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Heat denaturation of pepsinogen in a water–ethanol mixture

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Abstract The effect of ethanol and pH on thermodynamic parameters and cooperativity of pepsinogen heat denaturation was studied by scanning microcalorimetry. Addition of 20% ethanol decreases the protein denaturation temperature by 10.7°C at pH 6.4 and 15.8°C at pH 8.0. It also decreases the denaturation heat capacity increment from 5.8 to 4.2 kcal/K·mol. The dependences of calorimetric denaturation enthalpy on denaturation temperature both in water and 20% ethanol are linear and intersect at about 95°C. In 20% ethanol the pH shift from 5.9 to 8.0 results in a decreased number of cooperative domains in pepsinogen. This process causes no changes either in the secondary structure or in the local surroundings of aromatic amino acids. It is concluded that ethanol addition does not affect the cooperativity of pepsinogen denaturation substantially until the pH change provokes redistribution of charges in the protein molecule.

Key words: Pepsinogen; Heat denaturation; Ethanol; Scanning microcalorimetry

1. Introduction

It was shown earlier that alcohols decrease both protein denaturation temperature [1–5] and the difference of heat capacity between native and denatured states [3–5], and in some cases alter cooperativity of thermal denaturation process [2,4]. As a rule, these results were obtained for small globular proteins, the thermal denaturation of which in an aqueous medium followed the two-state transition model.

We have shown that 20% ethanol decreases the denaturation temperature of pepsin compared to that in aqueous solution, but has no effect on the calorimetric enthalpy of denaturation and the number of energetical domains (the regions melting according to the two-state model) [6]. This fact was observed only for neutral pH values at which the protein was not active. In 20% ethanol a pH decrease from 6.5 to 2.0 resulted in a decrease in the number of energetical domains in pepsin from four to two [6]. To determine the mechanism of ethanol effect on the thermal denaturation parameters at different pH values we have chosen to use pepsinogen, an inactive precursor of pepsin, differing from it by an additional positively charged 44-residue N-terminal prosegment. The polypeptide chain of the pepsin portion of pepsinogen folds in a fashion that is highly homologous to that of the active pepsin molecule, forming a similar hydrophobic core [7,8]. Acidification of the solution below pH 5 results in pepsinogen conversion to pepsin. At pH values higher than 8.5, pepsinogen denatures [9]. For this reason we have studied the ethanol effect on thermal denaturation of pepsinogen within the pH range 5.9–8.2.

2. Materials and methods

Grade I-S porcine pepsinogen, chromatographically free of pepsin activity, was purchased from Sigma (24F-8090). Protein solutions were prepared in 5 mM phosphate buffer or 20% (v/v) ethanol in buffer and, prior to calorimetric experiments, they were dialyzed against solvent for 24 h at 4°C. In the presence of ethanol, the reported pH values correspond to the apparent pH values [4]. Protein concentration was determined spectrophotometrically at 278 nm using the molar extinction coefficient of 51,700 $M^{-1} \cdot cm^{-1}$ [10].

Calorimetric measurements were carried out with a differential scanning microcalorimeter DASM-1M (NPO Biopribor, Pushchino, Russian Federation) in 1 ml cells at a heating rate of 1 K/min (in some experiments a heating rate of 2 K/min was used). Protein concentration varied from 0.3 to 0.9 mg/ml. The reversibility of heat denaturation was determined as a percentage ratio between the denaturation heat of a protein in its reheating after cooling and in the course of its first heating. Denaturation temperature (T_d), calorimetric denaturation enthalpy (ΔH_{cal}) and effective or van 't-Hoff denaturation enthalpy (ΔH_{eff}) were determined as described in [10]. To analyze functions of excess heat capacity we used the software package THERMCALC developed at the Institute of Protein Research (Pushchino, Russian Federation) [11].

Circular dichroism (CD) spectra were measured on a Jasco J-500A dichrograph (Japan). The results were expressed in units of ellipticity for the mean amino acid residue assuming its average mass for pepsinogen to be equal to 107. The calculation of protein secondary structure, based on CD spectra analysis, was carried out according to the method described in [12,13].

