis of agarose–trypsin showed no loss of amino acids. This finding is consistent with the proposed mechanism and linkage for cyanogen–bromide activation:



Acid hydrolysis of **III** would, of course, regenerate lysine. For trypsin bound to glass through the azo linkage, we observed losses in lysine and tyrosine. Notice that the losses are different for the *E* and *ES* forms of the enzyme (Table 1).

TABLE 1. **Amino Acid Analyses of Bovine Trypsin Reacted with Diazotized Arylamine Glass in the Presence (*ES*) and Absence (*E*) of Bz-Arg-OEt**

	Residues found (enzyme form)		
	Native	E	ES
Residue			
Lys	14.0	7.7	6.3
Tyr	10.0	5.7	3.6
Bonds/ enzyme molecule	-	10.6	14.1

Chemical Modification

The reactions of the E and ES forms of glass-bound trypsin with iodoacetamide are quite different (Fig. 3). A pseudo–first-order rate constant may be derived from Fig. 4, in which F is the percentage activity remaining at time t minus the percentage activity remaining at $t = \infty$. A second-order rate constant of $0.26\,M^{-1}\,h^{-1}$ results. Inagami and Hatano (11) found $0.02\,M^{-1}\,h^{-1}$ for the reaction of iodoacetamide with free trypsin, and $0.12\,M^{-1}\,h^{-1}$ for free trypsin plus methyl guanidine.

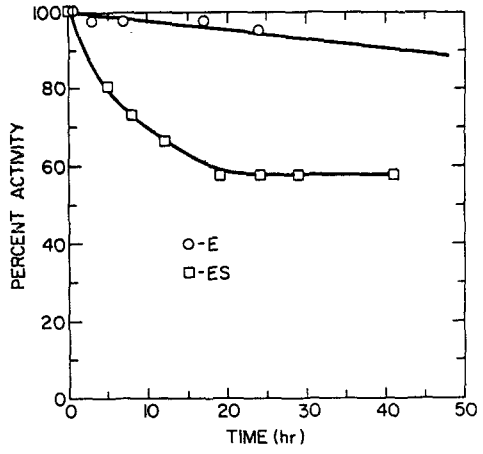


FIG. 3. Inactivation of glass-bound trypsin derivatives with iodoacetamide. E is the enzyme bound in the absence of Bz-Arg-OEt; ES is the enzyme bound in the presence of Bz-Arg-OEt.

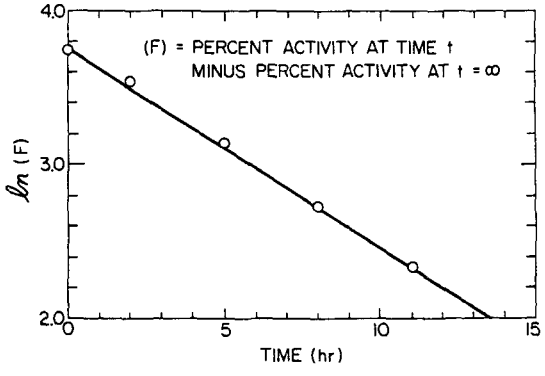


FIG. 4. First-order plot of the inactivation of the ES form of glass-bound trypsin with iodoacetamide.

TLCK inactivates trypsin by alkylation of His-46 (26), as does iodoacetamide (11). A difference between the reagents is that TLCK bears a strong structural similarity to a specific substrate. It may be seen in Fig. 5 that there is a small difference between the rates of inactivation of the *ES* and *E* forms of glass-bound trypsin with TLCK.

Bromoacetone bears little resemblance to trypsin substrates. It will, in fact, alkylate His-46 of DIP–trypsin (19). The rates of inactivation of the *E* and *ES* forms of glass-bound trypsin are virtually identical (Fig. 6). First-order plots of the data in Fig. 6 are straight lines for 90% of the reaction. A second-order rate constant of 2.28 M^{-1} h^{-1} results. With soluble trypsin

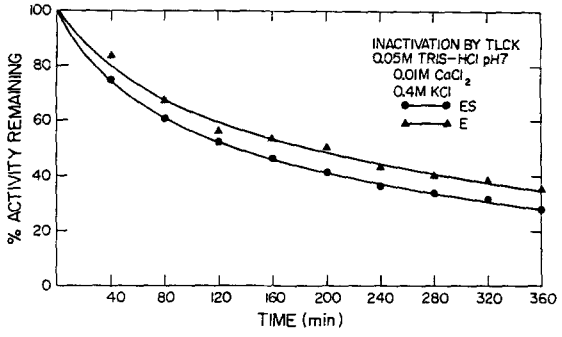


FIG. 5. Reaction of the *E* and *ES* forms of glass-bound trypsin with the active site-directed inhibitor TLCK.

