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Properties of an Insoluble Form of Trypsin*

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ABSTRACT: Insoluble trypsin preparations containing as high as 95% of the original soluble tryptic activity toward a small substrate have been prepared by attaching the enzyme onto aminoethylcellulose with the use of glutaraldehyde. These preparations are quite resistant to autolysis and suspensions of them are stable at room temperature for days or in the cold for months. Many properties of the insoluble trypsins, such as pH optima, activity in urea, and inhibition by certain low molecular weight compounds, are qualitatively similar to those of soluble trypsin. The main differences between the two forms of the enzyme, besides their relative stabilities, are the lack of complete inhibition of the insoluble form by excess diisopropyl phosphorofluoridate; the incomplete inhibition of the insoluble trypsin by protein inhibitors and an 80–100% reactivation by slow oxidation of insoluble trypsins which had been inactivated by reduction of the disulfide bonds. Preliminary studies of the action of pronase on insoluble trypsin indicate that a significant degradation of the trypsin molecule can occur without loss of enzymatic activity.

There are two main reasons for studying insoluble enzymes. According to Crook (1968) these are: first, to determine alterations in properties which result when enzyme molecules are immobilized on an insoluble matrix; and, second, to provide convenient insoluble catalysts which can be readily manipulated and easily removed from the reaction mixture. A further application is the possibility of determining the amino acid residues and conformation of the active center of an enzyme where this area has been relatively fixed in place. An additional application has resulted from the work presented here. The insoluble form of trypsin described in this paper is extremely resistant to autolysis and thus modification and degradation reactions can be performed at neutral or slightly basic pH values without the necessity of adding Ca^{2+} ions or inhibitors which sometimes are incompatible with other components in the reaction mixture. The stability of other forms of insoluble trypsin has been discussed in the literature (Weetall, 1970; Epstein and Anfinsen, 1962a,b; Epstein *et al.*, 1962; Levin *et al.*, 1964).

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Habeeb (1967) has reported an insoluble form of trypsin employing aminoethylcellulose as the insoluble matrix, glutaraldehyde as the cross-linking agent and crude trypsin. Haynes and Walsh (1969) described the cross-linking with glutaraldehyde of trypsin which had been adsorbed onto silica particles. The types of insoluble trypsin reported in this paper are modifications of those described by Habeeb (1967) but employing crystalline trypsin. This paper deals with the properties of these insoluble forms of trypsin and a comparison of them with trypsin in solution.

Experimental Section

Materials. Trypsin (twice crystallized, salt free), soybean trypsin inhibitor, lima bean trypsin inhibitor, and chicken ovomucoid were obtained from Worthington Biochemical Corp. Pronase, Grade B, was purchased from Calbiochem. Glutaraldehyde, 50% in water, was obtained from Fisher Scientific Co. AE-cellulose[1] was purchased from Bio-Rad Laboratories under the name of Cellex AE. Urea (Matheson Coleman and Bell, reagent grade) was recrystallized from hot 95% ethanol prior to use.

The casein used for assay of tryptic activity was obtained from Nutritional Biochemicals Corp., Hammersten quality. Benzoyl-DL-arginine-*p*-nitrophenylanilide hydrochloride (BAPA) also used for the assay of tryptic activity was obtained from either Schwarz-Mann or from Nutritional Biochemicals Corp.

p-Nitrophenyl *p*-guanidinobenzoate hydrochloride was purchased from Nutritional Biochemicals Corp.; α-*N*-*p*-tosyl-L-lysine chloromethyl ketone from Cyclo Chemical Corp.; phenylmethyl sulfonyl fluoride from Pierce Chemical Co.; and DFP from Schwarz-Mann. β-Mercaptoethanol (bp 153–157°) was obtained from Matheson Coleman and Bell.

Assay Procedures. Tryptic activity was determined with BAPA as the substrate by a modification of the method of Erlanger *et al.* (1961). After the 10-min incubation the reaction was stopped with 30% acetic acid and centrifuged at 3000 rpm for several minutes and the absorbance of the supernatant solution was measured at 410 nm. Tryptic activity toward casein as substrate was determined by the method of Kunitz (1947), except that a 2% stock casein solution and 20% trichloroacetic acid were used. In all cases except for pH–activity studies, assay reactions were carried out at room temperature in 0.1 M phosphate buffer (Sørensen) pH 8.0 with constant stirring.

