Journal of Thermal Analysis, Vol. 50 (1997) 73-80

Dedicated to Prof. Menachem Steinberg on the occasion of his 65th birthday

EFFECT OF HYDRATION AND *pH* ON THE THERMAL STABILITY OF PROTEINASE K

Wang Bangning, Han Buxing and Tan Fu

Institute of Chemistry, Academia Sinica, Beijing 100080, China

Abstract

The effect of hydration and *pH* on the thermal stability of proteinase K was studied in the temperature range 310–450 K by differential scanning calorimetry. The dependences of the denaturation temperature T_d, the specific enthalpy of denaturation ΔH_d and the maximum of excess apparent specific heat capacity C_{ex}^{max} upon the degree of hydration h and the *pH* of the buffers used are presented. The relation between T_d and h is of the Flory-Garrett's type. By means of Ooi's model, the two components of the denaturation enthalpy arising from hydration and conformational change, respectively, were estimated. The fact that the specific denaturation enthalpy of proteinase K is very low may be attributed to its very low enthalpy of conformational change per heavy atom.

Keywords: differential scanning calorimetry, hydration, *pH*, proteinase K, thermal stability

Introduction

The proteinase K (EC 3.4.21.14) from Tritirachium album Limber is the most active serine endopeptidase among the known proteases. The enzyme can hydrolyze native keratin, consequently it exhibits the strongest capability to hydrolyze proteins. It rapidly inactivates RNases and DNases thereby providing a possibility for the preparation of RNA and DNA in high purity. In the crystalline form it is stable during long-term irradiation by X-rays. Consequently, the enzyme is a new system which may be subjected to structural studies in itself or in the enzyme-inhibitor complex. From these specific properties of the enzyme, one may expect further theoretical and practical studies on the nature and properties of this enzyme. In addition, Betzel *et al.* [1] claimed, on the basis of structural analysis, that the proteinase K displayed favourable thermal stability and would offer a new system for studying the thermal stability. However, there has appeared no study in the literature on the thermal stability of the enzyme. The

0368–4466/97/ $ 5.00

John Wiley & Sons Limited
Chichester

purpose of this work is to investigate the thermal stability and the influence of hydration and *pH* upon the thermal stability of the enzyme by differential scanning calorimetry (DSC).

Experimental

Reagents

The proteinase K extracted from Tritirachium album Limber and purified chromatographically was a Merck product purchased from the Huamei Bioengineering Company. It was a white lyophilized powder with an activity of 27 mAnson U/mg. A set of three standard *pH* reagents was purchased from the Shanghai Aijian Ready-made Chemicals Factory. GR grade hydrochloric acid, biochemical reagent grade Tris, AR grade glacial acetic acid and CP grade sodium acetate were Chinese commercial reagents.

Instrumental

Samples were weighed on a Perkin-Elmer Model AD-2Z electromagnetic supermicrobalance. Calorimetric determinations were carried out by using a Perkin-Elmer Model DSC-2C differential scanning calorimeter. Control of the calorimeter was automatic by using a Perkin-Elmer software in a Model 3500 Thermal Analysis Data Station. The calorimetric system was calibrated prior to determination by high purity indium and redistilled water. The error in temperature measurement was $\pm 0.1^{\circ}$, and the relative error of the determination of energy was ±1%. The *pH* values of the buffers used in DSC studies were determined at room temperature by a Model PHS-2 *pH*-meter from the Shanghai Second Analytical Instruments Factory. The *pH*-meter was calibrated with three standard solutions (*pH* 4.01, 6.86 and 9.18), and had an accuracy of ±0.01 *pH*.