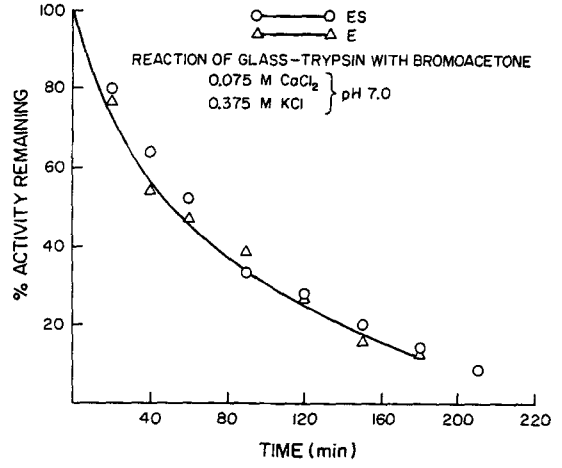


FIG. 6. Time course for inactivation of the *E* and *ES* forms of trypsin by bromoacetone.

(19), it is not possible to determine a corresponding rate constant; bromoacetone hydrolyzes and soluble trypsin autolyzes rapidly at 40°C and pH 7.0. The reaction rate of our bound trypsin, however, is not radically different from the rate for the soluble enzyme.

The reagents discussed above are directed at the imidazole group of His-46. It occurred to us that the reactivity of the Ser-183 would be worth investigating. The reaction rates of trypsin with DFP are quite different (Fig. 7). The *ES* form is inactivated at a faster rate, initially. After about 50 min, however, the rates appear to be about the same.

The effect of pH on the hydrolysis of Bz-Arg-OEt as catalyzed by the *E* and *ES* forms of Sepharose-bound trypsin is shown in Fig. 8. The pH optimum of the *E* form of Sepharose–trypsin is about 8.5. Knights and

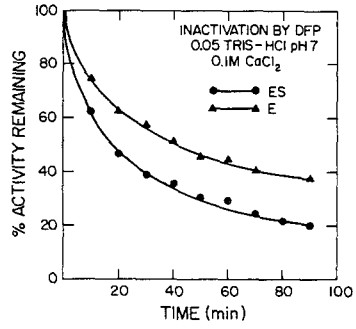


FIG. 7. Inactivation of the two enzyme forms by DFP.

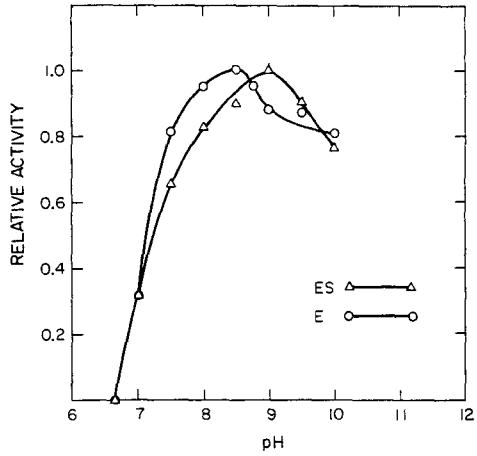


FIG. 8. Effect of pH on the hydrolysis of Bz-Arg-OEt as catalyzed by the *E* and *ES* forms of agarose-bound trypsin.

Light (20) reported for the Sepharose–trypsin, bound by the normal procedure, a pH optimum of 8.4. Note that the *ES* profile is shifted to the basic side. The pH optimum for the *ES* form is 9.0, or 0.5 unit greater than the *E* form. These experiments were done in buffer sufficiently strong to prevent a large pH gradient between the enzyme matrix and the bulk solution. It is assumed that the binding of enzyme in the presence of a specific substrate perturbs the pKa's of sidechains at or near the active site.