Preparation of Insoluble Trypsin. Insoluble trypsin was prepared by a modification of the method of Habeeb (1967). The AE-cellulose was first washed with 0.5 N NaOH (15 ml/g of matrix) then with water to remove all of the NaOH. In a typical experiment, 14 g of AE-cellulose was treated as above, then suspended in 200 ml of 1 M phosphate buffer, pH 7.0. To the suspension was added 56 ml of 50% glutaraldehyde in water and the covered mixture was stirred at room temperature for 2 hr. The ratio of aldehyde groups to amino groups was about 150:1. The mixture was then centrifuged at 4000 rpm and washed six times with the buffer to dilute out the glutaraldehyde. An aliquot of the suspension was treated with ninhydrin to determine whether there were any unreacted amino groups left on the AE-cellulose (Moore and Stein, 1948). Under the conditions employed all amino groups were apparently derivatized. The washed, aldehyde-treated matrix was then resuspended in 280 ml of the same buffer and to this was added the trypsin dissolved in 1–10 ml of water depending on the amount of enzyme to be added. The amount of trypsin added varied from 4 to 214 mg per g of AE-cellulose. The reaction was carried out for 2 hr at room temperature with constant stirring. The mixture was centrifuged and washed with water until the supernatant was free of soluble trypsin as indicated by no activity toward BAPA (Erlanger *et al.*, 1961). In some cases a fivefold molar excess of $NaHSO_3$ (based on fixed aldehyde) was added after the trypsin reaction to modify the unreacted aldehyde groups on the matrix. In some instances the trypsin was allowed to react for only 30 min and the unreacted soluble enzyme was washed out of the suspension by centrifugation. The packed material was then resuspended in 1 M phosphate buffer, pH 7.0, and allowed to incubate for another 90 min in order to promote further cross-linking with the matrix of the trypsin molecules already attached.

Determination of Amount of Trypsin on the Matrix. The minimum amount of insoluble trypsin which would correspond to 0.05–0.1 mg of protein was suspended in 10 ml of 6 N HCl in a glass-stoppered flask containing a Teflon sleeve. The suspension was kept at 110° for 20 hr and was stirred frequently for the first several hours until the suspension was dissolved. Any amount of insoluble trypsin higher than 15 mg resulted in considerable charring and in dark-colored hydrolysates. After 20 hr the HCl was removed on the flash evaporator using saturated NaOH in the receiver and the contents of the flask were taken to dryness. The residue was dissolved in the appropriate amount of 0.2 N citrate buffer, pH 2.2, and analyzed on the Beckman Model 120 amino acid analyzer according to the method of Moore *et al.* (1958). The amino acid analyses of the various insoluble trypsin preparations were similar to that of pure trypsin. The amount of trypsin was calculated from the amino acid content.

Results and Discussion

Several proteins and one diamine were employed as extenders before turning to AE-cellulose as a matrix in an attempt to produce an insoluble trypsin of maximum specific activity and dispersibility. The methods used were those of Habeeb (1967) using glutaraldehyde, but substituting the various compounds and crystalline trypsin for crude trypsin. The purpose was to obtain more homogeneity in the copolymer of trypsin–glutaraldehyde–amino compound than is obtained by using a mixture such as crude trypsin. The extenders tried were: insulin, α,ω-diaminododecane, bovine serum albumin, and crystalline trypsin alone. The first two substances were chosen because the amino groups are located at or near the termini of the molecule, thus keeping the trypsin molecules further apart in the copolymer. In none of the cases was the insoluble enzyme of appreciable specific activity, especially with casein as substrate, and in many cases the particles were too coarse to disperse uniformly for reproducible pipetting. In some cases the cross-linking was carried out in the presence of 0.3 M α-*N*-benzoylargininamide to protect the active center during the reaction. The insoluble products were gummy and could not be pipetted. Attempts were made to attach trypsin onto Amberlite IR 45 and onto Bio-Rad Cellex PAB (*p*-aminobenzylcellulose) after reaction with glutaraldehyde as insoluble matrices. The products had considerable tryptic activity but the particles were again too large to form uniform dispersion. Aminoethylcellulose proved to be the most acceptable matrix for the attachment of the trypsin. The products were

[1] Abbreviations used are: AE-cellulose, aminoethylcellulose; BAPA, *N*-α-benzoyl-DL-arginine-*p*-nitrophenylanilide hydrochloride; DFP, diisopropyl phosphorofluoridate.

TABLE I: Yields and Activities of Insoluble Trypsin Preparations.