Method

The buffers were prepared from 0.1 mol l^{-1} HAc and NaAc solutions, or from 0.1 mol l^{-1} HCl and Tris solutions. Their *pH* values at 25.5°C were 3.29, 4.67, 6.13 (for HAc–NaAc); 7.58 and 8.49 (for HCl-Tris). Each sample was encapsulated hermetically in a large-volume capsule of stainless steel with a Viton O-ring. Samples for the measuremen of hydration effects were composed of about 4.8 mg of the enzyme powder and a suitable amount of redistilled water. Samples for the measurements of the effect of *pH* were composed of about 3.21 mg of enzyme powder and 35 µl of the appropriate buffer solution. The sealed stainless steel capsules were stored in a refrigerator at 0–1°C for 8 days. Then the DSC determination was accomplished at a scan rate of 10 K min^{-1} in the temperature range 310–450 K. The method used for determining the water content of the enzyme has been described in previous papers [2, 3].

Results and discussion

Three thermodynamic parameters characterizing the thermal stability were obtained by DSC measurements on samples with different water contents h ($0.048 \leq h \leq 4.39$ g of water/g of dry enzyme; hereafter the unit of h will be either omitted or abbreviated to g/g). These were: (i) the denaturation temperature T_d, represented by the peak temperature of the endothermic peak of denaturation; (ii) the specific denaturation enthalpy ΔH_d, from the total area of a peak; (iii) the maximum of the excess apparent specific heat capacity C_{ex}^{max}, which was measured in the unit of specific heat capacity and on the basis of the maximum peak height relative to the baseline of a DSC curve. The latter can be used for calculating the van't Hoff enthalpy [4, 5]. It is also a measure of the sharpness of a peak. The dependence of these parameters upon the water content h is presented in Fig. 1. In the Figure, the four regions A, B, C and D have been divided by the dash lines according to the results of low temperature calorimetry [2]. It may be concluded from Fig. 1 that the three parameters change strongly with increasing degree of hydration h in the region A. This shows that the unfreezable water of primary hydration has a strong influence on the thermodynamic parameters of the enzyme. After the freezable water appears, however, T_d and C_{ex}^{max} change slowly with increasing h in the regions B, C and D. However, the change of ΔH_d is complex. In the region B, ΔH_d increases with increasing amount of the water of secondary hydration. But in the regions C and D, ΔH_d generally decreases as the water of intermediate hydration and the free water appear successively. It should also be noted that the specific denaturation enthalpy of the proteinase K

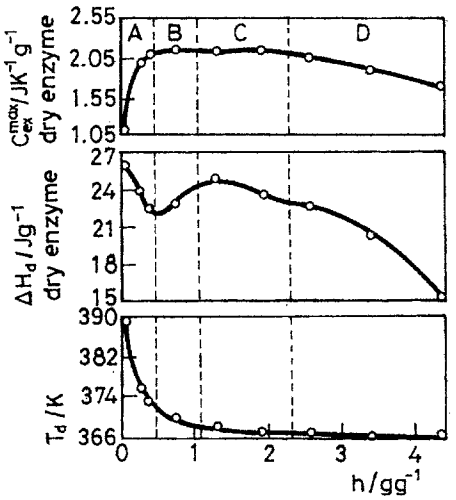

Fig. 1 Dependence of the maximum of excess apparent specific heat capacity C_{ex}^{max}, the specific enthalpy of denaturation ΔH_d and the denaturation temperature T_d upon the water content h of the proteinase K

is very low and its denaturation temperature is very high, in comparison with the corresponding dependences for globular proteins, double-stranded coiled-coils and triple-stranded coiled-coils [5]. This is obviously different from the dependence mentioned by Privalov [5], where ΔH_d increases with increasing T_d. It may also be seen from Fig. 1 that T_d decreases exponentially with increasing h. This resembles the results observed for the collagens from bovine achilles tendon and rat tail tendon by Flory *et al.* [6], for the β-lactoglobulin by Rüegg *et al.* [7] and for the tropocollagen of calf skin by Luescher *et al.* [8]. It may further be seen from Fig. 1 that T_d^0 being equal to 400 K is obtained by extrapolating h to zero. By using the crystal density of proteinase K 1.285 g cm^{-3} [9] and the water density 0.96120 g cm^{-3} at 369.15 K [10], the value of the water content h may be reduced to the value of the volume fraction of water υ_1. Following Eq. (3) of Flory-Garrett [6], an equivalent equation

