COOPERATIVE TRANSITIONS BETWEEN ACTIVE α- AND β-TRYPSIN CONFORMATIONS

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The temperature dependence of hydrolytic and autolytic reactions catalyzed by free and immobilized α- and β-trypsin has been studied. A reversible cooperative transition was found to occur in the temperature interval 40-45°. The transition is probably an unfolding - refolding process involving only part of the molecule, as the enzyme function is retained. This indicates that the complete unfolding of these enzymes consists of several independent unfolding processes - *multistate unfolding*. Some implications of multistate unfolding in proteins are discussed.

The activation energy for several enzyme catalyzed reactions has been shown to be temperature dependent (1,2). This has been explained in terms of: (i) the existence of cooperative conformation transitions where the catalytic activity is retained, i.e. the complete unfolding of the protein consists of several independent unfolding processes (*multistate unfolding* (3)); (ii) that different catalytic reactions are rate-limiting in different temperature regions. Studies on static protein properties also indicate the existence of multistate unfolding (4,5). The reported data did not allow unambiguous conclusions on the relative importance of the effects (i) and (ii) on the temperature dependence. Besides these another effect, (iii) enzyme heterogeneity, may influence the results for trypsin (6) as commercial trypsin samples contain the active forms α- and β-TRY* besides inactive autolysis products (7). The aim of this study was to investigate whether multistate unfolding transitions occur in homogeneous trypsins. The active forms α-TRY and β-TRY can be resolved (7,9) and the homogeneity checked by analytical affinity chromatography (8,9). Using immobilized trypsins the temperature dependence of the enzyme activity can be studied in the absence of autolysis.

*Abbreviations: TRY = trypsin; STI = Soybean trypsin inhibitor; TAME = *p*-tosyl-L-arginine methyl ester.

THEORY: REACTIONS AND RATE CONSTANTS

Trypsin-catalyzed reactions may be represented by the following scheme (10):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES + P_1 \xrightarrow{k_3} E + P_2 \qquad (1)$$

where S, P_1 , and P_2 are substrates and products, respectively. At high substrate content ($>>K_M$, the Michaelis-Menten constant) the specific rate of product formation is given by the catalytic constant $k_{cat}=k_2k_3/(k_2+k_3)$ where both the acylation k_2 and deacylation k_3 rate constants are temperature dependent. The apparent activation energy of k_{cat} should decrease with temperature when the acylation and deacylation rates are rate-determining in different temperature regions. A similar decrease should be observed when K_M increases with temperature.

Autolysis of β-TRY yields α-TRY (enzymatically active) and inactive TRY (9), as shown in the reaction scheme (2):

$$\begin{array}{ccccc} i & \overset{k_{EE}}{\searrow} & & \overset{k'_{EE}}{\nearrow} & i' \\ \alpha & \longleftarrow & \beta & \rightleftharpoons & \beta' \longrightarrow \alpha' \end{array} \qquad (2)$$

where we also have included a hypothetical reversible conformation change between two different enzymatically active structures of β-TRY. The prime indicates the structure that is stable at high temperatures. When β-TRY is immobilized to a gel matrix only intramolecular changes in the enzyme are possible. Thus when the apparent activation energy for k_{cat} is temperature dependent for both free and immobilized β-TRY the observed effects cannot be due to chemical (intermolecular) modifications of β-TRY. When a shift from one to another rate-limiting step can be excluded, the remaining cause for the change in activation energy should be the reversible conformation change indicated in scheme (2). β- and β'-TRY are expected to differ in both static and dynamic properties. A relation for k_{EE} in scheme (2) can be derived from reaction (1) with S=E and assuming steady-state conditions, the result is $k_{EE} = k_1k_2/(k_{-1}+k_2)$. This rate equals k_{cat}/K_M for the autolytic reaction, i.e. the specific rate for an enzyme catalytic reaction where the substrate content $<<K_M$.

