UNUSUAL (ZIG-ZAG) TEMPERATURE DEPENDENCE OF THE RATE CONSTANT FOR IRREVERSIBLE THERMOINACTIVATION OF HYDROPHILIZED ENZYMES

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Hydrophilized preparations of α-chymotrypsin and trypsin obtained by covalent modification of the enzymes with anhydrides of aromatic carboxylic acids (trimellitic, pyromellitic and mellitic) or with glyoxylic acid display an unusual temperature dependence of the rate constants of irreversible thermoinactivation: the linear plots (with positive and negative values of an effective activation energy) alternate in a zig-zag manner. A formal kinetic scheme describing this behaviour is suggested, involving the temperature-dependent conformational transition of modified α-chymotrypsin into a more stable conformation.

Recently, we have carried out the modification of enzymes by introducing a great number of highly hydrophilic amino[1,2] or carboxylic groups[2-4] into the surface layer of protein molecules. As was predicted by us[5,6], the hydrophilization of a non-polar surface area of proteins resulted in their essential stabilization against irreversible thermoinactivation, the stabilizing effect being thousand-fold and greater[2].

Now, we report that hydrophilized enzymes display an unusual ("zig-zag") temperature dependence of the rate constant of irreversible thermoinactivation in Arrhenius plots. Such unusual behaviour has been never observed for enzymes before.

EXPERIMENTAL

The catalytic activity of α-chymotrypsin (EC 3.4.21.1) and trypsin (EC 3.4.21.4) from bovine pancreas (Sigma) was evaluated by determining initial rates of enzymatic hydrolysis of specific substrates (5 mM N-acetyl-L-tyrosine ethyl ester or 5 mM N-benzoyl-L-arginine ethyl ester, respectively; both from Sigma) in 0·1M-KCl, pH 8·0, 25°C, using a Radiometer pH-stat.

Reductive alkylation of enzymes with glyoxylic acid[4] and acetic anhydride[7], *and acylation* with trimellitic, pyromellitic and mellitic anhydrides[2] was performed as previously described;

the enzyme preparations were characterized by a maximal degree of their modification. Yields of modified enzymes, as determined by active site titration[8], were never less than 25—30%. The k_{cat} value for the modified enzyme preparations, as determined by the rate of enzymatic hydrolysis of specific substrates, did not differ from k_{cat} for unmodified α-chymotrypsin and trypsin.

Thermoinactivation of the native and modified α-chymotrypsin and trypsin was studied in the temperature range 40—98°C, pH 8·0 (5 mM-KH_2PO_4 buffer in 0·1M-KCl, with initial concentration of enzymes being $0 \cdot 1^{-1}$ μ mol l^{-1}. Aliquots of the thermostated enzyme solution were withdrawn after certain time intervals, cooled down to room temperature with ice water, and the residual catalytic activity was assayed in a pH-stat. The processes of bimolecular autolysis and aggregation were negligible under the experimental conditions[9]; the thermoincativation rate constants were determined as previously described[9]; when the thermoinactivation kinetics was "biphasic", the stability was characterized by the rate constant for the second, more stable fraction.

Fluorescence spectra of chymotrypsin preparations were registered on a spectrofluorometer Hitachi 521, excitation wavelength 280 nm, spectral width 10 nm, in a thermostated cell (±0,5 °C).

RESULTS AND DISCUSSION

Kinetics of Irreversible Thermoinactivation of the Modified Enzymes

Fig. 1 shows the kinetics of thermoinactivation of native α-chymotrypsin and the enzyme acylated with pyromellitic dianhydride. As distinct from the native enzyme (for which the inactivation rate increases steadily with the temperature, Fig. 1a), the rate of incativation of modified α-chymotrypsin increases in the temperature range 45—55°C, then decreases between 55 and 75°C, and finally increases again at temperatures higher than 75°C (Fig. 1b). The same regularities were observed for other hydrophilized preparations studied, i.e. α-chymotrypsin and trypsin acylated with trimellitic, pyromellitic and mellitic anhydrides (two, three and five COO^- groups are introduced, respectively, per each modified NH_2 group), and α-chymotrypsin alkylated with glyoxylic acid (NH_3^+ groups are transformed into more hydrophilic NH_2^+-CH_2COO^- groups).