Preparation Number	mg of Trypsin[a]/g of AE-Cellulose	mg of Trypsin on Matrix/g of Insoluble Preparation	% Yield[b] Protein	% Yield[c] Activity BAPA	% Yield[c] Activity Casein
	Low Ratio[d]				
1	3.7	2.8	76	94	12
2	4.3	4.3	100	95	19
3	8.6	4.0	45	95	20
4	10.0	2.3	23	95	
	High Ratio[d]				
5	110	28	26	97	11
6	110	70	63	75	12
7	180	46	26	95	
8	200	20	10	95	15
9	214	26	12	88	24

[a] Milligrams of trypsin in the reaction mixture. [b] Milligrams of protein on the matrix as a percentage of the total mg of protein used in making the preparation. Values from column 3 divided by those in column 2 expressed as a percentage. [c] Activity toward BAPA or casein relative to that of an equal amount of soluble trypsin used for the preparation expressed as percentage. The amount of protein on the matrix was determined from the amino acid content of a hydrolysate of the preparation (see Experimental Section). [d] Ratio of trypsin amino groups to aldehyde groups on the matrix.

capable of being finely dispersed; reproducible pipeting could be easily carried out and the insoluble enzyme could be centrifuged down very rapidly at a low number of revolutions per minute.

In contrast to the method of Habeeb (1967) for preparing insoluble trypsin, the AE-cellulose was treated with a very large excess of glutaraldehyde prior to the addition of trypsin. The reaction was carried out in 1 M phosphate buffer, pH 7.0, to obtain a higher concentration of the buffer acid component for catalyzing the reaction. All of the excess glutaraldehyde was washed out of the aldehyde-treated AE-cellulose prior to the addition of the trypsin, hence the enzyme could couple or cross-link only to the matrix and could not cross-link with itself. The crystalline trypsin was added either in very small amounts (*low ratio* of trypsin amino groups to aldehyde groups on the matrix) or in very high amounts (*high ratio*). The former would supposedly give rise to a highly cross-linked, rather immobilized, insoluble trypsin, the latter to a very slightly cross-linked product of high molecular flexibility, the properties of which should be similar to those of soluble trypsin. Actually, as can be seen from the results of the experiments described below, little or no difference could be shown between the two types of preparations and any reference to cross-linked or non-cross-linked preparations is used in an operational sense only.

In some preparations the unreacted aldehyde groups on the matrix were derivatized by reaction with $NaHSO_3$; in most cases they were not so modified. No difference could be detected between the activities of the two types of preparations toward BAPA, casein, or soybean trypsin inhibitor.

The properties of the various insoluble trypsin preparations are discussed below.

Protein Content, Activity, and Yield. The protein content, activity relative to that of soluble trypsin toward BAPA and casein, and the yield of trypsin insolubilized of some insoluble trypsin preparations are shown in Table I. Variations in the volume of the reaction mixtures, ratio of derivatized AE-cellulose amino groups to trypsin, and time of reaction account for the observed differences in yield of protein on the matrix. For insoluble trypsin preparations of *high ratio* of trypsin to matrix, 2.5–7.0% of the total weight was protein. In the case of *low ratio* types only 0.2–0.4% of the total weight was protein. Amino acid compositions of the insoluble trypsin preparations were in most cases similar to that of pure trypsin. The tryptic activities of the insoluble preparations relative to that of the soluble trypsin ranged from 63 to 100% toward BAPA as substrate and from 6 to 30% toward casein as substrate. The commercial trypsin used to make the preparations was only 70–85% pure as determined by active-site titration (Chase and Shaw, 1967). No trend in the activities toward either substrate showed with respect to the supposed degree of cross-linking. It would appear that in both types of preparation the trypsin molecules are cross-linked to the same degree or are similarly confined by the matrix. The yield of commercial trypsin attached onto the matrix was 50–100% for the *low ratio* types and 10–26% for the *high ratio* preparations.

Stability at 4°, Room Temperature, and 100°. The insoluble trypsin preparations could be used repeatedly without loss of activity and could be left standing in pH 8 buffer at room temperature for 40 hr with only a 9% loss of activity. The insoluble trypsin preparations were stored wet (water) in the cold room (4°) for several months. In most cases there was at most a 15% drop in activity. The activity of the insoluble trypsin preparations was completely destroyed upon incubation at 100° in pH 8 buffer for 15 min.

