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The Preparation of a Chemically Cross-Linked Complex of the Basic Pancreatic Trypsin Inhibitor with Trypsin†

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ABSTRACT: The complex of the basic trypsin inhibitor of bovine pancreas with trypsin has been cross-linked chemically with dimethyl adipimidate. Both inter- and intramolecular cross-linking occur. In the monomeric complex, two to three pairs of lysine residues are cross-linked and about five lysine residues are monofunctionally substituted. Other amino acid residues are not affected. The cross-linked complex is not dissociated at pH 2 or during gel electrophoresis in the presence of sodium dodecyl sulfate. A variety of reaction conditions were studied to obtain the maximum yield of nonpolymerized cross-linked complex.

Studies of the mechanism of action of the basic pancreatic trypsin inhibitor with trypsin, based on X-ray (Huber *et al.*, 1971a,b; Stroud *et al.*, 1971; Blow *et al.*, 1972) and chemical (Chauvet and Acher, 1967; Kress and Laskowski, 1968; Fritz *et al.*, 1969) data, have shown that lysine-15 of the inhibitor binds to the active site serine-183 of trypsin. This binding alone does not account for the K_i of 10^{-11} M (Green, 1963; Pütter, 1967) to 6×10^{-14} M (Vincent and Lazdunski, 1972) or for the total free energy of 14 (Rigbi, 1971) to over 19 kcal/mol (Vincent and Lazdunski, 1972). The association of the inhibitor with trypsinogen (Dlouhá and Keil, 1969), with TLCK[1]-β-trypsin (Imhoff and Keil-Dlouhá, 1971) and with anhydrotrypsin (Ako *et al.*, 1972) and the fact that guanidinated or amidinated inhibitors, with lysine-15 modified, still inhibit trypsin (Kassell and Chow, 1966; Fritz *et al.*, 1969) indicate that other forces are important in the binding. From the X-ray studies, hydrogen bonds and van der Waal's contacts are known to occur in the region near lysine-15 of the inhibitor and the active site of trypsin (*e.g.*, Blow *et al.*, 1972).

The inhibitor complexes with trypsin over the broad range of pH from 3 to 10. Free trypsin undergoes conformational changes as the pH is decreased or increased (*e.g.*, Lazdunski and Delaage, 1965, 1967). The inhibitor's conformation is stable between pH 2 and 10 (Vincent *et al.*, 1971; Karplus *et al.*, 1973). Therefore, the surfaces of contact between the inhibitor and trypsin may change in the complex as the pH is changed, particularly at the extremes of pH where the association constant is decreased by several orders of magnitude (Green and Work, 1953; Vogel *et al.*, 1968). On the other hand, the trypsin may be stabilized by complex formation, as it is by a synthetic inhibitor (Bechet and D'Albis, 1969). The X-ray studies, for which the crystals were prepared at pH 7 (Rühlmann *et al.*, 1971), cannot detect the conformational changes that may occur.

We are approaching the elucidation of sites of contact by cross-linking the complex chemically with reagents of different specificities, chain lengths, and pH of reaction. This should help in understanding the alterations in the structure of the complex that may occur. The reagent dimethyl adipimidate, DMA, which is specific for lysine residues, was first studied by Hartman and Wold (1967); they were successful in cross-linking ribonuclease. The present paper describes the preparation of the first cross-linked inhibitor–trypsin complex and includes a study of the conditions required to apply the DMA reaction to this complex.