The values of thermoinactivation rate constants (k_{in}) were determined as described in Experimental, and the logarithmic values of k_{in} were plotted versus reciprocal temperatures (Fig. 2). For native (nonmodified) α-chymotrypsin ln k_{in} increases linearly with increasing temperature (Fig. 2a) which is typical of both native[10] and modified[11] enzymes. The same linear dependence of ln k_{in} on reciprocal temperature was observed for α-chymotrypsin alkylated with hydrophobic acetic aldehyde (Fig. 2a).*

* At temperatures higher than 55°C and up to 90°C, inactivation of both nonmodified α-chymotrypsin and the hydrophobic derivative of the enzyme proceeds very quickly and the value of k_{in} unfortunately cannot be determined experimentally. The maximal value of k_{in} which can be still experimentally determined, is shown (Fig. 2) in dotted line.

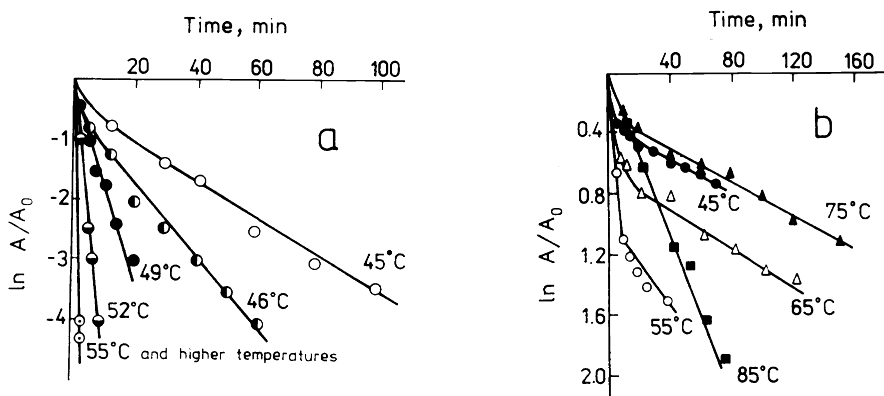


FIG. 1

Thermoinactivation kinetics (A and A_0 are the current and initial values of enzyme activity, respectively) of α-chymotrypsin preparations at pH 8·0 (5 mM-KH_2PO_4 buffer) in 0·1M-KCl; initial enzyme concentration 1 μmol l^{-1}. a Nonmodified α-chymotrypsin at temperatures given in the figure (⊙ for t = 55, 62, 70, and 80°C); b α-chymotrypsin modified with anhydride of pyromellitic acid at temperatures given in the figure

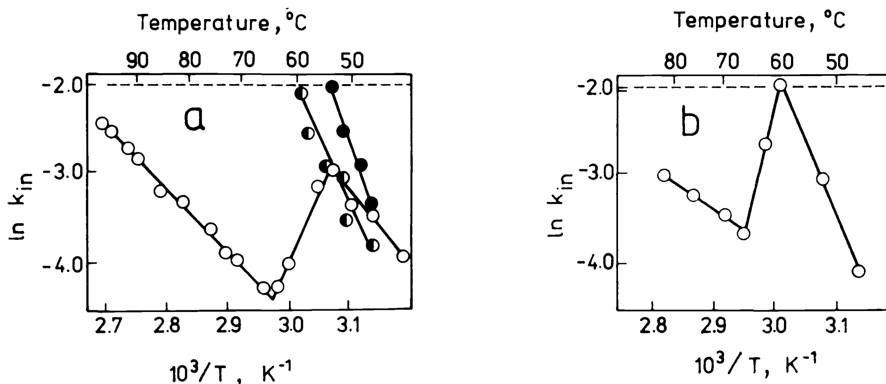


FIG. 2

Temperature dependence (in the Arrhenius plot) for the rate constant of irreversible thermoinactivation (k_{in}) of α-chymotrypsin preparations: a ● nonmodified α-chymotrypsin, ◐ α-chymotrypsin modified with acetic aldehyde, ○ α-chymotrypsin modified with dianhydride of pyromellitic acid; b α-chymotrypsin modified with glyoxylic acid. The maximal value of k_{in} which can be still experimentally determined, is shown in the dotted line