Effect of pH on Activity. The activity toward BAPA at various pH values of two insoluble trypsin preparations was compared with the activity of crystalline trypsin. There was a slight shift in pH maxima of all insoluble trypsin preparations to around pH 9 compared to the pH maximum of 8.5 for crystalline trypsin and a somewhat broader pH curve for the former from pH 8 through pH 10. With casein as substrate the pH maxima were also around pH 9 and the pH curves

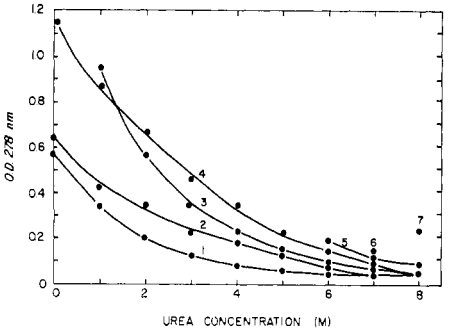

FIGURE 1: Activity in urea solutions of soluble and insoluble trypsin on BAPA. All reactions were carried out in 0.1 M phosphate buffer, pH 8.0. Ordinate is optical density at 410 nm of *p*-nitrophenol released from BAPA (see Experimental Section): curve 1, soluble trypsin, 40 μg; curve 2, preparation 7 (Table I), high ratio, equivalent to 60 μg of trypsin; curve 3, soluble trypsin, 160 μg; curve 4, preparation 7, 120 μg; curve 5, preparation 7, 240 μg; point 6, soluble trypsin, 2560 μg; point 7, preparation 7, 960 μg.

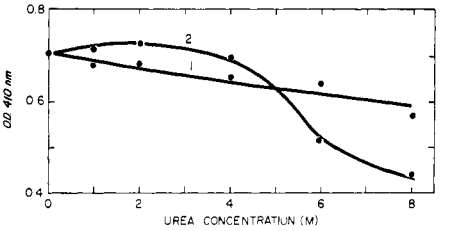

FIGURE 2: Stability of insoluble trypsin in urea. Incubations of insoluble trypsin preparations were carried out in 0.1 M phosphate buffer, pH 8.0. Ordinate is optical density at 410 nm of *p*-nitrophenol released from BAPA (see Experimental Section): curve 1, preparation 9 (Table I), equivalent to 70 μg of trypsin, preincubated in urea for 10 min; curve 2, preparation 9, 70 μg; preincubated in urea for 4 hr. The urea was washed out of the preincubation mixture by centrifugation prior to assay.

were identical for both insoluble and crystalline trypsin. The pH activities determined in this study for soluble trypsin were identical with those reported for casein as substrate (Northrop *et al.*, 1948) and with BAPA as substrate (Erlanger *et al.*, 1961). Levin *et al.* (1964) and Goldstein *et al.* (1964) have reported the shift in pH optimum to 9–10.5 of insoluble polyanionic-trypsin copolymers and have attributed the shift to the negative charges in the microenvironment of the trypsin. The insoluble trypsin preparations used in the present study showed no evidence of carboxyl groups when titrated with NaOH.

Effect of Urea on Activity and Stability. The effect of urea concentration on the activity of insoluble and soluble trypsin toward BAPA is shown in Figure 1. Notice that the effect of urea was dependent on the concentration of enzyme even though this was extremely small in comparison to the concentration of urea. The effect of urea was identical in both the soluble and insoluble form of the enzyme and was similar to that reported with *N*-α-*p*-tosyl-L-arginine methyl ester as substrate (Harris, 1956). It was anticipated that urea would have only a slight effect or no effect on the activity of the *low ratio*, supposedly highly cross-linked type of insoluble trypsin because of a relatively fixed conformation expected for this form of the enzyme. However, this was not observed.

The denaturing effect of urea on any given amount of insoluble trypsin depended on the urea concentration and the time of exposure. The results are shown in Figure 2. In 8 M urea for 10 min at pH 8.0 there was 18% inhibition (assayed after urea was removed), and 38% inhibition after 4-hr preincubation. After removal of the urea this loss of activity could not be recovered even after 8-hr stirring at room temperature or 40-hr standing at 4°. In 6 M urea the irreversible loss of activity was 9% after 10 min and 26% after 4-hr incubation. In contrast to this, soluble trypsin is 45% irreversibly denatured in 8 M urea, pH 7.7, for 4 hr, but 80% irreversibly denatured in 5 M urea for this same time and pH. This has been explained by Riordan *et al.* (1960) as the digestion of the denatured form by the still active form of trypsin in 5 M urea, whereas in 8 M urea there is essentially no active form. Since insoluble trypsin does not autolyze during the times of urea exposure and since it is essentially inactive in 8 M urea, the observed losses in activity were presumably due to irreversible denaturation. It cannot be readily determined whether insoluble trypsin autolyzes in 6 M urea or is irreversibly denatured at this concentration. It has considerable activity in 6 M urea but normally does not autolyze at least in the absence of urea.