Materials and Methods

Bovine trypsin from Novo Industri A/S, Copenhagen, was purified further by chromatography on SE-Sephadex C-25 (Papaioannou and Liener, 1968). The basic pancreatic trypsin inhibitor was the same as that described earlier (Kassell *et al.*, 1963), except that CM-cellulose was used for the final puri-

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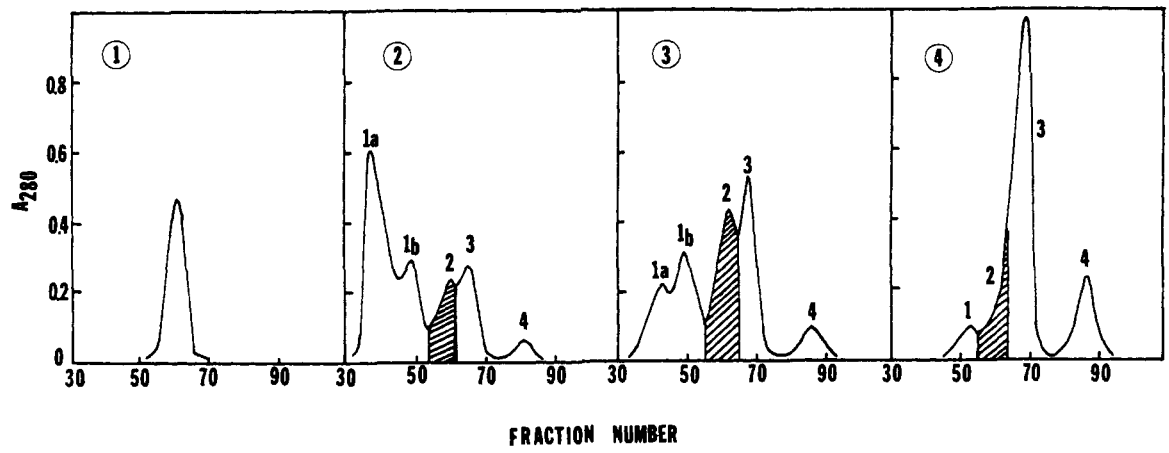
[1] Abbreviations used are: DMA, dimethyl adipimidate; TLCK-, tosyllysine chloromethyl ketone derivative of; the complex, the basic pancreatic trypsin inhibitor–trypsin complex.



FIGURES 1–4: The elution position of the monomeric cross-linked complex at pH 2 (Figure 1) and the effect of variation in concentration of reagents on the yield of cross-linked complex. Column: Sephadex G-75, 1 × 150 cm, flow rate 5 ml/hr. For Figures 2–4, peak 1 subfractions are polymerized material; peak 2 (crosshatched) is an approximation of the monomeric cross-linked complex; peak 3 is trypsin; peak 4 is inhibitor. The figures correspond to the experiments of Table I as follows: Figure 2, no. 6 (high concentration of reactants); Figure 3, no. 1 (moderate concentration of reactants); Figure 4, no. 5 (low concentration of reactants).

fication (Avineri-Goldman *et al.*, 1967). [^{14}C]Dimethyl adipimidate (DMA) was synthesized by the method of Hartman and Wold (1967), using Na^{14}CN, specific activity 60.6 Ci/mol, from Amersham-Searle, Arlington Heights, Ill., and 1,4-dichlorobutane from Aldrich Chemical Co., Milwaukee, Wis. The product contained 1.5 × 10^6 cpm/mg and melted at 220–221°.

Tryptic and inhibitory activities were measured by the method of Erlanger *et al.* (1961) as described previously (Kassell and Chow, 1966), using benzoyl-DL-arginine *p*-nitroanilide as substrate. Scintillation counting was carried out as described by Meitner (1971). Gel electrophoresis in the presence of sodium dodecyl sulfate was conducted by the method of Weber and Osborn (1969).

