Journal of Thermal Analysis and Calorimetry, Vol. 62 (2000) 317–324

EFFECT OF SOLUTION pH AND COMPOSITION ON HORSE LIVER ALCOHOL DEHYDROGENASE THERMOSTABILITY

B. N. Wang, B. X. Han and F. Tan

Center for Molecular Science, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, China

(Received April 8, 1999; in revised form January 14, 2000)

Abstract

The influence of solution composition (pH, salts, and chelant) on the thermostability of horse liver alcohol dehydrogenase was studied by differential scanning calorimetry (DSC) in the pH range from 7.51 to 9.50 and showing the enzyme catalytic activity. The experiments demonstrated that the effect of increasing pH on the heat denaturation temperature of the enzyme was slight, but the denaturation enthalpy was considerably increased, indicating the enzyme conformation alteration by changing pH and the presence of enthalpy-entropy compensation. The effect of ionic strength on thermostability was not noticeable, i.e., the electrostatic interactions were not a dominant factor for the thermostability. The anions Cl^- and SCN^- imposed diverse influence upon the enzyme thermostability, and SCN^- can reduce the thermostability considerably. The chelant 1,10-phenanthroline, which can reversibly bind together with the zinc ions functioning the catalytic action in the enzyme molecules, increases the thermostability considerably. The hydration of the enzyme plays an important role to the thermostability.

Keywords: alcohol dehydrogenase, differential scanning calorimetry, heat denaturation, thermostability

Introduction

The horse liver alcohol dehydrogenase (HLADH) is an important enzyme among the alcohol dehydrogenases, its thermostability is much higher than the yeast alcohol dehydrogenase. It is a dimer enzyme composed of two identical subunits, each of them contains two zinc atoms. One zinc performs the catalytic function in the catalytic domain of a subunit, and causes various primary and secondary alcohols to be oxidized into corresponding aldehydes and ketones and vice versa, and the other zinc exists in the coenzyme-binding domain [1]. Recently, it has been used in asymmetric syntheses because its high specificity and stereospecificity for the substrates [2]. Different techniques have been used to investigate the thermostability of the enzyme, such as calorimetry [3], phosphorescence [4], fluorescence and NMR [5], EPR [6].

1418–2874/2000/ $ 5.00

Akadémiai Kiadó, Budapest
Kluwer Academic Publishers, Dordrecht

Calorimetry can give a detailed picture of heat denaturation of an enzyme and its thermodynamic parameters. However, the only calorimetric study of the thermostability of HLADH was performed at pH 8.1 [3]. In this work, the effect of pH, ionic strength, Cl^- and SCN^-, and chelant 1,10-phenanthroline on the thermostability of HLADH was studied by DSC.

Experimental

Reagents

The HLADH (EC1.1.1.1.) (A6128, Lot 86H7010) used was purchased from the Sigma Chemical Co., possessing the activity 1.6 U mg^{-1} protein with a purity of >98%. It was used without purification. Backing for 3.5 h at 105°C, and then immediately putting into a desiccator with anhydrous $Mg(ClO_4)_2$, re-weighing on the next day, 6.8 mass% water content of the enzyme was obtained. The dry mass of the enzyme was calculated using the water content. The reagents, Na_2HPO_4, NaCl, NaSCN, 1,10-phenanthroline, NaOH, and H_3PO_4 used were A.R. grade or better, and the NaH_2PO_4 was C.P. grade.

Instrumental

The instruments used were a Model DSC-2C differential scanning calorimeter equipped with a Model 3700 data station and a model AD-2Z electromagnetic supermicrobalance, all of them were supplied by Perkin Elmer Company. The calorimetric system was calibrated by indium and lead of high purity before used. Its thermometric accuracy was ±0.1 K, the enthalpy-determining relative accuracy was ±1%. Upon checking by a standard mass, the weighing error of the AD-2Z balance was ±0.01 mg or less.

The acidimeter used was a Model SPM-10A digital pH meter from Xiaoshan Instrument Factory. Upon calibrating by a set of three pH standard chemicals from Shanghai Aijian Chemical Factory, the pH-determining error at room temperature was ±0.01 pH.

Method

The various solutions containing 0.1 mol dm^{-3} sodium phosphate were prepared by normal methods. The pH value of each solution was adjusted to the required value at room temperature by H_3PO_4 or NaOH. The white downlike solid enzyme about 2.37 mg and 35 μl of required solution were mixed in a stainless steel capsule, immediately sealed by means of a Viton O-ring and a special sealing tool, and then maintained at room temperature overnight. Then the DSC determination was performed. The reference was the same type of stainless steel capsule containing 35 μl of 0.1 mol dm^{-3} sodium phosphate buffer at pH 7.99. All the DSC determinations were performed in a flow of N_2 gas at heating rate 10 K min^{-1}.