The slight increase in activity of the insoluble trypsin preincubated for 4 hr in 1–4 M urea (curve 2, Figure 4) might be real. Small increases in activity have been observed for all insoluble trypsin preparations upon stirring in buffer for an hour or more after having been stored for some time under water, even though the temperature had previously been raised to 25°.

Action of Mercaptoethanol. Treatment of the insoluble trypsin preparations for 30 min with 10^4 molar excess of β-mercaptoethanol resulted in around 50% loss of activity. Longer treatments did not increase the amount of inactivation. The limited loss of activity was not caused by a deficiency of mercaptoethanol because after 4-hr incubation additional insoluble trypsin could be added to the reaction mixture with resultant loss of 30–40% of its activity. After removal of the mercaptoethanol and subsequent stirring for several hours open to the air, 80–100% of the original activity was regained. When the reduction was carried out in the presence of 7 M urea for 4 hr all of the activity was lost irreversibly. As shown in Figure 2 only about 32% of the activity was lost after 4-hr incubation in 7 M urea alone.

It thus appears that there is at least one somewhat inaccessible and crucial disulfide bond in the insoluble trypsin preparation. Whether this is a matter of heterogeneity or one molecular species with a lowered enzymatic efficiency could possibly be determined by an all-or-none assay. As is shown below, DFP cannot be used for this purpose and the results with *p*-nitrophenyl guanidinobenzoate (Chase and Shaw, 1967) gave doubts as to the applicability of this inhibitor to the determination of active sites in the insoluble trypsins used in the present study. Soluble trypsin similarly treated lost activity irreversibly. Epstein and Anfinsen (1962a,b) have reported the reduction with loss of activity and reoxidation with slight regain of activity of two forms of insoluble trypsin.

Effect of Protein Trypsin Inhibitors. The degree of inhibition of insoluble trypsin by protein inhibitors depended among other factors on the molecular weight of both the inhibitor and of the substrate. This was also shown by Haynes and Walsh (1969) for the type of insoluble trypsin studied by them. For any given inhibitor the degree of inhibition depended on the amount of inhibitor, the amount of enzyme, and on the time of preincubation with the inhibitor. The inhibition on BAPA as substrate by soybean trypsin inhibitor was not instantaneous as it was for soluble trypsin. The inhibition reached its maximum in 10 min for a 1:1 ratio of soybean trypsin in-

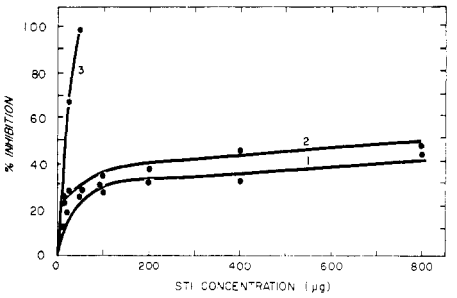

FIGURE 3: Effect of concentration of soybean trypsin inhibitor on the inhibition of soluble and insoluble trypsin. Preincubation with inhibitor was carried out in 0.1 M phosphate buffer, pH 8.0. The inhibited enzymes were assayed with BAPA: curve 1, preparation 5 (Table I), *high ratio*, equivalent to 40 μg of trypsin, 30-min preincubation; curve 2, preparation 2, *low ratio*, 40 μg, 30-min preincubation; curve 3, soluble trypsin, 50 μg, 10-min preincubation.

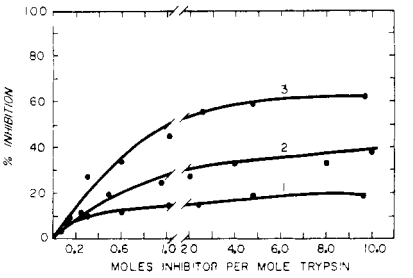

FIGURE 4: Inhibition of insoluble trypsin by protein trypsin inhibitors. Insoluble trypsin preparation 9 (Table I) was used. Enzyme and inhibitor were preincubated 30 min (soybean trypsin inhibitor was 10 min) in 0.1 M phosphate buffer, pH 8.0, and then assayed with BAPA: curve 1, chicken ovomucoid; curve 2, soybean trypsin inhibitor; curve 3, lima bean trypsin inhibitor.