Amino acid analyses were performed as previously described (Kassell and Chow, 1966). N^{ϵ},N^{ϵ}-Adipamidinobislysine was determined on the short column of the Beckman-Spinco analyzer by changing the buffer to pH 9.7 (Hartman and Wold, 1967). N^{ϵ}-4-Carboxybutyroamidinolysine (formed by acid hydrolysis of the derivative of adipimidate with one lysine residue) was determined according to Niehaus and Wold (1970) with another aliquot of the same hydrolysate. In this analysis, involving the use of the long column with short column buffer (pH 5.28), the derivative appears after phenylalanine. For calculation, the factors for phenylalanine and lysine were determined with standard amino acid solutions under the same conditions. The factors for lysine (short and long column, respectively) were used to calculate the adipamidinobislysine (Hartman and Wold, 1967) and the monosubstituted derivative (F. Wold, personal communication). The correction of 15% for conversion to lysine during hydrolysis, given by Hartman and Wold (1967) for the amidinobislysine, was used for both derivatives. The amount of phenylalanine was used in place of an internal standard to relate the amount of the monosubstituted derivative to the other amino acids found in the previous run under standard conditions.

The complex was prepared by mixing a slight excess of inhibitor (1.5 mg/ml in 50 mM borate buffer (pH 8)) with trypsin (4 mg/ml in the same buffer) at about 25° and subjecting the product to gel filtration on Sephadex G-100 in the same buffer at 4°.

To separate and identify the reaction products, a column of Sephadex G-75 (1 × 150 cm) was first equilibrated with 50 mM ammonium bicarbonate buffer (pH 7). The elution positions of the pure inhibitor–trypsin complex, of trypsin, and of inhibitor were separately determined. The column was then equilibrated with HCl at pH 2.0. It was tentatively assumed, for selecting the monomeric cross-linked complex of the reaction mixtures, that the elution position of this product would be nearly the same at pH 2 as the native complex at pH 7. The locations of free trypsin and of free inhibitor were confirmed by their activities. At the completion of the experiments to be described below, when the monomeric cross-linked complex had been purified, gel filtration on the same column at pH 2 confirmed the correctness of this assumption. The position of the purified monomeric cross-linked complex is shown in Figure 1.

Results

It is known (Wold, 1967, 1972) that not only intra- but also intermolecular cross-linking occurs with this reagent, the amount of each type depending on protein concentration, and that the degree of cross-linking is also dependent on the concentration of the reactants. The objective of these experiments was to obtain as high a yield as possible of monomeric cross-linked complex. To find the best conditions for the inhibitor–trypsin complex, the pH, time, temperature, and the the concentrations of reactants were varied.

Cross-Linking of the Inhibitor–Trypsin Complex with [^{14}C]*-Dimethyl Adipimidate.* The general procedure, similar to Hartman and Wold (1967), was to dissolve 18 mg of complex in 50 mM sodium borate buffer at room temperature (about 25°). Solid DMA was added to the gently stirred solution in 2-mg portions at 2-min intervals. The pH was maintained by adding 0.1 M NaOH as required. After 1 hr, or overnight when indicated, the pH was adjusted to 6.5. The reaction mixture was concentrated in a 60-ml Amicon ultrafilter (Amicon Corp., Cambridge, Mass.) using a UM-2 membrane. The concentrated solution, about 0.5 ml, was adjusted to pH 2 with 0.1 M HCl; this acidification dissociated non-cross-linked complex. The solution was applied to the column of Sephadex G-75 equilibrated with HCl at pH 2.0.

A typical elution pattern is shown in Figure 3; the data are taken from experiment 1 of Table I. The cross-hatched area, representing approximately the monomeric cross-linked com-

TABLE I: Reaction of 18 mg (22.5 A_{280} units) of Inhibitor–Trypsin Complex with Dimethyl Adipimidate. The Effect of pH and Reactant Concentrations. Minimum Values for Polymer and/or Inhibitor Indicate the Best Conditions.