3. Results and discussion

Fig. 1 shows temperature dependences of pepsinogen excess heat capacity at pH 6.4 in aqueous solution and 20% ethanol. Transition parameters in aqueous solution coincided with those published in [10] and practically did not change upon a heating rate increase up to 2 K/min. The reversibility of denaturation was about 80–90% at all studied pH values and was the same in the presence of ethanol. As is seen from Fig. 1, ethanol addition has no effect on the shape of calorimetric curve, but results in the denaturation peak shift towards lower temperatures. The latter agrees with the results obtained for pepsin [6], ribonuclease A [1,2], lysozyme [3], cytochrome *c* [4] and ubiquitin [5]. It has been suggested that alcohols destabilize proteins by weakening hydrophobic interactions between non-polar residues as well as by disturbing the specific water structure around the protein molecule [3].

The results of quantitative analysis of calorimetric curves of pepsinogen melting within the pH range 5.9–8.2 are given in Table 1. Addition of ethanol lowers pepsinogen T_d, and the difference in T_d for aqueous and water–ethanol solutions becomes greater as pH increases (Fig. 2), i.e. it correlates with the distance from the protein isoelectric point (3.7 [9]). Without

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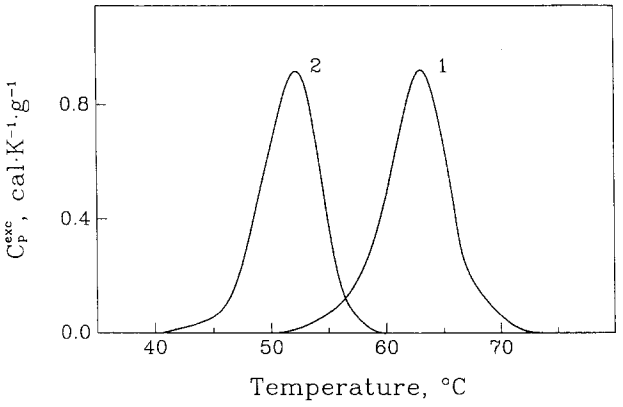

Fig. 1. Transition excess heat capacity vs. temperature for pepsinogen at pH 6.4: (1) 5 mM Na-phosphate buffer; (2) 5 mM Na-phosphate buffer, 20% ethanol.

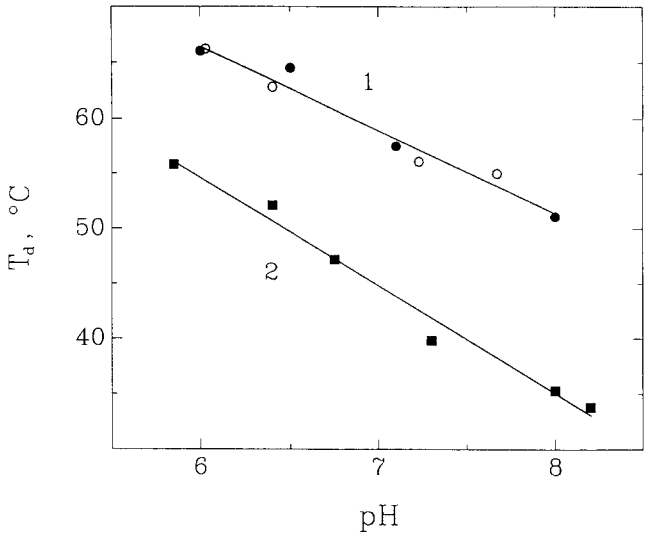

Fig. 2. pH dependence of the denaturation temperature of pepsinogen in aqueous solution (1) and 20% ethanol (2). (○) Data from [10].

ethanol, a T_d shift per one pH unit is 7.7°C, whereas in the presence of ethanol this value is equal to 10°C. In water–ethanol solutions water is bound to both protein and alcohol molecules. For this reason the dielectric constant of the medium decreases in comparison to that of aqueous solution. Thus, in ethanol all kinds of charge interactions are stronger. As a result, along with the shift from the protein isoelectric point, intraglobular charge repulsion in ethanol will be stronger compared to that in water. This is what could be seen in the case of T_d dependence on pH (Fig. 2). So, ethanol stimulates an increased electrostatic energy contribution to the total interaction energy.