hibitor to enzyme, or a higher maximum in 30 min for an 8:1 ratio, and no appreciable increase occurred thereafter even up to 280-min incubation. The effect of concentration of soybean trypsin inhibitor on the inhibition of two insoluble trypsin preparations and on soluble trypsin is shown in Figure 3. At a 20-fold molar excess of soybean trypsin inhibitor over enzyme the inhibition was only 40–50%. It was shown that the excess soybean trypsin inhibitor was not nonspecifically adsorbed onto the matrix or protein, but was free in solution. Soluble trypsin was 100% inhibited by a stoichiometric amount of soybean trypsin inhibitor. With casein as the substrate the inhibition was 88% at 0.5% final substrate concentration. This concentration of casein was about two times the molar concentration of soybean trypsin inhibitor and ten times the concentration of trypsin. An increase in the final casein concentration to 2% caused a small decrease in inhibition to around 78%. Thus, the higher concentration of casein did not result in a significant reversal of inhibition by soybean trypsin inhibitor. The slight increase in activity was due to the higher substrate concentration which was observed in the absence of inhibitors.

The maximum amount of inhibition of insoluble trypsin by soybean trypsin inhibitor could not be attained by stepwise addition of stoichiometric amounts of inhibitor. The amount of inhibition against BAPA was 30% after the first treatment with soybean trypsin inhibitor and remained at this figure after five treatments with inhibitor. However, when a tenfold amount of soybean trypsin inhibitor was added initially, the maximum of 50% inhibition was obtained in one treatment.

The manner of soybean trypsin inhibitor inhibition of insoluble trypsin was similar to that of the inhibition of soluble trypsin insofar that the inhibitor could be dissociated from the soybean trypsin inhibitor–insoluble trypsin complex (Finkenstadt and Laskowski, 1965) by lowering the pH to around 3.5 followed by washing with 0.001 N HCl. The regenerated insoluble trypsin possessed 85% of its original activity toward BAPA.

In Figure 4 is shown a comparison of the inhibition with BAPA as substrate by ovomucoid (molecular weight 33,000), soybean trypsin inhibitor (molecular weight 22,000), and lima bean inhibitor (molecular weight 9000). These inhibitors inhibit soluble trypsin 100% in stoichiometric amounts. The degree of inhibition was inversely related to the molecular weight of the inhibitor, a result observed by Haynes and Walsh (1969) for their insoluble trypsin preparation. It can be seen that there are some trypsin molecules that are not inhibited by protein inhibitors even when they are preincubated with a large molar excess of inhibitor. These trypsin molecules are either attached to the inside of pockets on the matrix or are attached to the matrix in a conformation that cannot bind protein inhibitors. The already mentioned fact that insoluble trypsin has only 6–30% of the activity toward casein as soluble trypsin has is in agreement with the existence of inner molecules of trypsin within the matrix as postulated by Haynes and Walsh (1969). However, there are some trypsin molecules on the matrix that are not inhibited by lima bean inhibitor, soybean trypsin inhibitor, or ovomucoid but are able to hydrolyze casein. For example, the maximum inhibition by these inhibitors toward casein was not 100%, but was 93, 88, and 76%, respectively. This could be a result of heterogeneity of the casein although ultracentrifugal analysis of this protein showed no molecules with molecular weights in the range of those of the inhibitors.

Reaction of Insoluble Trypsin with p-Nitrophenyl Guinidinobenzoate. An attempt was made to titrate the active sites of insoluble trypsin with *p*-nitrophenyl *p'*-guanidinobenzoate. However, this could not be done quantitatively. The addition of the titrant to a suspension of insoluble trypsin did not result in an immediate "burst" of *p*-nitrophenol, as is the case for soluble trypsin (Chase and Shaw, 1967). The insoluble trypsin showed a gradual, uniform increase in the production of *p*-nitrophenol with time. Thus, the end of enzymatic hydrolysis and the beginning of nonspecific hydrolysis of this inhibitor could not be resolved. Nevertheless, the insoluble trypsin was 100% inhibited after about 5-min reaction when assayed with both casein and BAPA. This behavior toward *p*-nitrophenyl *p'*-guanidinobenzoate is different from that observed by Haynes and Walsh (1969) who reported the titration of their insoluble trypsin preparation with this inhibitor.

Inhibition by Arginine. Insoluble trypsin preparations were inhibited toward BAPA by arginine to a lesser extent but otherwise in the same way as soluble trypsin is inhibited.

It is puzzling why *p*-nitrophenyl *p'*-guanidinobenzoate reacted at a lower rate and why arginine reacted to a lesser extent with insoluble trypsin than with soluble trypsin whereas in most cases BAPA reacted at the same rate with the two different forms of the enzyme.