Expt No.	Volume (ml)	Complex (mg/ml)	DMA (mg)	DMA (mg/ml)	pH	Polymer Peak 1a and 1b (A_{280} units)	Inhibitor[a] Peak 4 (A_{280} units)
1	25	0.72	15	0.60	10.0	6.84	0.98
2[b]	25	0.72	15	0.60	8.5	5.28	1.48
3	25	0.72	15	0.60	8.0	1.34	2.06
4	15	1.20	15	1.00	10.0	9.02	1.34
1	25	0.72	15	0.60	10.0	6.84	0.98
5	50	0.36	15	0.30	10.0	1.06	2.04
6	3.6	5.00	8	2.20	10.0	11.10	0.58
7	15	1.20	8	0.53	10.0	5.90	1.04
8	50	0.36	8	0.16	10.0	1.62	1.66

[a] If no cross-linking occurred, the inhibitor peak would contain 3.20 A_{280} units. [b] 18 hr reaction time; all others 1 hr.

plex, was selected by its corresponding position to the peak of Figure 1 (within 1 to 2 fractions) and by determination of trypsin activity in the overlapping peaks 2 and 3. From Figure 3 it is clear that the single gel filtration was inadequate to separate the monomeric cross-linked complex (peak 2) from trypsin (peak 3). Some tryptic activity was detectable in the complex peak and the activity on the ascending side of the trypsin peak was low.

The other two peaks, however, could be evaluated. The separation of polymeric material (peaks 1a and 1b) from the monomer (peak 2), although not perfect, permitted a reasonable calculation of the quantity of material in the polymer peak. The separation of free inhibitor (peak 4) was very good. In finding the best conditions for production of monomeric complex, we have therefore evaluated the experiments by considering the size of either or both of the two *undesired* peaks 1 and 4. The polymer peak is a measure of material lost by cross-linking between molecules. The inhibitor peak is a measure of the amount of complex that failed to be cross-linked between trypsin and the inhibitor, and was therefore dissociated in the acid solution.

Variation of pH. In experiments 1–3 of Table I, the pH was lowered from 10.0 to 8.5 and 8.0. The amount of free inhibitor increased with lower pH, showing that less cross-linking occurred, even though the reaction at pH 8.5 was continued overnight. Dissociation of the complex above pH 10 prevented the testing of the reaction at higher pH values. On the basis of these results, further experiments were conducted at pH 10.0.

Variation of Concentrations of Reactants. In experiments 4–8 of Table I, the volume was varied, keeping the absolute amount of complex constant at 18 mg and using two amounts of DMA, 15 and 8 mg. When the concentration of reactants was increased, as in experiment 6 (illustrated in Figure 2) there was little free inhibitor, showing good cross-linking, but a large amount of polymeric material formed. As the concentration of reactants decreased, less cross-linking occurred, as shown by the increase in the amount of free inhibitor. At the same time, however, the amount of polymeric material also decreased. Figures 2 and 4 show the contrast between extremely high and extremely low concentrations of reactants. As a compromise between excessive polymer formation and too little cross-linking, experiments 1 and 7 gave similar results and were the best in giving reasonably small amounts of undesired by-products. This agreed with our qualitative judgment of the relatively large size of the peak in the position of the monomeric cross-linked complex in these two experiments, illustrated in Figure 3. Thus, pH 10, complex concentration about 0.7–1.2 mg/ml and DMA concentration 0.5–0.6 mg/ml were the most desirable conditions.

Because of the small amount of each monomeric complex peak, this fraction from all the preparations, including many repetitions, were combined and further purified by repeated fractionation on Sephadex G-75. The last contamination with trypsin was removed by passage through a column of Sepharose–ovomucoid trypsin inhibitor (Robinson *et al.*, 1971). After the final purification, the product gave a single, not quite symmetrical peak on Sephadex G-75 (Figure 1). Following each purification step, a narrow cut of the monomeric complex peak was taken for the next step to minimize contamination by neighboring peaks. Under these circumstances, the final yield of purified material was low, 2.5%.

Other Conditions. Attempts to minimize polymerization by the addition of urea or by lowering the temperature of the reaction to 4° were not successful. The yields of monomeric complex were decreased.

Characterization of the Monomeric Cross-Linked Complex. Amino acid analyses of the individual monomeric peaks of the experiments showed the presence of N^{ϵ},N^{ϵ}-adipamidinobislysine, but quantitatively the results were difficult to interpret because of contaminating trypsin.