Unlike denaturation temperature, the pepsinogen denaturation enthalpy in the presence of ethanol is practically the same as in aqueous solution at the same pH values (Table 1). The effect of ethanol on pepsin [6] and methanol on ribonuclease A [2] is similar. The ΔH_{cal} in both aqueous and water–ethanol solutions appears as a linearly increasing function of T_d (within the studied pH range; Fig. 3), and extrapolation of these dependences allows us to determine the intersection temperature at about 95°C. This value is close to the temperature of convergence of $\Delta H_{cal}/T_d$ dependence for different globular proteins in aqueous solutions and for cytochrome *c* at different methanol concentrations [4]. As is seen from Fig. 3, for pepsinogen, like for cytochrome *c* [4], the denaturation enthalpy at the same denaturation temperatures is lower in water than in ethanol solution. The slope of the dependence of calorimetric enthalpy on denaturation temperature coincides with the denaturation heat capacity increment ($\Delta_d C_p$) determined directly from the calorimetric curve, and is equal to 5.8 ± 0.4 kcal/K·mol for aqueous solution and to 4.2 ± 0.4 kcal/K·mol for 20% ethanol, which is 1.4 times lower. A $\Delta_d C_p$ decrease upon alcohol addition was also observed for pepsin [6] and some other proteins [3–5].

The conformation of pepsinogen was analysed by a new method of protein secondary structure determination from experimental CD curves [12,13]. The method accounts for the contribution of aromatic and dicarbonic residues to CD spectra in the peptide region and hence allows us to follow secondary structure alterations upon changes of pH, temperature, solvent and upon amino acid substitutions. Fig. 4 presents the

Table 1
Parameters of pepsinogen thermal denaturation at different pH values (5 mM Na-phosphate buffer)

Conditions	T_d (°C)	ΔH_{cal} (kcal/mol)	ΔH_{eff} (kcal/mol)	R^a
pH 6.0	66.2	254	132	1.9
pH 6.4	62.8	248	131	1.9
pH 6.4[b]	64.5	269	132	2.0
pH 7.2	56.1	195	107	1.8
pH 7.7	55.0	173	101	1.7
pH 8.0[c]	51.1	182	122	1.5
pH 5.9, 20% ethanol	55.8	245	106	2.3
pH 6.4, 20% ethanol	52.1	248	118	2.1
pH 6.4[b], 20% ethanol	53.0	260	131	2.0
pH 6.8, 20% ethanol	47.2	221	108	2.0
pH 7.3, 20% ethanol	39.8	192	116	1.7
pH 8.0, 20% ethanol	35.3	169	125	1.4
pH 8.2, 20% ethanol	33.8	170	125	1.4

The relative error of the given ΔH_{cal} and ΔH_{eff} values did not exceed ±6%; the absolute error of the given T_d values did not exceed ±0.4°C.
[a] $R = \Delta H_{cal}/\Delta H_{eff}$.
[b] Heating rate 2 K/min.
[c] Data from [10].

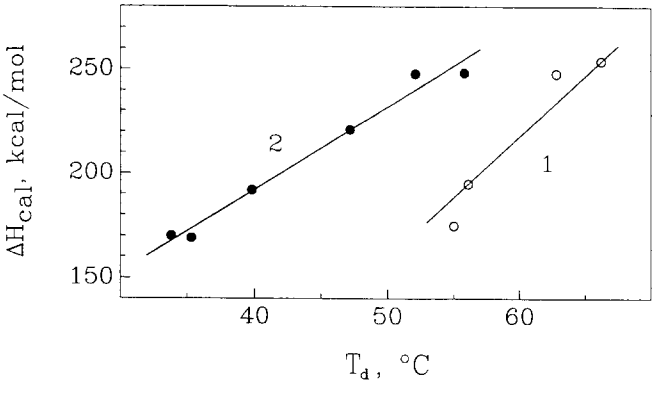

Fig. 3. Dependence of pepsinogen calorimetric denaturation enthalpy on denaturation temperature for aqueous solution (1) and 20% ethanol (2).

