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Autolysis of β-Trypsin

Influence of Calcium Ions and Heat

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The effect of calcium ions and heat on the autolysis of β-trypsin has been investigated. A considerable decrease in the rate constant of autolysis was observed in the presence of calcium. Below 40°, 0.02 M $CaCl_2$ reduced the velocity of autolysis by a factor of 100, as compared to the velocity recorded in the absence of Ca^{2+}. At about 40° a change in the apparent activation energy for the reaction was found both in the presence and absence of calcium. This has been attributed to reversible cooperative transitions in β-trypsin and the β-trypsin-calcium complex. Conformational transitions in the different trypsins were also manifested by changes in the activity towards *N*-*p*-tosyl-L-arginine methyl ester, but not towards *N*-benzoyl-L-arginine ethyl ester. β-Trypsin immobilized on Sephadex was more heat stable than β-trypsin bound to agarose.

The kinetics of activation of trypsinogen has been investigated to a considerable extent.[1–3] It has been shown that the velocity of activation as well as the amount of inactive material formed depend on the concentration of calcium ions present.[1,2] Two highly active forms of trypsin, α-trypsin and β-trypsin, are formed during the activation,[4] besides a less active form, ψ-trypsin.[5] These enzyme species, together with inactive material, are found in commercial enzyme preparations.[4]

A conformational change between different active forms at about 45° has been shown to occur in commercial enzyme preparations in the presence of Ca^{2+} (Refs. 6, 7). This change was attributed to the formation of a stable Ca-trypsin complex, as no similar conformational change was observed in the absence of Ca^{2+} (Ref. 7). Recently, reversible cooperative transitions between enzymatically active conformations have been observed in pure preparations of free and immobilized α- and β-trypsin in the absence of Ca^{2+} (Ref. 8). It is considered that the discrepancy between these findings may be partly due to autolysis and the use of heterogeneous enzyme preparations.

Although it has been known for a long time that trypsin solutions are stabilized by calcium ions, presumably by a reduction of the rate of autol-

ysis,[9-11,32-34] no extensive quantitative investigations on this phenomenon on pure enzyme preparations have been made. In the present study we found a considerable stabilization of pure β-trypsin towards autolysis at all temperatures up to 65°, indicating the existence of Ca-trypsin complexes. These complexes could not be isolated by gel chromatography.

MATERIALS AND METHODS

Bovine trypsin was a gift from Novo A/S, Copenhagen. It was separated into α- and β-trypsin by chromatography on SE-Sephadex [4] and the components were recovered by lyophilization after dialysis against 0.001 M HCl. The β-trypsin sample was found to contain ≃10 % α-trypsin and ≃10 % inactive material, as determined by analytical bioaffinity chromatography on soy bean trypsin inhibitor agarose (STI-agarose).[12] As rechromatography of the β-trypsin peak on the same column gave similar results, it was concluded that some autolysis during the bioaffinity chromatography procedure is unavoidable (as observed for α-chymotrypsin [13]) and that the original sample contained less α-trypsin and inactive trypsin. The α-trypsin sample contained more (≃40 %) inactive trypsin. STI-Sepharose 4B, α- and β-trypsin-Sephadex G-200, and β-trypsin-Sepharose 2B were prepared using the cyanogen bromide method.[13-15] *N*-Tosyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Sigma, Cleveland, Ohio, and used without further purification. Casein (Hammarsten quality) was from Merck and Co., Darmstadt, Germany.