Amino acid analysis of a sample from the center of the purified peak was that expected from the known composition of the complex, except for a reduced amount of lysine (Table II). The amount of adipamidinobislysine corresponded to 2.7 cross-links when corrected for reconversion to lysine during acid hydrolysis. In addition, approximately five residues of lysine were converted to the monofunctionally substituted derivative, carboxybutyroamidinolysine. The results account for the total number of lysine residues of the complex. Radioactivity measurements showed that 8.9 mol of DMA was incorporated. This compares reasonably well with 10.2 residues of modified lysine found by amino acid analysis, especially if one considers that the factors used for calculation of the derivatives and for correction for hydrolytic destruction may not be exact.

The trypsin derived from the treated complex (peak 3) contained approximately 1 cross-link per mole, as measured by amino acid analysis. The inhibitor (peak 4) did not contain a cross-link, although it incorporated radioactivity.

TABLE II: Amino Acid Composition of the Cross-Linked Inhibitor–Trypsin Complex.

Amino Acid	Residues per Molecule		
	Found[a]	Lysine Equiv[b]	Calcd[c]
Lysine	9.2	7.6	
Adipamidino-bislysine	2.2	5.3	
Carboxybutyro-amidinolysine	4.2	4.9	
Total lysine residues		17.8	18
Histidine	3.0		3
Arginine	8.2		8
Aspartic acid	27.1		27
Threonine[d]	14.0		13
Serine[e]	33.5		34
Glutamic acid	17.1		17
Proline	12.5		13
Glycine	30.7		31
Alanine	19.5		20
Half-cystine	17.3		18
Valine[f]	13.0		18
Methionine	2.9		3
Isoleucine[f]	15.0		17
Leucine	16.1		16
Tyrosine	13.4		14
Phenylalanine	6.0		7
Tryptophan	Not det.		4

[a] Based on a single 24-hr hydrolysate. In this column, the values for lysine and its derivatives are uncorrected. [b] Corrected values. Part of the lysine was derived by breakdown of mono- and bifunctionally amidinated lysine during HCl hydrolysis (Hunter and Ludwig, 1962; Hartman and Wold, 1967). The values for the two derivatives are corrected for 85% recovery (Hartman and Wold, 1967), and these corrections are subtracted from the lysine. [c] The composition of trypsin (Walsh and Neurath, 1964) plus inhibitor (Kassell *et al.*, 1963). [d] Corrected 5% (Moore and Stein, 1963). [e] Corrected 10% (Moore and Stein, 1963). [f] The six peptide bonds in which valine and isoleucine are linked to themselves or to each other are extremely resistant to hydrolysis, accounting for the low values in the 24-hr hydrolysate.

Of the two to three cross-links, at least one must be between the inhibitor and trypsin to prevent dissociation of the complex under the acid conditions of gel filtration. One cross-link is likely to be within the trypsin part of the molecule.

Confirmation of the conclusions derived from gel filtration was obtained by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 5). The monomeric complex appeared on the curve (point 5) just below pepsin (molecular weight, 34,000) and had a molecular weight of 32,500, in good agreement with the calculated molecular weight of 31,600 for the modified complex containing ten substituted lysine residues. The native complex partially dissociated in sodium dodecyl sulfate solution and two bands appeared (points 6 and 8) in the positions for complex and for trypsin; the inhibitor portion did not give a clear band. The fact that the cross-linked complex did not dissociate in sodium dodecyl sulfate is further evidence that a cross-link was inserted between the trypsin and the inhibitor portions of the complex molecule.