Inhibition by N-α-p-Tosyl-L-lysine Chloromethyl Ketone and by Phenylmethylsulfonyl Fluoride. These two inhibitors (Shaw *et al.*, 1965; Fahrney and Gold, 1963) reacted similarly with soluble and insoluble trypsin. Both forms of the enzymes at 10^{-6} M concentration were completely inhibited toward BAPA after 30-min treatment with 10^{-4} M tosyl-L-lysine

chloromethyl ketone at pH 7.0. At 10^{-6} M enzyme concentration nearly complete inhibition was attained with 10^{-3} M phenylmethylsulfonyl fluoride at pH 8.0 after 10-min incubation.

Inhibition by DFP. The reaction of DFP with insoluble trypsin was significantly different than the reaction of tosyl-L-lysine chloromethyl ketone, *p*-nitrophenyl *p'*-guanidinobenzoate, and phenylmethylsulfonyl fluoride. Figure 5 shows that the insoluble trypsin was not inhibited by a stoichiometric amount of DFP as is the case for soluble trypsin (Jansen *et al.*, 1949) and that the inhibition could not be increased above 75% by the addition of a fourfold molar excess of DFP. This possibly indicates the presence of different species of enzymes present on the matrix. DFP does not have a lysyl or a guanido group to direct it to the active center of the enzyme. Thus, the reaction with DFP may reflect the different specific reactivies of the serine hydroxyl groups in the active centers of the different species of enzymes present on the matrix.

Action of Proteases on Insoluble Trypsin. Preliminary experiments were done to determine if the insoluble trypsins were susceptible to hydrolysis by various proteases and, if so, whether they lost activity as a result of protease action.

Insoluble trypsin was incubated separately with pronase (Nomoto and Narahashi, 1959), chymotrypsin, or trypsin. At various time intervals the reaction mixture was centrifuged and the absorbancy of the supernatant solution was read at 280 nm and 225 nm to determine the amount of peptides cleaved from the insoluble trypsin. An aliquot of the suspension was also taken at the corresponding time intervals, and assayed with BAPA to determine any loss of activity.

In one experiment 1 mg of insoluble *high ratio* trypsin was reacted with 80 μg of pronase for 6 hr with constant stirring at room temperature. It was calculated from the 225-nm readings that 35% of the insoluble protein was solubilized as peptides. The starting crystalline trypsin was only 80% pure so some of the material solubilized from the matrix could have been inert protein present in the commercial trypsin. The original activity (100%) of the insoluble phase remained. The above experiment was also performed using trypsin and chymotrypsin as the degradation proteases. There was no significant increase in the 225- and 278-nm readings, and the activity of the insoluble trypsin remained the same.

The results from the pronase action are very significant. Further experimentation is needed to determine exactly what portion of the trypsin molecule is still attached to the matrix but remaining 100% active, and whether conditions can be found to degrade even more of the molecule with continued retention of activity.

The main aspects of this study are summarized below. The method of preparation of the insoluble trypsin described here was designed to produce either highly flexible molecules similar to trypsin in solution or highly cross-linked, immobilized molecules where the conformation would be relatively fixed. It was thought that the properties of the two types of enzyme would be quite different and an assessment of these differences would possibly yield information regarding the nature of the active center of trypsin. Actually very little difference in the behavior of the two forms of insoluble trypsin was observed and it appears that the nature of the insoluble matrix (aminoethylcellulose) is such that all the trypsin molecules lie in indentations either on the surface or within the folds of the matrix. Once on the matrix, the trypsin molecules then further cross-link onto it to about the same extent.

Although the insoluble trypsin obtained by the present method possessed nearly the same specific activity as the crystalline trypsin used to prepare it, several of its properties were significantly different from those of the latter. The main difference in the two forms of this enzyme is that insoluble trypsin is completely free of autolysis. Also, although the activity of both insoluble and soluble trypsin was lost upon reduction of the disulfide bridges with mercaptoethanol only the former could be reactivated by oxidation in air. Whereas protein trypsin inhibitors inhibit soluble trypsin in a one to one molar ratio, these substances did not completely inhibit insoluble trypsin even at a 20-fold molar excess. This fact together with the observed low activity of insoluble trypsin toward casein indicates that there are some molecules of trypsin within the matrix which are relatively inaccessible to large substrates or inhibitors. However, these buried molecules are completely accessible to most small molecular weight substrates and competitive inhibitors. An unusual observation was the lack of complete inhibition of insoluble trypsin by DFP even up to a fourfold molar excess, whereas this form of the enzyme was fully active toward BAPA. It would appear that whatever the conformation of soluble trypsin is that imparts a peculiar reactivity to the active-site serine, this conformation is not entirely essential for the catalytic efficiency of the enzyme.