$$(1/T_d - 1/T_d^0)/\upsilon_1 = A - A\,\chi_1\,\upsilon_1 \qquad (1)$$

may be presented. The data of T_d and h below 375.87 K have been treated by Eq. (1). The results obtained are presented in Fig. 2. The excellent linear relationship in Fig. 2 shows that the action of hydration water on the proteinase K is the same as that of a diluent on a polymer. By use of the slopes and intercepts of lines 1 and 2, the enzyme-water interaction parameter χ_1 was calculated to be equal to 1.027 and 0.576. These show that the enzyme-water interaction is stronger when the water content is lower. From the intercept A of line 1, the Flory-Garrett specific melting enthalpy Δh of pure proteinase K was calculated to be 409.8 J g^{-1} dry enzyme at υ_1=0. Witnauer *et al.* [11] have given the values 321 and 356 J g^{-1} for bovine skin collagens. Flory *et al.* [6] stated that the error of this quantity is ±20%. Therefore, the value of Δh from the present paper is reasonable.

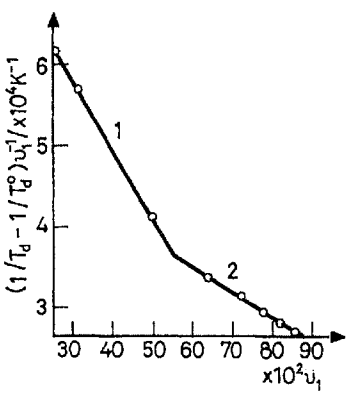

Fig. 2 Dependence of the denaturation temperature T_d upon the volume fraction υ_1 of the water in the hydrated proteinase K. Correlation coefficient: 0.999 for line 1; 0.996 for line 2

For the reversible two-state denaturation of globular proteins, the van't Hoff enthalpy or effective enthalpy ΔH_{vH} may be calculated by the Equation [12]

$$\Delta H_{vH} = 2R^{1/2} T_d \, (MC_{ex}^{max})^{1/2} \tag{2}$$

and the ratio $\Delta H_{cal}/\Delta H_{vH}$ should be equal to one. For the proteinase K, both the calorimetric enthalpy ΔH_{cal} and the van't Hoff enthalpy ΔH_{vH} are calculated by use of the molecular mass M=28900 [2] and the related DSC data. Besides the sample with a water content of h=4.39, the ratio is slightly larger than 1.2. Moreover, the ratio is much larger than one for the samples in region A. Therefore, it may be concluded that the thermal denaturation of the proteinase K is not amenable to the two-state model. In fact, the thermal denaturation of the enzyme is irreversible and the endothermic peak of denaturation does not reproduce when the denatured samples with different water contents are reheated in situ in the calorimeter.

The effect of hydration water and the denaturation process may be analysed theoretically using the cycle

$$\begin{array}{ccc} \text{N(non-hydrated)} + \text{water} & \xrightarrow{\Delta H_d^c} & \text{D(non-hydrated)} + \text{water} \\ \uparrow\ -\Delta H_N^h & & \Delta H_D^h \downarrow \\ \text{N(hydrated)} & \xrightarrow{\Delta H_d} & \text{D(hydrated)} \end{array}$$

$$\Delta H_d = \Delta H_d^c + (\Delta H_D^h - \Delta H_N^h) = \Delta H_d^c + \Delta H_d^h$$