Since the number and nature of sidechains of trypsin bound to agarose cannot be readily determined, we sought an alternative method that would reflect structural differences or similarities between the two enzyme forms. If the *ES* forms have more or less points of attachment between enzyme and the agarose matrix, it would not be unreasonable to expect a difference in the rate of denaturation. Both enzyme forms were stable up to 60°C. The temperature of 70°C produced a rate of denaturation that was convenient to follow. The bound enzymes were heated at 70°C for timed intervals; at the end of each interval, the samples were cooled to 25°C and assayed. No renaturation was evident during the course of the assay. The retention of activity as a function of time for the two enzyme forms is shown in Fig. 9. The *ES* form of the Sepharose–trypsin is more stable than the *E* form. First-order plots of the data shown in Fig. 9 are linear and biphasic. The rate constants for the fast reaction are 40×10^{-3} min^{-1} for *E* and 25×10^{-3} min^{-1}

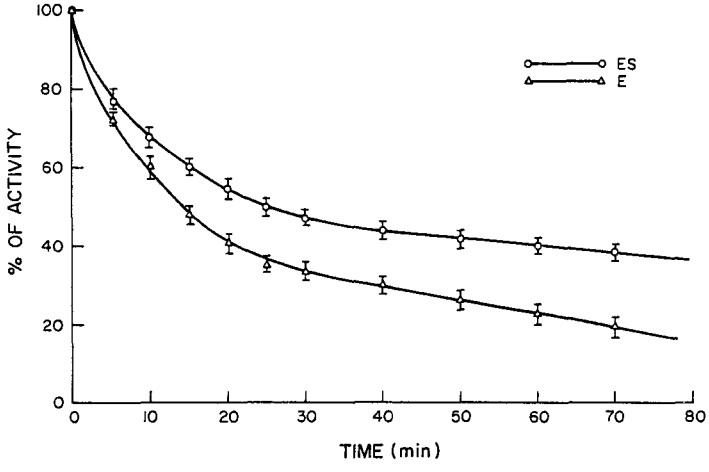


FIG. 9. Thermal inactivation of the two forms of agarose-bound trypsin. The bound enzyme samples were incubated at 70°C for the periods indicated and brought to 25°C for assay against Bz-Arg-OEt. During the assay, the appearance of product with time is linear, indicating that no renaturation occurs.

for *ES*. For the slower reaction, the constants are 12×10^{-3} min^{-1} and 4×10^{-3} min^{-1}, respectively. It is not our purpose to study the mechanism of the denaturation, but the results are interesting in that no protein–protein interaction can occur. This fact eliminates the complications of enzyme autolysis and precludes the possibility of a bimolecular mechanism. For soluble enzymes, kinetic orders (with respect to time) of >1 have been explained by cooperative mechanisms and the presence of impurities (27 and references therein). It appears that the bound forms of trypsin denature in two separate stages.

The hydrolysis of Bz-Arg-OEt and Bz-Arg-NH_2 in the presence of free trypsin follows Michaelis–Menten kinetics; deacylation and acylation are the respective rate-determining steps. The agarose-bound forms of trypsin, which catalyze the hydrolysis of these substrates, also show Michaelis–Menten kinetics; apparent K_m values appear in Table 2. Notice that K_m for Bz-Arg-OEt is 50–100 times greater when the enzyme is matrix bound. Immobilization of trypsin does not drastically raise the apparent K_m for Bz-Arg-NH_2, however.

DISCUSSION

The immobilization of an enzyme in the presence of a specific substrate may be visualized as a three-step process (Fig. 10). First, the enzyme and substrate are mixed at the top of the column under conditions favoring formation of the enzyme–substrate complex. Some of these complexes react with the activated solid support during passage through the column. Attachment occurs with varying numbers of bonds per enzyme molecule and with the imposition of varying three-dimensional stabilization. After removal of

TABLE 2. **Michaelis Constants for Agarose-Bound and Free Trypsin (mM)**

	E	ES	Free
Bz-Arg-OEt	0.41 ± 0.02	0.24 ± 0.01	0.004[a]
Bz-Arg-NH_2	2.4 ± 0.2	1.6 ± 0.1	3.3[b] 6.8[c]

[a] Value of BAINES, N. J., BAIRD, J. B., and ELMORE, D. T. (1964) Biochem. J. 90, 470–476.
[b] CHEVALLIER, J., and YON, J. (1966) Biochem. Biophys. Acta 122, 116–121 (pH 7.9, 35°C).
[c] GOLDSTEIN, L., LEVIN, Y., and KATCHALSKI, E. (1964) Biochemistry 3, 1913–1919 (pH 7.6, 25°C).