MATERIALS AND METHODS

Materials. α- and β-TRY were prepared from commercial bovine trypsin (NOVO A/S,Copenhagen) by chromatography on SE-Sephadex (7) and recovered by lyophilization after dialysis. The β-TRY sample was found to contain ≈10 % α-TRY and ≈10 % inactive TRY by analytical affinity chromatography on STI-agarose (9). As rechromatography of the β-TRY peak on the same column gave similar results it was concluded that some autolysis during the affinity chromatography procedure is unavoidable (as observed for α-chymotrypsin (8)) and that the original sample contained less α-TRY and inactive TRY. The α-TRY sample contained more (≈40%) inactive TRY. STI-Sepharose 4B and β-TRY-Sephadex G-200 were prepared using the CNBr method (8,11). TAME (Sigma, Cleveland, Ohio) was used as purchased. Protein concentrations were determined by absorbance measurements using the molar absorptivity $\varepsilon_{280,TRY} = 3.42 \cdot 10^4\ M^{-1}cm^{-1}$(8).

Determination of enzyme activity: Free enzyme. Stock solutions of the enzyme in 1 m*M* HCl were prepared the day they were used and stored on ice. The activity was determined under nitrogen after addition of 50 µl enzyme solution to 2.0 ml 0.1 *M* NaCl, 0.01 *M* TAME equilibrated in the thermostated titration vessel of a Radiometer pH-stat. The temperature of the assay solution was determined with a calibrated thermistor; the precision was ±0.2°. The production of acid at pH 8.0 was followed and corrected for non-enzymatic alkali uptake. The precision in the rates of quinduplicates was 3%. *Conjugated enzyme.* The procedure described elsewhere for chymotrypsin-Sephadex (11) was adapted with following modifications: A final NaCl concentration of 0.1 *M* was used and a thermostated solution of TAME was added to give a final concentration of 0.05 *M*. The alkali uptake at pH 9.3 was recorded and adequate blank corrections were made. At the high substrate concentration used the recorded rates are approximatively independent of the substrate concentration and proportional to k_{cat} (12). The precision of quinduplicates (8%) was not as good as in the case of the free enzyme, mainly due to a higher rate of non-enzymatic hydrolysis.

Autolysis of β-TRY. At zero time known amounts of β-TRY were dissolved in

buffer of pH 8.1 (0.01*M* $NaBO_3$-HCl, 0.1*M* NaCl) kept at different temperatures ($\pm 0.2^{o}$) in a thermostated water bath. The protein content in these solutions was 8-30 μM. At different times of incubation samples were withdrawn for determination of the remaining content of β-TRY by enzyme kinetics and determination of the amount of enzymatically inactive autolysis products by analytical affinity chromatography. In the former case the TAME activity was determined at 25.0^{o} immediately after withdrawal as described above. The activity was considered to be directly proportional to the remaining β-TRY content. In the latter case 1.0 ml of the incubated sample was cooled in an ice-water bath immediately after withdrawal and then introduced in a STI-agarose column (6x150 mm) equilibrated with buffer of pH 8.0 (Tris-HCl, I=0.05; 0.2 *M* NaCl, 0.1 *M* $CaCl_2$) and eluted with the same buffer at room temperature. The amount of protein eluted with this buffer was used as a measure for the amount of inactive autolysis products. An untreated sample was eluted to obtain a blank correction. The active enzyme was eluted with a pH-gradient from pH 5.0 to pH 3.0 (HOAc-NaOH, I=0.05; 0.2 *M* NaCl). From the elution diagrams the product ratio inactive TRY/α-TRY was determined.

Fluorescence measurements were performed as described earlier (11).