Here ΔH_d represents the denaturation enthalpy determined by calorimetry; ΔH_d^c represents the contribution of the conformation change itself to ΔH_d; ΔH_d^h is the difference between the hydration enthalpy of the native state ΔH_N^h and that of the denaturated state ΔH_D^h, ΔH_d^h is another term contributed to ΔH_d. For the sample with a water content of h=4.39, the values T_d=366.03 K and ΔH_d=15.2 J g^{-1} dry enzyme were obtained by DSC determination. As the atomic coordinates of the enzyme were not available, we used the primary structure of the enzyme given by Gassen *et al.* [13] and the enthalpy and heat capacity of hydration of amino acid residues from Ooi [14], and then assumed residue additivity and made allowance for the N-terminal and C-terminal residues Ala's. The result ΔH_d^h=−978×4.184 kJ mol^{-1} at T_d=366.03 K was thus obtained. Using the value of ΔH_d mentioned above, we obtained the value 1083×4.184 kJ mol^{-1} for the ΔH_d^c of the proteinase K at T_d=366.03 K. There are 2030 heavy atoms in each molecule of the enzyme. We find, by comparison with the results of the fourteen proteins in Ooi's Table 10 [14], that the value of ΔH_d/NA of the proteinase K is the smallest and the value of its ΔH_d^c/NA is almost the smallest but the value of its $|\Delta H_d^h|$/NA is not very low. Furthermore, the molecular mass of a protein is directly proportional to its number of heavy atoms NA. Therefore it may be con-

cluded that the very low value of specific denaturation enthalpy of the proteinase K has to be attributed mainly to its very low value of the enthalpy of conformational change per heavy atom. This conclusion may be seen more clearly from Fig. 3 which shows that the increase of ΔH_d^c value of proteinase K is situated far below the line representing the dependence of ΔH_d^c on the number of heavy atoms NA. This shows that the increase in ΔH_d^c with the increase in the number of heavy atoms is not sufficient at least for the proteinase K, resulting in a very low value of its specific denaturation enthalpy.

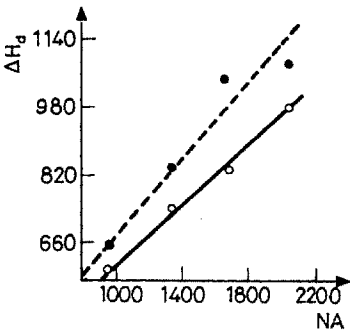

Fig. 3 Dependence of the two components of the denaturation enthalpy, ΔH_d^c and ΔH_d^h, upon the number of heavy atoms NA. ΔH_d^c (- - -) ; $-\Delta H_d^h$ (——); proteinase K (o); the other three data points on each line correspond to the three globular proteins, 5RSA, 2LZM and 8PAP from Ooi [14]. One unit of ordinate represents 4.184 kJ mol^{-1}

Crystals of the proteinase K are insensitive to *pH* changes in the range from 4°C to room temperature and from *pH* 3.5 to 9 [15]. The enzyme displays the optimum activity in the *pH* range from 7.5 to 12.0 [16], and it is autolyzed and degraded when its concentration is very low [17]. Therefore, in order to inspect the effect of *pH* change on the thermal stability of the enzyme, the samples were composed of about 3.21 mg of the enzyme powder and 35 μl of the required buffers with *pH* 3–9, i.e. the concentration of the enzyme was slightly lower than its saturation concentration, and autolysis was negligible. The DSC data are presented in Fig. 4. The Fig. shows that the three thermodynamic parameters ΔH_d, C_{ex}^{max} and T_d show abrupt change when the *pH* is near neutral. For the HAc–NaAc buffers, C_{ex}^{max} and ΔH_d increase with increasing *pH* of the buffers used. For the HCl-Tris buffers, C_{ex}^{max} and ΔH_d decrease with increasing *pH* of the buffers used. For the HCl-Tris buffers C_{ex}^{max} and ΔH_d decrease with increasing *pH* of the buffers used. The curve representing the dependence of T_d on the *pH* is sigmoid and is similar to the sigmoid curve of calf skin collagen of Dick *et al.* obtained with non-calorimetric method [18]. However, the temperature step of the proteinase K is much smaller, as found in this work. The abrupt change in the thermodynamic parameters at near neutral *pH* may be attributed to the change of the ionization state of imidazole rings of four His's in its molecule. Indeed, Betzel *et al.* [1] indicated that the central core of the three-dimensional structure