Determination of esterase activity. (a) *Free enzyme.* Stock solutions of the enzyme in 0.001 M HCl were prepared the day they were used and stored on ice. Activity measurements were performed under nitrogen in the thermostatted titration vessel of a Radiometer pH-stat. A correction for nonenzymatic alkali uptake was applied. The temperature of the assay solution was determined with a calibrated thermistor; the precision was ±0.2°. 50μl of the enzyme stock solution was added either to 2.0 ml 0.1 M NaCl, 0.01 M BAEE and the activity at pH 8.0 recorded immediately, or added to 1.9 ml 0.11 M NaCl adjusted to pH 8.0 After 30 sec, 0.1 ml of a prethermostatted 0.2 M BAEE solution (giving a final concentration of 0.01 M) was added and the activity recorded immediately. The activity in the presence of calcium ions (the assay solution then contained 0.02 M $CaCl_2$ and 0.04 M NaCl) was independent of the order of addition. (*b*) *Conjugated enzyme.* The procedure described elsewhere [8] was adapted. When BAEE was the substrate, a final concentration of 0.03 M was used; with TAME the final concentration was 0.05 M.

Determination of catalytic constants. The activity assay was carried out at 25.0° in a pH-stat in a total volume of 10 ml. β-Trypsin was added to the assay solution containing Tris-HCl ($I = 0.0025$), 0.25 M NaCl and TAME in the concentration range 0.04 to 15 mM. The activity was recorded at pH 8.0. K_M and k_{cat} were determined from an Eadie-Hofstee plot, using the activity values at substrate concentrations below 4 mM. Above that concentration, substrate activation was observed.[16]

Determination of protease activity. A procedure described elsewhere (Gabel and von Hofsten [22]) was followed, with the modification that 0.1 M $NaBO_3$-HCl buffer, pH 8.1, was used.

Autolysis of β-trypsin. A β-trypsin solution of suitable concentration in 0.01 M $NaBO_3$-HCl buffer, 0.02 M $CaCl_2$, 0.04 M NaCl, pH 8.1, was incubated at the desired temperature in a water bath. Samples were withdrawn at regular time intervals and tested for their activity as described above, or introduced into a STI-agarose column (1.4 × 10 cm) equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 0.5 M NaCl. After washing, a linear gradient of the initial buffer and 0.05 M glycine-HCl buffer, pH 2.7, containing 0.5 M NaCl (100 ml of each) was applied. From the chromatograms obtained the relative amounts of inactive, α- and β-trypsin were determined. Apparent second order rate constants k_{EE} were determined from the obtained residual activity or the amount of β-trypsin.[8]

Calcium dependence of autolysis. The calcium dependence at 50° of the rate constant of autolysis was determined in 0.01 M $NaBO_3$-HCl buffer, pH 8.1, containing 10^{-6} to 10^{-2} M $CaCl_2$ and NaCl to give an ionic strength of 0.1. 100 μl of a suitable β-trypsin solution

was added to 10 ml of the prethermostatted buffer. Samples were withdrawn at time intervals and tested for their activity as described above. The apparent second order rate constants were plotted against the logarithm of the calcium concentration for determination of the binding constant.

Measurements of tryptophan fluorescence were performed in a Turner Spectro 210 spectrofluorometer (Palo Alto, California). The excitation bandwidth was 10 nm, the excitation wavelength 280 nm. The emission bandwidth was 2.5 nm. The reproducibility of the spectra was estimated to ±1 nm. All measurements were done in 0.01 M $NaBO_3$-HCl buffer, pH 8.1, containing either 0.1 M NaCl or 0.04 M NaCl and 0.02 M $CaCl_2$. In some cases the buffer contained 0.01 M BAEE in addition to the salts. 100 µl of the enzyme solution (approximately 1 mg/ml in 0.001 M HCl) were added to 1.9 ml of the prethermostatted buffer, and the fluorescence spectrum was recorded after 1 min. The wavelength of maximum emission was reached after about 5 min. The temperature inside the cuvette was monitored after each run with a thermistor.