RESULTS AND DISCUSSION

Arrhenius plots for the activities (mean of five determinations) for α-TRY, β-TRY, and β-TRY-Sephadex are given in Fig.1 a-c. The activities were determined under conditions where they are directly proportional to the catalytic constant. Experimental data for the autocatalytic disappearance of β-TRY at different temperatures are given in Fig. 2. These show that the decay, as is expected from the scheme (2), is a second order process. The second order decay rate obtained by enzyme kinetic measurements and the corresponding rate obtained by analytical affinity chromatography agree within the experimental error, estimated to 20% based on reproducibility tests. These data indicate that the rate for the autolysis reaction leading to inactive TRY is much larger than the rate for the re-

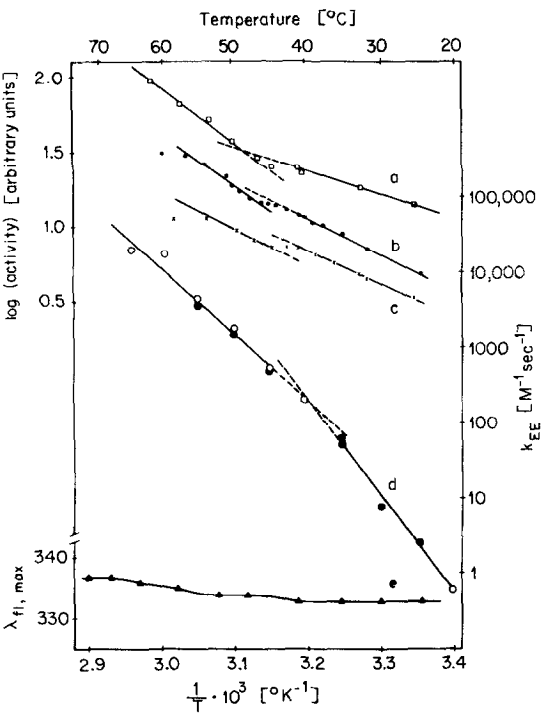

Fig. 1.

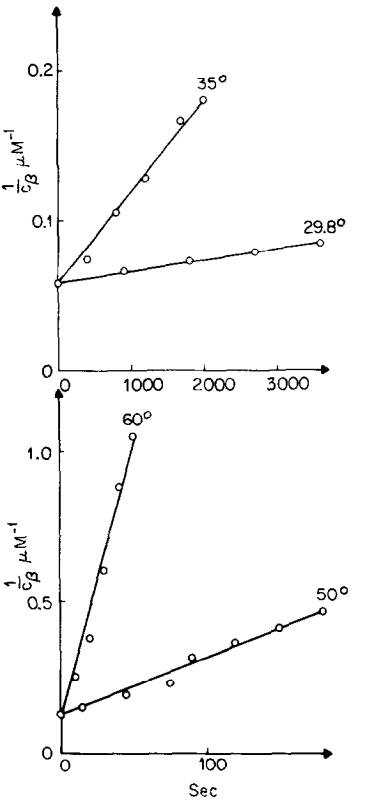

Fig. 2.

Fig. 1, Curves a-d: Arrhenius plots for activity and inactivation of different trypsins. Curves a-c (left ordinate): Logarithm of rate of TAME hydrolysis (arbitrary units) by β-TRY-Sephadex (a, □), β-TRY (b, ●), and α-TRY (c, x). The semi-filled point in Curve a (◪) represents the activity of β-TRY-Sephadex at 25° after heating to 70° for 15 min. Curve d (right ordinate): second order decay rate for β-TRY, measured by activity assay (o) and affinity chromatography (●). Curve e (▲): Wavelength of maximum tryptophan fluorescence emission for β-TRY-Sephadex as function of temperature.

Fig. 2. Second-order reaction plot of the autocatalytic disappearance of β-TRY at the temperatures indicated in the diagram, determined by affinity chromatography (29.8°, 35°, initial enzyme concentration 19 μM) and activity assay (50°, 60°, initial enzyme concentration 8 μM).