However, the temperature dependences of k_{in} for hydrophilized preparations of α-chymotrypsin in Arrhenius plots are unusual; for instance, see Fig. 2a (modification with dianhydride of pyromellitic acid) and Fig. 2b (modification with glyoxylic acid). They can be subdivided into three linear portions. In the range 40 – 50°C the value of ln k_{in} increases linearly, the apparent value of activation energy (E_a) being 50 kcal/mole (210 kJ/mole) and higher as one could expect for the denaturation o-proteins[12]. In the range 55 – 65°C the value of k_{in} decreases with increasing temperaf ture, which gives the negative E_a values. Finally, at temperatures higher than 70°C the value of ln k_{in} again increases linearly with E_a value being 25 – 40 kcal/mole (105 – 165 kJ/mole).

It has been previously stated[10,13] that reversible binding of ligands or conformational changes preceeding irreversible inactivation result in *nonlinear* temperature dependences of experimentally determined values of ln k_{in} versus T^{-1}. We suppose that reversible conformational transition of hydrophilized α-chymotrypsin at 50 – 60°C is the reason of unusual "zig-zag" temperature dependence of k_{in}.

Reversible Conformational Transition of the Hydrophilized Preparations

To prove the existence of a conformational transition we used fluorescence spectroscopy. Fluorescence spectra were registered during an initial period of incubation at elevated temperature (first several minutes) which enabled us to exclude irreversible changes in spectra resulting from the "slow" irreversible thermoinactivation observed by us (Figs 1 and 2).

Figure 3 shows the dependence on temperature of the wavelength of maximal

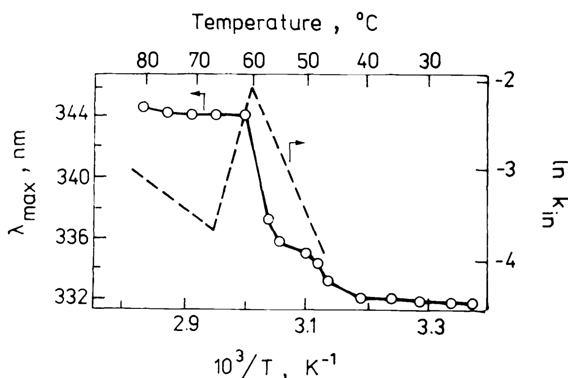


FIG. 3

Temperature dependence of the maximal wavelength of the fluorescence spectrum of α-chymotrypsin modified with glyoxylic acid. In comparison (cf. Fig. 2b), temperature dependence of k_{in} for the modified enzyme is shown in the broken line (in Arrhenius plot)

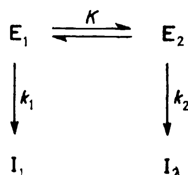
intensity (λ_{max}) in the fluorescence spectrum of α-chymotrypsin alkylated with glyoxylic acid. In the range of 45–60°C an essential increase of λ_{max} from 332 nm to 345 nm occurs, which is the evidence of the temperature-dependent conformational transition in the protein molecule. The transition proceeds at the same temperatures where the nonlinearity of the dependence of k_{in} in Arrhenius plots appears (cf. the broken line in Fig. 3).

The conformational transition is reversible since after cooling the enzyme solution preheated at 60–70°C to 20°C, the value of λ_{max} regains its initial value (332 nm).

For the native (nonmodified) α-chymotrypsin some structural changes registered by fluorescent spectroscopy proceed at 45–60°C as well. However, as distinct from the modified enzyme, these changes are irreversible and result from rapid thermoinactivation of the nonmodified enzyme at elevated temperatures.