Binding of $^{45}Ca^{2+}$ to β-trypsin. 10 mg β-trypsin (this special preparation contained ≃20 % α-trypsin) was incubated at 50° for 2 min in 1 ml 0.1 M $NaBO_3$-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ with 10^7 dpm $^{45}Ca^{2+}$ (Radiochemicals, Amersham). The solution was cooled immediately in an ice bath and applied to a Sephadex G-25 column (dimensions 1.4 × 50 cm) equilibrated with the same buffer. The effluent was collected in 4.5 ml fractions. Enzymatic activity was measured as described above. 1 ml of each fraction was added to 10 ml scintillation fluid (70 % (v/v) toluene, 30 % (v/v) Triton X-100 with 7 g 2,5-diphenyloxazole and 0.35 g dimethyl-1,4-di[2-(5-phenyloxazolyl)]benzene per liter solution) and radioactivity measured with a Mark II Liquid Scintillation Counter (Nuclear Chicago). The counting efficiency was 50 %.

RESULTS

Dependence of activity on calcium. The recorded activity of β-trypsin-Sephadex towards BAEE as substrate was not dependent on the presence of calcium ions at all temperatures. The activity increased up to 70° with a constant value of the activation energy, and then decreased rapidly with increasing temperature; but incubation at 95° for 15 min did not lead to an irreversible inactivation, as complete recovery of activity was observed after cooling to room temperature.

The activity of free β-trypsin towards BAEE had a similar temperature dependence as observed for β-trypsin-Sephadex up to 70°, when calcium was present in the assay solution (Fig. 1). When no calcium was present, the recorded specific activity at temperatures above 50° depended on the order of addition of enzyme and substrate, as well as on the amount of enzyme added. When the enzyme was added to the thermostatted salt solution prior to the substrate, a considerably lower activity was recorded than when the order of addition was reversed. This is shown in Fig. 1. From the concentration of BAEE present (0.01 M) and the K_M value for trypsin-BAEE (10^{-5} M)[35] it can be calculated that about 0.1 % of the total trypsin is not in complex with BAEE under these conditions and thus has an unoccupied active site. Therefore the rate of autolysis may be expected to be considerably lower. When relatively high amounts of trypsin were assayed in the absence of calcium, a progressive inactivation was observed even during the activity measurement at temperatures above about 50°. Increased amounts of β-trypsin in calcium solutions above 65° showed a similar behaviour.

The esterolytic activity of α-trypsin in free and Sephadex-bound form decreased at temperatures above 55° both in the presence and absence of

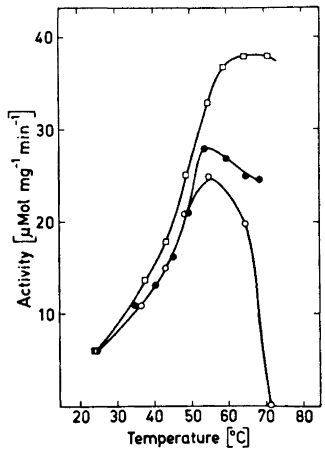

Fig. 1. Dependence of activity towards BAEE on temperature for β-trypsin. The initial activity at the temperatures indicated was measured at pH 8.0 in the presence of 0.1 M NaCl (●, ○) or 0.02 M $CaCl_2$ and 0.04 M NaCl (□). The closed circles represent values obtained when the enzyme stock solution was added after the substrate. For the open circles, the substrate stock solution was added to the salt solution 30 sec after the addition of the enzyme. In the presence of calcium the order of addition was of no importance for the recorded activity.

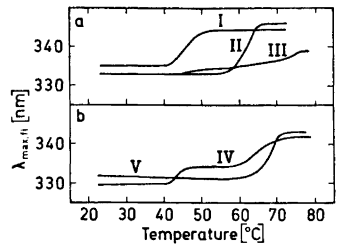

Fig. 2. Fluorescence of β-trypsin at different temperatures. Part a shows values obtained at pH 8.1 in 0.1 M NaCl (curve I) or in 0.02 M $CaCl_2$ and 0.04 M NaCl (curve II) for free β-trypsin (final concentration 2 μM) after 5 min. Curve III shows the curve for β-trypsin-Sephadex in 0.1 M NaCl. Part b shows values obtained at pH 8.1 in the presence of 0.01 M BAEE in 0.1 M NaCl (curve IV) or 0.02 M $CaCl_2$ and. 0.04 M NaCl (curve V). All measurements were made in 0.01 M $NaBO_3$-HCl buffer.

calcium. The peptide bond cleavage leading to the formation of α- from β-trypsin,[4] which has no influence on the rate of urea denaturation,[15] seems to decrease the heat stability when compared to β-trypsin.