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action leading to α-TRY. Consequently the second order rate constant observed here can be considered to be practically equal to k_{EE}. The Arrhenius plot for this rate constant is also given in Fig.1. Below 40° all Arrhenius plots show good linearity. Above ≈45° the data may also be represented in linear form, except at the highest temperatures, but the slopes differ from the slopes of the

corresponding curves for the data below 40^{0}. This indicates that different processes govern the temperature dependence of the observed properties in the following temperature regions: I, $20\text{-}40^{0}$; II, $40\text{-}45^{0}$; III, $45\text{-}55^{0}$; IV, $>55^{0}$.

The slope of the Arrhenius plots for the activity of β-TRY and β-TRY-Sephadex is larger in region III than in region I. In the 'break' region II, k_{cat} has a much smaller temperature coefficient than in regions I and III. The magnitude of the rate constant k_{EE} excludes any appreciable autolytic decrease in β-TRY in region III during the activity determination. These observations exclude autolysis of β-TRY and a shift from one rate determining catalytic reaction to another as the cause for the different temperature dependence in regions I and III.

The negligible change in k_{cat} with temperature observed in region II for α-, and free and immobilized β-TRY; the complete temperature reversibility of the enzymatic activity of β-TRY-Sephadex; the difference in temperature dependence of the observed quantities in regions I and III, indicate that at least two different conformations of α- and β-TRY account for these findings. This is also supported by the observation that the breaks in the different Arrhenius plots for β-TRY occur at approximatively the same temperature. The further discussion will be limited to β-TRY which is the major active trypsin species (7,9). However, similar conclusions as those obtained for β-TRY are expected to be valid also for α-TRY due to the similarity in the observed temperature dependence.

Based on these results we conclude that the observed phenomena are due to the reversible transition $\beta \rightleftharpoons \beta'$ (scheme (2)). The deviations from linear Arrhenius plots for the activities in regions IV are due to the autolytic reactions that interfere at these temperatures. The transition $\beta \longrightarrow \beta'$ shows a high degree of cooperativity, i.e. occurs over a narrow temperature interval ($\simeq 5^{0}$). This follows from the small change in k_{cat} in the temperature interval $40\text{-}45^{0}$ where both β and β' are present simultaneously, compared to the temperature dependence in the regions with linear Arrhenius plots. Weber (13) has recently stressed the fact that a protein in solution has no unique conforma-

tion, but exists in a large number of conformations, mainly different states of protonation. Temperature-dependent changes in the distributions of such conformations should have transition regions much larger than 5^{o}, as the activation energies involved in transitions between these conformations are small (1).

Immobilization of enzymes has been shown to alter the ease with which conformational changes occur (11). In this case,however, immobilization has a negligible influence on the reversible conformational transition $\beta \rightleftharpoons \beta'$.

The data for k_{EE} do not allow a conclusion whether the complex formation or the acylation reaction is rate-controlling. The marked decrease in activation energy for k_{EE} (60 kcal/mol for β-TRY and 40 kcal/mol for β'-TRY) may indicate less steric hindrance in the hydrolysis of β'-TRY. The bond hydrolyzed in β'-TRY should then be more accessible than the corresponding bond in β-TRY due to the conformation change. The latter is also observed as a change in fluorescence maximum (Fig. 1 e). The change observed in the $\beta \longrightarrow \beta'$ transition is, however, small compared to the change observed in the complete unfolding of β-TRY. Thus the $\beta \longrightarrow \beta'$ transition is similar to the 'predenaturation' transitions observed in trypsinogen, ribonuclease, or myoglobin (14).

The existence of multistate unfolding where the partially unfolded proteins retain their biological activity, as observed here for α- and β-TRY, has important consequences. It should be considered in kinetic studies on protein reactions where the T-jump technique is used (15). In X-ray crystallographic studies on protein structure and its relation to the function in solution it should be important to study whether the different folded states show differences in crystal structure. It has been shown that different crystals are formed from lysozyme crystallized at different temperatures (16). The transition temperatures for the unfolding processes have been shown to depend on pH (4) and solvent composition (5). Further studies on cooperative unfolding transitions, where the biological function is retained, should be of interest for our understanding of protein structure-activity relations *in vitro* and *in vivo*.

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