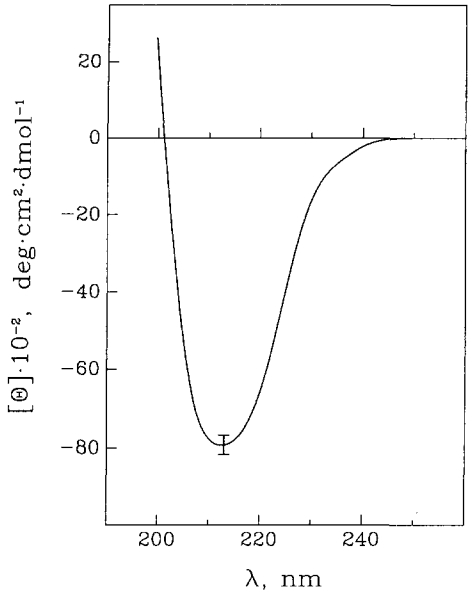

Fig. 4. CD spectrum of pepsinogen in the far UV region at pH 6.4 (5 mM Na-phosphate buffer).

pepsinogen CD spectrum in the far UV region at pH 6.4 and Table 2 gives the determined secondary structure. Within the pH range 6–8, as well as after addition of 20% ethanol, no changes in CD spectra were observed. The calculated secondary structure composition coincides well with the X-ray data (Table 2). The assignment of secondary structure regions according to the 'rigid' method [12] was carried out using two sets of refined atomic coordinates of pepsinogen that are available from the Brookhaven Protein Data Bank [15,16]. The pepsinogen CD spectra in the near UV region were also independent of pH and the presence of ethanol.

The effect of ethanol addition on thermodynamic parameters of pepsinogen denaturation is similar to that found earlier for a number of proteins [1–5]. However, the heat denaturation of these comparatively small proteins follows a two-state transition model, whereas the pepsinogen melting is characterized by the ratio $R = \Delta H_{cal}/\Delta H_{eff}$ close to 2, i.e. by a relatively independent melting of two cooperative units [10]. We have shown earlier that the addition of 20% ethanol does not affect the number of energetical domains in pepsin, but a change in pH of the water–ethanol mixture has a strong effect on this number [6].

To study the effect of a change in the solvent hydrophobicity on the pepsinogen energetical domains, we have compared the criterion for the heat denaturation cooperativity (R) in the presence of ethanol and without it (Table 1). It is evident that at equal pH values, the R ratio for a water–ethanol mixture, as a rule, is close to that for aqueous solutions. Hence, we can conclude that the number of energetical domains in pepsinogen (as in pepsin [6]) does not change upon ethanol addition.

However, as was shown in [2,6,14], the number of energetical domains may be changed following a pH shift. A similar conclusion can be drawn for pepsinogen (Table 1). An increase in pH of the aqueous solution results in an R decrease from 1.9 at pH 6.0 to 1.5, at pH 8.0. A decrease in R is more prominent upon ethanol addition: from 2.3 at pH 5.9 to 1.4 at pH 8.0. These results suggest that the number of energetical domains in pepsinogen is pH dependent and hence is specified by the electrostatic potential of the globule. It should be noted that the decrease in the number of energetical domains in pepsin occurs as the pH approaches the protein's isoelectric point [6], whereas in pepsinogen, in contrast, this takes place upon moving away from it. Probably, this can be explained by an essential difference in the charge distribution in the enzyme and its zymogen, due to the presence of an additional polypeptide chain fragment in pepsinogen, carrying a strong positive charge.