Dependence of fluorescence on calcium. Fig. 2 shows the influence of temperature on the tryptophan fluorescence of trypsin. A considerable shift of the maximum of fluorescence emission to longer wavelengths occurred in the presence of calcium at 60°. A similar shift occurred at 40° when only sodium chloride was present. (In the temperature range of 50 – 60° and in the absence of calcium, precipitation was sometimes observed.) In the presence of BAEE (Fig. 2b) this shift did not occur until the temperature was raised to 65°. In this case, the spectrum below 40° was slightly shifted to shorter wavelengths as compared to the spectra measured without BAEE. Changes in the exposure of tyrosyl and tryptophyl residues in β-trypsin due to competitive inhibitors have been described recently.[17] Above 45° a slight but significant shift to longer wavelengths was observed, so that the wavelengths of maximum intensity was identical to that in the presence of $CaCl_2$, but in the absence of substrate. This may reflect a minor opening of the protein structure, as observed for β-trypsin-Sephadex.[8] The curve for the insoluble enzyme is also included in Fig. 2.

The results depicted in Figs. 1 and 2 can be caused by autolysis, as can be shown from the rates of autolysis published.[8] The concentration of trypsin in the fluorescence measurements was about 2 μM. At 50° and in the absence of calcium and substrate the rate constant of autolysis is $\simeq 2 \times 10^3$ $sec^{-1}M^{-1}$. The half life for β-trypsin is then ≃200 sec. The maximum of the fluorescence spectrum was, however, not reached until after 300 sec. Considerable autolysis has thus occurred during the measurement. The same may be true for the measurements over 60° in the presence of calcium or in the presence of substrate only, and over 70° in the presence of both calcium and substrate.

Autolysis of β-trypsin. The effect of temperature on the apparent bimolecular rate constant for the autolysis of β-trypsin in the presence of

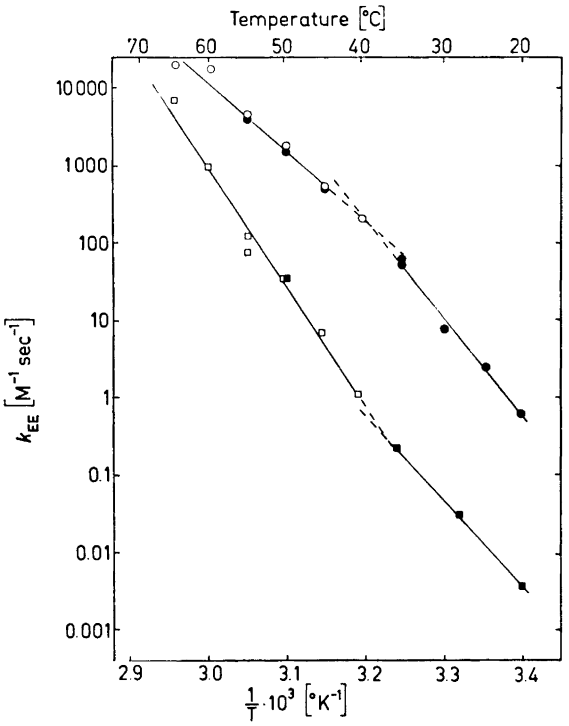

Fig. 3. Influence of calcium ions and temperature on the autolysis of β-trypsin. The logarithm of the apparent rate of autolysis of β-trypsin k_{EE} as determined by activity assay (open) or bioaffinity chromatography (filled symbols) is plotted against the reciprocal of the absolute temperature. The measurements were made in 0.01 M $NaBO_3$-HCl buffer, pH 8.1, containing either 0.1 M NaCl (circles) or 0.02 M $CaCl_2$ and 0.04 M NaCl (squares).