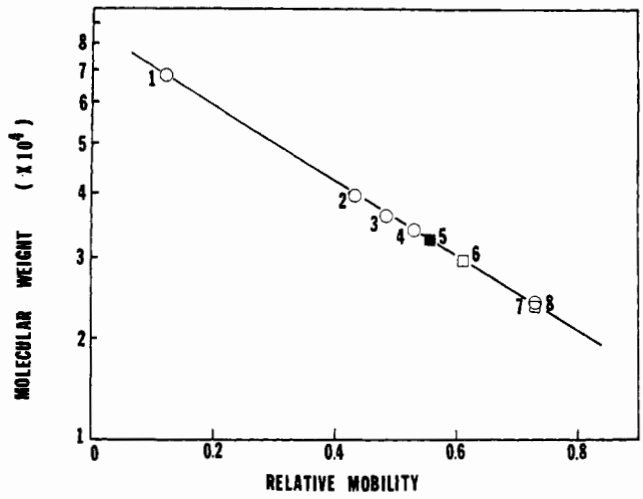


FIGURE 5: Semilog plot of molecular weight determined by sodium dodecyl sulfate gel electrophoresis. Electrophoresis was conducted in 0.01 M sodium phosphate buffer (pH 7.1), 0.1% in sodium dodecyl sulfate, in 9.5 cm of 10% acrylamide gels for 3 hr at 8 mA per gel. O markers: (1) bovine serum albumin, 68,000; (2) aldolase, subunit weight 39,500; (3) glyceraldehyde phosphate dehydrogenase, 36,000; (4) pepsin, 34,000; (7) trypsin, 24,000. Experimental: (■) (5) monomeric cross-linked complex, Figure 1, 32,500; (□) native inhibitor–trypsin complex; (6) undissociated, 29,500 (theory 30,500); (8) dissociated free trypsin.

Discussion

Cross-linking reactions offer interesting possibilities for studying the distances between specific amino acid residues within proteins and in protein–protein interactions (Wold, 1967), of which inhibitor–trypsin reactions are good examples. The DMA reaction was chosen for several reasons. Following the demonstration by Hunter and Ludwig (1962) that imido esters react only with the amino groups of proteins, it was shown that amidination of all the lysine residues does not inactivate the pancreatic inhibitor (Kassell and Chow, 1966); the ϵ-amino groups of trypsin are not required for activity (SriRam *et al.*, 1954); the reaction is reversible (Ludwig and Byrne, 1962; Hunter and Ludwig, 1972). This type of reagent also has the advantage that the length of the carbon chain between the two functional groups can be varied; diimidates containing one to seven carbon atoms in the chain have been used with other proteins, for example, diethyl malonimidate (Dutton *et al.*, 1966) and dimethyl suberimidate (Davies and Stark, 1970). Although bond distances cannot be calculated from a single flexible reagent, the use of a series of diimidates of varying chain length would show the minimum number of carbons in the chain that are required to form the cross-link.

We hope by a large scale reaction to prepare enough of the monomeric cross-linked complex to isolate radioactive peptides that will contain a piece of inhibitor and a piece of trypsin. Such peptides can then be located in the known sequences of trypsin (Walsh and Neurath, 1964) and of the inhibitor (Kassell and Laskowski, 1965). This should yield information regarding the relationship of these areas of trypsin and of the inhibitor when the complex is in solution at pH 10.

It has been suggested (Kassell, 1968; Vincent and Lazdunski, 1972) that after formation of the complex, a secondary interaction occurs by interchange of disulfide linkages be-

tween the inhibitor and trypsin, forming two covalent bonds. These suggestions were based on indirect evidence, slowing of the reaction of the inhibitor with trypsin by sulfhydryl reagents in one case (Kassell, 1968) and a decrease in the association constant by several orders of magnitude when a single disulfide bond of either the inhibitor or trypsin was reduced in the other (Vincent and Lazdunski, 1972). Recent X-ray data (Rühlmann *et al.*, 1973, 1974) shows, however, that disulfide interchange is improbable. From the digestion products of a cross-linked complex, it may be possible to isolate a peptide containing one of the crucial disulfide bonds near the reactive site of the inhibitor or the active site of trypsin, which would then provide direct evidence as to whether or not disulfide exchange occurs.

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