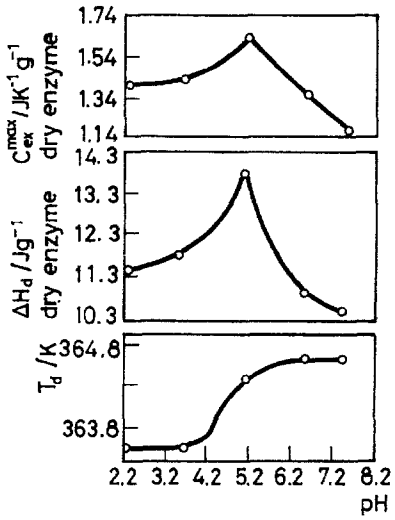

Fig. 4 Dependence of the maximum of excess apparent specific heat capacity C_{ex}^{max}, the specific enthalpy of denaturation ΔH_d and the denaturation temperature T_d upon the *pH* of the buffers used

of the enzyme molecule is a β-sheet consisting of parallel β-strands which is decorated by six α-helices, three short antiparallel β-sheets and 18 β-turns. Both His 69 and His 72 exist in the α_2-helix. The positive charges on their imidazole rings are absent in the HCl-Tris buffers. The electrostatic repulsion between them and among them and Arg 52 and Lys 57 in the neighbouring β-strand βII2 are absent in the HCl-Tris buffers. Obviously, this is favourable to the compact three-dimensional structure of the enzyme molecule. Similar interpretation is applicable to the His 46 in the β-turn $t_4t_5t_6t_7$ and the His 229 in the α_5-helix. Therefore, the thermal stability of the enzyme is higher in the HCl-Tris buffers.

For the samples used in the investigation of the effect of *pH*, the endothermic peak of denaturation is not reproduced when the samples having undergone thermal denaturation are reheated in situ in the calorimeter. The ratio $\Delta H_{cal}/\Delta H_{vH}$ for the samples is about 0.8. Hence, the denaturation of the enzyme in the buffers is not amenable to the two-state model, either.

* * *

This major project was supported by the National Natural Science Foundation of China.

References

1 C. Betzel, G. P. Pal and W. Saenger, Eur. J. Biochem., 178 (1988) 155.
2 B.-N. Wang and F. Tan, Chinese J. Biochem. Biophys., 25 (1992) 213.
3 B.-N. Wang and F. Tan, Acta Biochim. Biophys. Sinica (in Chinese), 22 (1990) 189.
4 J. M. Sturtevant, Ann. Rev. Phys. Chem., 38 (1987) 463.

5 P. L. Privalov, Advances in Protein Chemistry, Academic Press, New York 1982, p. 1.
6 P. J. Flory and R. R. Garrett, J. Am. Chem. Soc., 80 (1958) 4836.
7 M. Rüegg, U. Moor and B. Blanc, Biochim. Biophys. Acta, 400 (1975) 334.
8 M. Luescher, M. Rüegg and P. Schindler, Biopolymers, 13 (1974) 2489.
9 C. Betzel, G. P. Pal and W. Saenger, Acta Crystallogr., Sect. B: Struct. Sci., 44 (1988) 163.
10 J. A. Dean (ed.), Lange's Handbook of Chemistry, McGraw-Hill Book Company, New York 1985 (13th ed.), P. 10–91.
11 L. P. Witnauer and J. G. Fee, J. Polymer Sci., 26 (1957) 141.
12 P. L. Privalov and N. N. Khechinashvili, J. Mol. Biol., 86 (1974) 665.
13 F. A. Gunkel and G. Gassen, Eur. J. Biochem., 179 (1989) 185.
14 M. Oobatake and T. Ooi, Prog. Biophys. Mol. Biol., 59 (1993) 237.
15 J. K. Dattagupta, T. Fujiwara, E. V. Grishin et al., J. Mol. Biol., 97 (1975) 267.
16 W. Ebeling, N. Hennrich and M. Klochow, Eur. J. Biochem., 47 (1974) 91.
17 J. Bajorath, W. Saenger and G. P. Pal, Biochim. Biophys. Acta, 954 (1988) 176.
18 J. P. Dick and A. Nordwig, Arch. Biochem. Biophys., 117 (1966) 466.