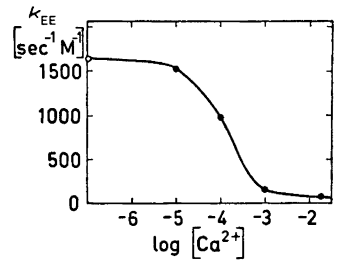

Fig. 4. Influence of calcium ions on the rate of inactivation at 50°. The inactivation rate as measured by activity assay was determined in 0.01 M $NaBO_3$ buffer, pH 8.1, containing the indicated amount of calcium ions and sodium chloride to give an ionic strength of 0.1. The open circle represents the rate measured in the absence of Ca^{2+}.

sodium chloride has been described before.[8] Fig. 3 compares these results with the autolysis in the presence of calcium chloride. Good agreement was found between the rate constants based on activity measurements and those obtained by bioaffinity chromatography. Bioaffinity chromatography was used to determine the relative amounts of α-, β-, and inactive trypsin present. Below 45° a steady state concentration of α-trypsin, amounting to about 10 % of the originally present β-trypsin, was found until about half of the β-trypsin had been degraded, whereafter even the concentration of α-trypsin decreased. Above 45°, negligible amounts of α-trypsin were observed. A markedly reduced rate of autolysis in the presence of calcium was found at all temperatures up to 65°, the highest temperature investigated. Whereas at temperatures below 40° the activation energy for the reaction was not influenced by calcium as compared to the value obtained with sodium chloride, both being around 60 kcal/mol, the presence of calcium ions above this temperature resulted in a considerable increase in the activation energy observed in the absence of calcium, from 40 to 80 kcal/mol. The apparent rate constant of autolysis at 50° was influenced by calcium as expected for the presence of a single metal ion binding site. The dissociation constant determined from the rate constants was 10^{-4} M (Fig. 4). At this temperature the ratio of the rate constants in the presence and absence of Ca^{2+} was much larger than the ratio of free β-trypsin to the total enzyme content. Therefore the observed temperature dependence of k_{EE} cannot be due to autolysis of free β- or β′-trypsin and a temperature dependent dissociation of the enzyme-calcium complex.

As pointed out previously,[8] "breaks" in the Arrhenius plot for k_{EE} do not alone provide sufficient evidence for the existence of conformational transitions. Additional evidence is provided by the temperature dependence of the rate constant for TAME hydrolysis.[6-8] We therefore concluded that the "break" in the Arrhenius plot for k_{EE} in the presence of Ca^{2+} is due to a conformational change in the calcium-trypsin complex similar to that observed in the absence of Ca^{2+}.[8]

Calcium-trypsin complex. The calcium-trypsin complex formed above 45° has been proposed to be so stable that it can be separated from other trypsin forms by chromatography.[7] We therefore attempted to isolate a β-trypsin-calcium complex by gel filtration. Fig. 5 shows the chromatogram obtained, after β-trypsin had been incubated at 50° for 2 min in the presence of $^{45}Ca^{2+}$. The specific enzyme activity (enzyme activity was found only in fractions 6 and 7) of the blank was slightly higher than that of the heated sample, probably due to some autolysis during the heating period. A small amount of radioactivity emerged before the total column volume (which corresponded to fraction 18) in both the blank and the heated sample, but no radioactivity was associated with the protein peak. A 1:1 complex of trypsin and calcium would have had a radioactivity of about 5×10^4 dpm, considerably more than that observed in all fractions before the total volume.