In the presence of ethanol the dependence of R on pH is significantly higher than in its absence, 0.43/pH unit and 0.2/pH unit, respectively. This correlates with the data on ribonuclease A melting in 50% methanol, which is a two-state transition at pH 3.8 and above [2]. However, a decrease in pH of the water–methanol mixture to 2.1 results in the appearance of two heat absorption peaks on the melting curve, each peak corresponding to the single cooperative transition in one of the two ribonuclease A domains [2].

We consider that changes in solvent dielectric properties, caused by ethanol, do not affect the cooperativity of pepsinogen denaturation substantially until the pH shift provokes redistribution of charges in the protein molecule. Ethanol enhances charge interactions in pepsinogen and makes thermostability (T_d) and cooperativity (R) parameters more sensitive to pH.

Table 2
Determination of pepsinogen secondary structure using CD spectra (pH 6.4) and X-ray ternary structure [15,16]

	f_α	f_β	$f_{\beta\text{-turn}}$	f_u
CD spectra analysis[a]	0.15	0.30	0.17	0.38
X-ray structure analysis[b]	0.15	0.32	0.16	0.37

Molar fractions of amino acid residues in α-helices (f_α), β-structures (f_β), β-turns ($f_{\beta\text{-turn}}$) and in unordered regions (f_u).
[a]The relative error of the given values did not exceed ± 10%.
[b]The assignment of secondary structure using X-ray data was carried out according to the 'rigid' method [12].

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References

[1] Schrier, E.E. and Scheraga, H.A. (1962) Biochim. Biophys. Acta 64, 406–408.
[2] Brandts, J.F., Hu, C.Q., Lin, L.N. and Mas, M.T. (1989) Biochemistry 28, 8588–8596.
[3] Velicelebi, G. and Sturtevant, J.M. (1979) Biochemistry 18, 1180–1186.
[4] Fu, L. and Freire, E. (1992) Proc. Natl. Acad. Sci. USA 89, 9335–9338.
[5] Woolfson, D.N., Cooper, A., Harding, M.M., Williams, D.H. and Evans, P.A. (1993) J. Mol. Biol. 229, 502–511.
[6] Makarov, A.A., Protasevich, I.I., Frank, E.G., Grishina, I.B., Bolotina, I.A. and Esipova, N.G. (1991) Biochim. Biophys. Acta 1078, 283–288.
[7] Sielecki, A.R., Fedorov, A.A., Boodhoo, A., Andreeva, N.S. and James, M.N.G. (1990) J. Mol. Biol. 214, 143–170.
[8] Sielecki, A.R., Fijinaga, M., Read, R.J. and James, M.N.G. (1991) J. Mol. Biol. 219, 671–692.
[9] Northrop, J.H., Kunitz, M. and Herriot, R.M. (1948) Crystalline Enzymes, 2nd ed., Columbia University Press, NY.

[10] Privalov, P.L., Mateo, P.L., Khechinashvili, N.N., Stepanov, V.M. and Revina, L.P. (1981) J. Mol. Biol. 152, 445–464.
[11] Filimonov, V.V., Potekhin, S.A., Matveyev, S.V. and Privalov, P.L. (1982) Molekulyarnaya Biologiya (Moscow) 16, 551–562.
[12] Bolotina, I.A. and Lugauskas, V.A. (1985) Molekulyarnaya Biologiya (Moscow) 19, 1409–1421.
[13] Morozov, Yu.V., Chekhov, V.O. and Bazhulina, N.P. (1993) Molekulyarnaya Biologiya (Moscow) 27, 1032–1038.
[14] Protasevich, I.I., Platonov, A.L., Pavlovsky, A.G. and Esipova, N.G. (1987) J. Biomol. Struct. Dynamics 4, 885–893.
[15] Hartsuck, J. and Remington, S. Set 1PSG in Brookhaven Protein Data Bank.
[16] Sielecki, A.R. and James, M.N.G. Set 2PSG in Brookhaven Protein Data Bank.