Kinetic Model

The irreversible thermoinactivation of hydrophilized enzymes is depicted by the kinetic scheme 1,



SCHEME 1

where E_1 and E_2 are "low-temperature" and "high-temperature" conformers of the modified enzyme, and I_1 and I_2 their irreversibly inactivated forms, respectively; k_1 and k_2 are the corresponding rate constants of irreversible inactivation of E_1 and E_2, and K is the equilibrium constant for a rapid transition of the protein conformation; that is, $K = [E_2]/[E_1]$. Then the experimentally determined value of k_{in} is equal to:

$$k_{in} = \frac{1}{1 + K} k_1 + \frac{K}{1 + K} k_2 \,. \qquad (1)$$

The zig-zag character of temperature dependence of the apparent value of k_{in} (Fig. 2) is caused by different sensitivity to temperatures of the true constants: k_1, k_2, and K. At low temperatures where conformational equilibrium in Scheme 1 is shifted to the left ($K \ll 1$), $k_{in} = k_1$. At temperatures in the region of conformational transition ($K \approx 1$), the value of k_{in} depends on all the true constants. In this case, the first term in Eq. (*1*) is the product of two factors, i.e. k_1 rising with temperature and

$1/(1 + K)$ decreasing with temperature; the latter factor determines the negative value of E_a. Finally, at high temperatures, the enzyme is in conformation E_2 $(K \ll 1)$ and $k_{in} = k_2$. This qualitative analysis is also supported by computer simulation.

CONCLUSION

The results presented here show that linear extrapolation of the temperature dependences of k_{in}, which is often used for comparison of stabilities of different enzyme preparations[6], may not be always valid. This comparison is informative only if there is an evidence that inactivation of enzymes under consideration is described by the same mechanism in the whole temperature range.

Of course, the true reasons of great difference in stability of the conformations E_1 and E_2 of the hydrophilized enzyme preparations should be studied in detail in future. Nevertheless, it is highly probable that the enzyme in the conformation E_2 is stabilized to a great extent against irreversible conformational changes, i.e. against unfolding with subsequent scrambling. Therefore, its inactivation may be caused by one of the chemical mechanisms stated by us[14,15] and later studied in detail[16,17].

REFERENCES

1. Mozhaev V. V., Melik-Nubarov N. S., Martinek K., Berezin I. V.: Dokl. Akad. Nauk SSSR *288*, 1006 (1986).
2. Mozhaev V. V., Šikšnis V. A., Melik-Nubarov N. S., Galkantaite N. Z., Denis G. J., Butkus E. P., Zaslavskii B. Yu., Mestechkina N. M., Martinek K.: Eur. J. Biochem. *173*, 147 (1988).
3. Šikšnis V. A., Galkantaite N. Z., Denis G. J., Butkus E. P., Mozhaev V. V., Martinek K., Berezin I. V.: Dokl. Akad. Nauk SSSR *288*, 1508 (1986).
4. Melik-Nubarov N. S., Mozhaev V. V., Šikšnis V. A., Martinek K.: Biotechnol. Lett. *9*, 725 (1987).
5. Mozhaev V. V., Martinek K.: Enzyme Microb. Technol. *6*, 50 (1984).
6. Mozhaev V. V., Berezin I. V., Martinek K.: CRC Crit. Rev. Biochem. *23*, 235 (1988).
7. Torchilin V. P., Maksimenko A. V., Smirnov V. N., Berezin I. V., Klibanov A. M., Martinek K.: Biochim. Biophys. Acta *567*, 1 (1979).
8. Schonbaum G. R., Zerner B., Bender M. L.: J. Biol. Chem. *236*, 2930 (1961))
9. Martinek K., Klibanov A. M., Goldmacher V. S., Berezin I. V.: Biochim. Biophys. Acta *485*, 1 (1977).
10. Laidler K. J.: *The Chemical Kinetics of Enzyme Action*. Clarendon, Oxford 1958.
11. Martinek K., Klibanov A. M., Berezin I. V.: J. Solid-Phase Biochem. *2*, 343 (1977).
12. Joly M.: *A Physicochemical Approach to the Denaturation of Proteins*. Academic Press, London 1965.
13. Zale S. E., Klibanov A. M.: Biotechnol. Bioeng. *25*, 2221 (1983).
14. Mozhaev V. V., Martinek K.: Enzyme Microb. Technol. *4*, 299 (1982).
15. Mozhaev V. V., Šikšnis V. A., Torchilin V. P., Martinek K.: Biotechnol. Bioeng. *25*, 1937 (1983).
16. Ahern T. J., Klibanov A. M.: Science *228*, 1280 (1985).
17. Zale S. E., Klibanov A. M.: Biochemistry *25*, 5432 (1986).

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