The complex of β-trypsin and calcium therefore has a life time considerably shorter than one hour, the time needed for the experiment shown in Fig.5. One can also exclude that the α-trypsin-calcium complex is stable enough to allow a chromatographic separation, as the preparation used contained about

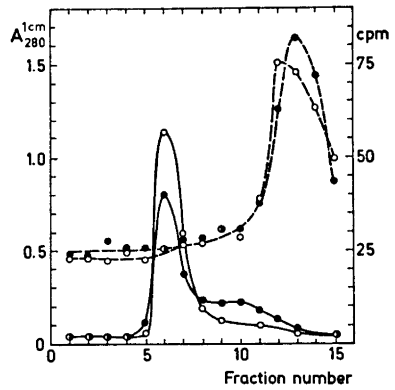

Fig. 5. Binding of $^{45}Ca^{2+}$ to β-trypsin. 10 mg β-trypsin in 1 ml 0.1 M $NaBO_3$-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ with 10^7 dpm $^{45}Ca^{2+}$ were incubated at 50° for 2 min and gel filtered on a Sephadex G-25 column in 0.1 M $NaBO_3$-HCl buffer 0.02 M $CaCl_2$, pH 8.0 (●). A blank run on the same column, where the heating step has been omitted, is shown by open circles. The fractions were analysed for A_{280} and radioactivity (– – –). The total volume of the column emerged at fraction 18. Enzyme activity was found only in fractions 6 and 7.

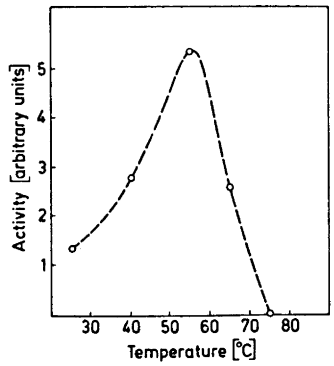

Fig. 6. Activity of β-trypsin-Sephadex against casein at different temperatures. A 1 % solution of casein in 0.1 M $NABO_3$-HCl buffer, pH 8.0, was digested with the immobilized enzyme and analysed for trichloroacetic acid soluble material.

20 % of this enzyme species. This behaviour is expected on the basis of published binding constants [3] and rates for protein-ligand reactions.[18] Our results are supported by the observation that a specific calcium complex has not been found in the X-ray crystallographic study of this enzyme.[19] A very stable complex of the type found in thermolysin,[20,21] where the calcium ion is deeply buried in the inner part of the molecule, is therefore not formed in the present case.

Proteolysis of casein at elevated temperatures. The temperature dependence of the activity of β-trypsin-Sephadex towards casein as substrate differed from that towards esters at higher temperatures. An apparent hydrolysis maximum was found at 55°, and the activity decreased again at higher temperatures, as shown in Fig. 6. This is probably due to a conformational change of the substrate, leading to its exclusion from the relatively tight gel. Conformational changes in the substrate other than expansion, making it a less good substrate, or conformational changes in the enzyme in addition to the one observed at 45° could also account for the observed phenomenon. Although more permeable matrices such as agarose gels should allow a further investigation of the different possible explanations, the properties of the conjugate described below disqualified these gels for the purpose.

Esterolytic activity of β-trypsin-agarose at elevated temperatures. β-Trypsin covalently bound to agarose exhibited below 50° a catalytic behaviour compar-

able to that of free and Sephadex-bound β-trypsin, when BAEE or TAME were used as substrates. The cooperative transition observed at 40° with TAME as substrate [8] was found also with the agarose-bound enzyme (Fig. 7). At temperatures above 55°, however, irreversible inactivation occurred, and after heating to 75° for 15 min the conjugate showed only very little activity at room temperature, in contrast to the Sephadex-bound enzyme. Although the treatment with cyanogen bromide at alkaline pH renders the gel stable towards melting even at 100°, presumably by cross-linking,[22] minor changes in the conformation of the supercoiled agarose chains [23] may directly or indirectly impair the function of the enzyme.