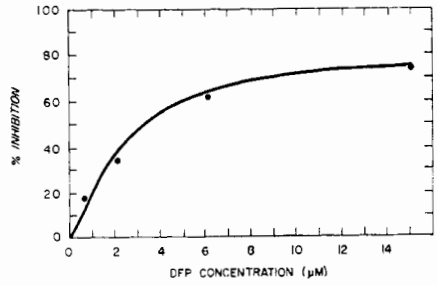

FIGURE 5: Inhibition of insoluble trypsin by DFP. All reactions were carried out in 0.1 M phosphate buffer, pH 8.0. The insoluble trypsin used was preparation 9 (Table I) at a concentration equivalent to 4 μM trypsin. The DFP was impure (partially hydrolyzed) and the values on the ordinate are effective concentrations determined by titration against a known concentration of crystalline trypsin.

The stability of insoluble trypsin proved to be advantageous because the preparation could be treated with proteases for sufficiently long periods of time to obtain a significant degradation without the interference of autolytic products. The preliminary studies with pronase indicated that perhaps as much as 15% of the trypsin molecule is unnecessary for enzymatic activity.

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Kinetic Aspects of Conformational Changes in Proteins. I. Rate of Regain of Enzyme Activity from Denatured Proteins*

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ABSTRACT: The enzymes fumarase, enolase, aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase, denatured in 6 M guanidine hydrochloride, were renatured *in vitro* and the effects of environmental conditions on the kinetics of reactivation examined. The presence of substrate or cofactor was found to increase both the rate and final extent of reactivation for all six enzymes. Changes in the ionic strength and protein concentration of the renaturation medium also influenced the regain of biological activity, however, both the direction and magnitude of these effects varied from enzyme to enzyme. The rate of equilibration between the different conformational species formed during renaturation was relatively rapid for the enzymes fumarase, enolase, and aldolase, but very slow for glyceraldehyde phosphate dehydrogenase and lactic dehydrogenase.

This result suggests that for the former group of enzymes the denatured protein refolds to final conformational state(s) which are determined solely by thermodynamic considerations, whereas for the latter group of enzymes the denatured protein refolds to metastable conformational state(s) specified by kinetic factors.

Sela, White, and Anfinsen (1957) and Anfinsen and Haber (1961) first demonstrated that reduced and denatured ribonuclease could be refolded to a conformational state possessing full biological activity. Since these pioneering studies, many other proteins have been shown to refold spontaneously from a denatured to the native state upon removal of the denaturing conditions (*cf.* review by Sund and Weber, 1966). These experiments provide convincing evidence that the information needed to specify the three-dimensional structure of a protein resides within its primary amino acid sequence.

Further study of this important phenomenon has inevitably raised questions about other facets of the folding problem. A recent investigation of the renaturation of a large number of enzymes, for example, revealed that the per cent regain of biological activity varied dramatically from enzyme to enzyme even when they were renatured under identical conditions (Cook and Koshland, 1969). Also, some enzymes when refolded in the presence of their substrates or cofactors yield a significantly higher level of enzymatic activity than that found when renaturation occurs in the absence of metabolite (Hill and Kanarek, 1964; Chilson *et al.*, 1965; Deal, 1969). These facts raise questions concerning the role of the environment in the refolding process and the extent to which refolding might be affected by specific metabolites in the media.

In addition several enzymes possessing the same amino acid sequence have been found to refold into different conformational forms. Thus, it has been suggested that different conformers of malic dehydrogenase have been found *in vivo* even though their primary covalent structures appear to be identical (Kitto *et al.*, 1966, 1970). Also lactic dehydrogenase (Chilson *et al.*, 1966), ribonuclease (Pflumm and Beychok, 1969), and creatine kinase (Dawson *et al.*, 1965), refolded *in vitro*, have been reported to regain biological activity but may possess different structural properties than their original native conformation. These observations have been contested by others (Schechter and Epstein, 1968; Mann and Vestling, 1968) but they raise questions as to whether a single conformational species is predetermined by a single sequence and, further, if such a species is so predetermined, whether kinetic or thermodynamic factors are more important in specifying the final conformational state.

An investigation of some of the kinetic aspects of protein renaturation was therefore undertaken in an effort to throw

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