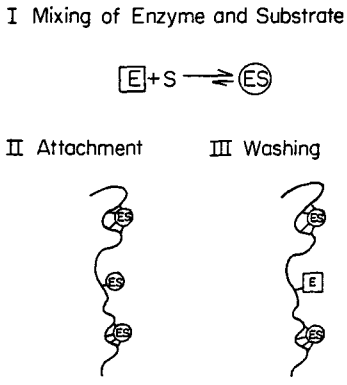


FIG. 10. Schematic illustration of our conception of the stabilization of substrate-induced conformation by covalent binding of enzyme to a solid matrix.

the substrate, those molecules that are not sufficiently cross-linked revert to the native form.

The data in Table 1 show that the *E* and *ES* forms are attached differently to the porous glass matrix. At least three additional side chains of trypsin react when the specific substrate is present. The numbers of lysine and tyrosine groups available for reaction are consistent with the X-ray structure of Stroud et al. (28) and the solution studies of Villaneuva and Herskovits (29,30). Also, a recent report suggests perturbation of tyrosine as a result of the binding of *N*-α-acetyl-L-lysine-methyl ester (31). The conditions for these latter studies are −56° and pH 4.6.

As shown in Fig. 3, about 40% of the *ES* form of trypsin bound to glass has an enhanced susceptibility to inactivation by iodoacetamide. The second-order rate constant of 0.26 M^{-1} h^{-1} is double the value for soluble trypsin in the presence of methyl guanidine. We interpret this as strong evidence that Bz-Arg-OEt induces a conformational change on binding to trypsin, and that this substrate-induced conformation is preserved by attachment of the enzyme to the solid matrix. The data on other histidine-directed reagents support this inference. TLCK, which bears a strong resemblance to a substrate, can induce the conformational change itself, at least to some extent. The rate of inactivation of the *E* form therefore approaches that of *ES*.

The mechanism of alkylation of His-46 with bromoacetone is evidently different from that of iodoacetamide. Beeley and Neurath (19) stated that the rate of reaction of bromoacetone does not depend on the nature of the inhibitor they used to retard autolysis. Similar rates of trypsin inactivation were obtained in the presence of benzamidine, methylguanidine, and even *N*-α-benzoyl-L-arginine. In contrast, the iodoacetamide reaction is activated by methylguanidine by 6-fold, whereas butyl guanidine reduced the rate (11). We would therefore ascribe the similarity in rates of akylation of

the *E* and *ES* forms by bromoacetone to the insensitivity of this reagent to the presence of guanidine compounds or substrate-induced structural changes. The data in Fig. 6 are significant in one other respect. Beeley and Neurath were not able to demonstrate first-order kinetics for the reaction of bromoacetone with trypsin because of autolysis and hydrolysis of bromoacetone. With the immobilized enzyme system, the bromoacetone solution is renewed at each assay, and the trypsin does not autolyze. We can therefore derive a meaningful rate constant. Also, the observation of first-order kinetics of inactivation is consistent with the loss of one type of residue that Beeley and Neurath showed by structural studies.

Inagami and Hatano (11) found that methyl guanidine *prevented* the phosphorylation of the active-site serine of trypsin by DFP. The inference is that the "inducer" enhances the reactivity of one part of the active site, but protects another. In our case, the "inducer," a specific substrate, is not present during the chemical modification study. As shown in Fig. 7, DFP reacts significantly faster with the *ES* form than the *E* form, which suggests that the binding of substrate affects Ser-183 as well as His-46.

In the consideration of Michaelis constants for bound enzymes, there are a number of complications that must be taken into account (32–34). The substrate may be attracted or repelled by the matrix. The inherent binding ability of the enzyme can be changed as a result of attachment to the support. External or internal diffusional limitations can raise the apparent K_m. A change in the K_m's of the *E* and *ES* forms of agarose-bound trypsin could result from any of the factors discussed above except external diffusion, which was eliminated as described earlier. If partitioning of substrate and internal diffusion effects of the matrix were similar for the *E* and *ES* forms of the enzyme, the lower K_m of the *ES* form could be ascribed to better binding. If the *ES* form is stabilized by the attachment to the matrix, less free energy of substrate binding would be needed for the conformational change, which would result in a decrease in the apparent K_m. In the case of Bz-Arg-NH_2, this analysis appears more attractive, since the K_m's of the bound enzyme forms do not differ greatly from the K_m of the soluble enzyme, which suggests the absence of diffusional and partitioning effects.

In conclusion, we present here a number of experiments that suggest that the presence of a specific substrate brings about a conformational change of trypsin, which may be preserved by attachment of the enzyme to a solid support. The experimental approaches seem sufficiently varied to make explanation of our findings by an artifact unlikely. Two support materials were used. Points of reaction of the two enzyme forms were shown. A number of chemical modification studies were done. Thermal stability, pH dependence, and kinetic data also suggest the preservation of the induced conformer.