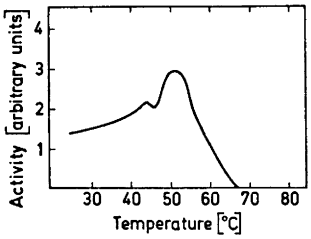

Fig. 7. Dependence of the activity of β-trypsin-agarose against TAME on temperature. The activity at pH 9.3 was measured in 0.1 M NaCl against 0.05 M TAME after preincubation of the enzyme for 15 min at the desired temperature.

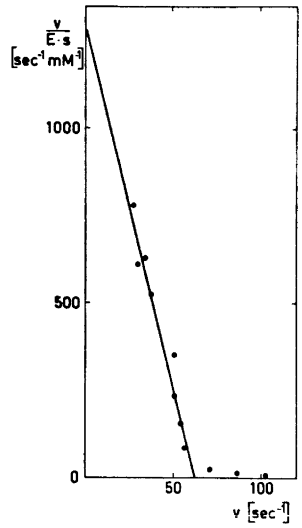

Fig. 8. Eadie-Hofstee plot of β-trypsin/TAME. The activity was measured at pH 0.8 in 0.0025 M Tris-HCl, 0.25 M NaCl at 25° in a pH-stat. The concentration of TAME varied between 0.04 and 15 mM. The enzyme concentration was 0.50×10^{-8} M.

Catalytic constants of β-trypsin. The constants for hydrolysis of TAME by the β-trypsin used here were determined at 25° from an Eadie-Hofstee plot (Fig. 8) and were: $K_M = 0.05$ mM, and $k_{cat} = 62$ sec^{-1}. If a correction was applied for the possible presence of 20 % impurities (10 % α-trypsin and 10 % inactive material, as determined by bioaffinity chromatography and discussed in Materials and Methods), a value for k_{cat} of 77 sec^{-1} was obtained. The corresponding constants for β-trypsin prepared by us from Worthington trypsin were: $K_M = 0.12$ mM, and $k_{cat} = 88$ sec^{-1}.[24] The differences in K_M are considered to be significant as they are much larger than the experimental error, estimated to be ≃20 % from the Eadie-Hofstee plot.

DISCUSSION

The transitions between the different molecular forms of the proteins derived from trypsinogen are shown in Fig. 9. Reversible temperature-induced transitions are indicated by double arrows, autolytic inactivations by arrows pointing backwards. Those reactions, for which no direct experimental evidence could be found, are indicated by dashed arrows. The subscript of rate constants refers to the corresponding reactions in Fig. 9.

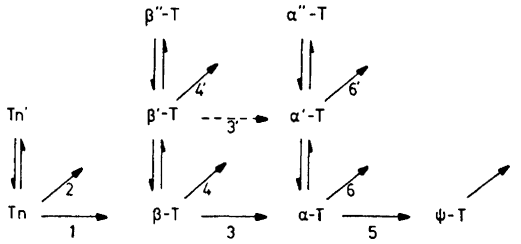

Fig. 9. Arrangement of the proteins derived from trypsinogen. For a description of this scheme, see Discussion. Tn designates trypsinogen, T trypsin.

The reversible transition from native trypsinogen to a more unordered structure at low pH has been described by Pohl.[25] The corresponding reactions leading to β′-trypsin and α′-trypsin have been described in a previous paper [8] as well as the reactions 3,4,4′,6, and 6′. There is no experimental evidence for reaction 3′; this will be discussed below.

The temperature-induced transitions leading to the doubleprimed forms of α- and β-trypsin have been observed only in the immobilized derivatives of these enzyme. These forms seem to have negligible enzyme activity. Investigation of the soluble derivatives is obscured by the rapid autolysis, and it could not be unambiguously established whether this reaction in the free enzyme leads to a reversibly inactivated form.

The most striking effect of calcium on β-trypsin is the great stabilization towards autolysis. The addition of calcium reduces the observed velocity of autolysis at room temperature by a factor of 100. This means that it is advisable for practical purposes to add calcium to the incubation mixtures which are to be digested by the enzyme, as has been observed before.[9–11] When pure β-trypsin is used, there is little risk of "parasitic splitting" which is observed with the commercial enzyme preparations.[26,27] Complete avoidance of the type of cleavage apparently caused by ψ-trypsin can be achieved by the immobilization of β-trypsin.

It has been shown before that calcium ions reduce the apparent rate constant k_2 (Ref. 1) (Fig. 9) and increase the constant k_1 (Ref. 3). In the present work it is established that calcium also decreases the rate constants k_4 and k_6. k_3 seems equally to be decreased, as a steady state concentration of α-trypsin is observed both in the presence and absence of calcium ions. The apparent constant k_4' is also decreased, whereas the experiments conduc-

ted here do not allow a similar conclusion for k_6'. As, however, no steady state concentration of α-trypsin is observed in the presence of calcium at higher temperatures, k_6' should be considerably larger than k_3'.

The calcium-trypsin complex which seems to be formed at temperatures both below and above 40° (as deduced from the altered rate of autolysis) is not sufficiently stable to allow isolation by chromatographic procedures. This is in contrast to previous results.[7] The critical dependence of the separation on SE-Sephadex on ionic strength, temperature, and concentration of the protein applied may have caused the observed phenomenon. The two peaks observed by Sipos and Merkel [7] could probably be α- and β-trypsin.

The results obtained here and elsewhere permit the following conclusions about the nature of the β-trypsin-calcium complex. Fluorescence measurements (presented here), difference spectra and optical rotatory measurements (described by Sipos and Merkel [7]) suggest that the trypsin molecule acquires a more compact structure upon binding of calcium. Below 40° the presence of BAEE in the absence of calcium causes, however, a more pronounced shift of the fluorescence spectrum to shorter wavelengths than calcium does. Above that temperature, a more open structure is found. Owing to the interference of autolysis with the fluorescence measurements nothing can be said about the fluorescence properties of trypsin in the absence of both calcium and substrate. Fluorescence measurements as well as the temperature dependence of the autolysis rate of free β-trypsin indicate a more loose structure above 40° (Ref. 8).

The cooperative transitions described here and in a previous paper [8] cannot always be inferred from activity measurements alone. Only with TAME as substrate was a "break" in the Arrhenius plot and a minimum in the curve activity *vs.* temperature observed. BAEE did not yield such results. The importance of a constant conformation of the substrate throughout the interval of conditions investigated is stressed by the fact that casein is not hydrolyzed by the Sephadex-bound enzyme above 60°, which may be ascribed to an extension of the molecular dimensions of casein at higher temperatures, hindering the penetration of the molecule into the gel. Therefore the temperature dependence of several molecular properties must be studied to establish the possible existence of a reversible cooperative transition.[8]

Intermolecular reactions as those observed with free enzymes are unlikely to occur in immobilized proteins. In a number of cases, however, the immobilized products have properties which are not found in the free proteins, and which have not been expected by exclusion of intermolecular reactions.[15,28–31] In the present investigation, the properties of the matrix are found to be transferred to the protein. Melting of agarose at higher temperatures is prevented by the reaction with cyanogen bromide.[22] This reaction apparently does not restrict the mobility of the matrix chains completely, and some changes in its structure seem still to occur. Similar effects will have to be considered when the molecular properties of immobilized proteins are to be elucidated.

The enzyme kinetic characteristics k_{cat} and K_M found in this work for β-trypsin obtained from Novo differed significantly from those found for β-trypsin from Worthington.[24] The reason can be genetic differences between

these two β-trypsins. Similar differences in k_{cat} and K_M have been found for π-chymotrypsin from different mice strains (H. Amnéus and V. Kasche, manuscript in preparation). We therefore suggest that minor differences, presumably in the primary sequence, exist between these two trypsin preparations.

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