ACKNOWLEDGMENTS

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REFERENCES

1. BERNHARD, S. A., and GUTFREUND, H. (1958) Proc. Intern. Symp. Enzyme Chem. Tokyo–Kyoto, p. 124.
2. SPENCER, T., and STURTEVANT, J. M. (1959) J. Am. Chem. Soc. 81 : 1874–1882.
3. HEIN, G. E., and NIEMANN, (1962) J. Am. Chem. Soc. 84 : 4495–4503.
4. NIEMANN, C. (1964) Science 143 : 1287–1296.
5. HAMILTON, C. L., NIEMANN, C., and HAMMOND, G. S. (1966) Proc. Natl. Acad. Sci. U.S.A. 55 : 664–669.
6. KOSHLAND, D. E., JR. (1958) Proc. Nat. Acad. Sci. U.S.A. 44 : 98–104.
7. THOMAS, J. A., and KOSHLAND, D. E., JR. (1960) J. Am. Chem. Soc. 82 : 3329–3333.
8. KOSHLAND, D. E., JR. (1970) Adv. Enzymol. 22 : 45–97.
9. KOSHLAND, D. E., JR., and NEET, K. E. (1968) Annu. Rev. Biochem. 37 : 359–410.
10. INAGAMI, T., and MURACHI, T. (1964) J. Biol. Chem. 239 : 1395–1401.
11. INAGAMI, T., and HATANO, H. (1969) J. Biol. Chem. 244 : 1176–1182.
12. WALSH, K. A., and NEURATH, H. (1964) Proc. Natl. Acad. Sci. U.S.A. 52 : 884–889.
13. ROYER, G. P., and GREEN, G. M. (1971) Biochem. Biophys. Res. Commun. 44 : 426–432.
14. CUATRECASES, P. (1970) J. Biol. Chem. 245 : 3059–3065.
15. GUTFREUND, H. (1955) Trans. Faraday Soc. 51 : 441–446.
16. ROYER, G. P., and UY, R. (1973) J. Biol. Chem. 248 : 2627–2629.
17. FEISER, L. F., and FEISER, M. (1967) *In* Reagents for Organic Synthesis, John Wiley and Sons, New York, p. 393.
18. LEE, P. P., and WESTHEIMER, H. G. (1966) Biochemistry 5 : 834–837.
19. BEELEY, J. G., and NEURATH, H. (1968) Biochemistry 7 : 1239–1251.
20. KNIGHTS, R. J., and LIGHT, A. (1974) Arch. Biochem. Biophys. 160 : 377–386.
21. KASCHE, V., LUNDQUIST, H., BERGMAN, R., and AXEN, R. (1971) Biochem. Biophys. Res. Commun. 45 : 615–621.
22. LILLY, M. D., HORNBY, W. E., and CROOK, E. M. (1966) Biochem J. 100 : 718–724.
23. KEYES, M. H., and SEMERSKY, F. E. (1972) Abstract No. B101.007, 164th Meeting of the American Chemical Society, New York.
24. WILKINSON, G. M. (1961) Biochem. J. 80 : 324–329.
25. MOORE, S. (1968) J. Biol. Chem. 243 : 6281–6283.
26. SHAW, E., and SPRINGHORN, S. (1967) Biochem. Biophys. Res. Commun. 27 : 391–397.
27. LAIDLER, K. J., and BUNTING, P. S. (1973) *In* The Chemical Kinetics of Enzyme Action, Clarendon Press, Oxford, p. 416.
28. STROUD, R. M., KAY, L. M., and DICKERSON, R. E. (1971) Cold Spring Harbor Symp. Quant. Biol. 36 : 125–140.
29. VILLANEUVA, G. B., and HERSKOVITS, T. T. (1971) Biochemistry 10 : 4589–4594.
30. VILLANEUVA, G. B., and HERSKOVITS, T. T. (1971) Biochemistry 10 : 3358–3365.

31. FINK, A. L. (1974) J. Biol. Chem. 249 : 5027–5032.
32. GOLDMAN, R., and KATCHALSKI, E. (1971) *In* STARK, G. R. (ed.), Biochemical Aspects of Reactions on Solid Supports, Academic Press, New York, p. 59.
33. THOMAS, D., BROWN, G., and SELEGNY, E. (1972) Biochimie 54 : 229.
34. BUNTING, P. S., and LAIDLER, K. J. (1972) Biochemistry 11 : 4477.