Results and discussion

The results of the DSC determinations of HLADH in various solutions are summarized in Table 1. Some typical DSC curves are shown in Fig. 1.

Table 1 The DSC determination results of HLADH in various solutions

m/mg	Solution composition	pH	T_i/K	T_p/K	T_f/K	ΔH/J g^{-1}
2.19	A	7.51	342.97	356.57	363.88	6.91
2.21	A+0.01 mol dm^{-3} OP	7.51	354.19	366.57	373.70	4.40
2.21	A	7.99	342.65	354.68	365.12	7.59
2.21	A	8.41	342.35	356.28	363.42	7.86
2.22	A+0.5 mol dm^{-3} NaCl	8.41	341.43	356.60	363.90	7.77
2.22	A+0.5 mol dm^{-3} NaSCN	8.41	339.07	348.66	359.67	5.25
2.21	A+0.5 mol dm^{-3} NaSCN	8.41	329.25	338.28	353.59	3.64
2.22	A	9.01	341.73	354.53	363.42	8.62
2.22	A	9.50	342.21	354.55	365.15	10.2

where m represents the dry mass of the enzyme reduced by its water content; A is 0.1 mol l^{-1} sodium phosphate solution; OP represents 1,10-phenanthroline

In Table 1, T_i is the initial temperature of an endothermic peak of heat denaturation; T_p is the peak temperature of the peak and taken as the heat denaturation temperature; T_f is the end temperature of the peak; ΔH is the specific denaturation enthalpy calculated by the dry mass of the enzyme. All the samples were heated from 290.0 to 376.5 K or higher, and then cooled down to 290.0 K at the rate 10.0 K min^{-1}. When the cooled samples were re-heated, the DSC curves without any peak were obtained. Therefore, the heat denaturations of the enzyme were irreversible. The results shown in Table 1 and Fig. 1 were the determined results in the first heating.

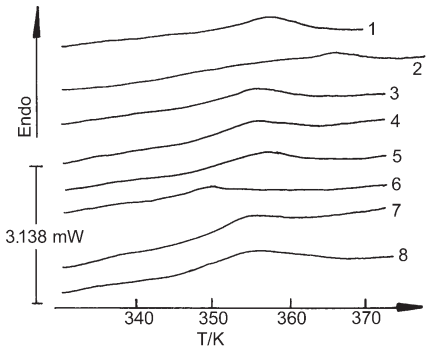

Fig. 1 Typical DSC curves of HLADH in various solutions. The solution compositions corresponding to the curves are as follows: 1 – 0.1 mol dm^{-3} sodium phosphate (A) at pH 7.51; 2 – (A+0.01 mol dm^{-3} OP) at pH 7.51; 3 – A at pH 7.99; 4 – A at pH 8.41; 5 – (A+0.5 mol dm^{-3} NaCl) at pH 8.41; 6 – (A+0.2 mol dm^{-3} NaSCN) at pH 8.41; 7 – A at pH 9.01; 8 – A at pH 9.50, respectively

It may be seen from Table 1 and Fig. 1 that as far as the solutions containing only sodium phosphate are concerned, the heat denaturation peaks of HLADH in these solutions occurred in the nearly same temperature range. The differences between the heat denaturation temperatures represented by T_p are about 2 K or less, but ΔH increases steadily when the pH values of these solutions are increased from 7.51 to 9.50. These facts imply that the pH change results in the alteration of enzyme conformation, thereby ΔH is increased. However, the denaturation entropy changes with the alteration of enzyme conformation. As far as the contribution to the denaturation free energy is concerned, the change of denaturation entropy caused by the pH change compensates largely for the change of denaturation enthalpy. Therefore the denaturation temperatures are nearly the same. Recently, Lee [7] studied the enthalpy–entropy compensation in the transfer process of hydrophobic molecules from non-aqueous phase to aqueous phase, and indicated that most subprocesses involved in the transfer process of hydrophobic molecules are the enthalpy-entropy compensation processes, and that the thermodynamic state functions are composed of the uncompensating parts and compensating parts, and as concerns the compensating parts of the thermodynamic state functions (marked by the superscript c) there is a relationship $\Delta G^c=\Delta H^c-T\Delta S^c\approx 0$. According to these results it may be deduced that one reason of the enthalpy–entropy compensation in the heat denaturation processes of HLADH observed by the pH change may be attributed to the exposure of the hydrophobic residues from the interior of hydrophobic nuclei (seen as non-aqueous phase) to the aqueous solution during the unfolding of the polypeptide chains of HLADH, i.e. the transfer to the aqueous phase. Furthermore, it may be said that the changes of denaturation enthalpy and denaturation entropy ($\Delta\Delta H$ and $\Delta\Delta S$) are caused by the pH change, these portions of variation stem from the compensating parts of the corresponding thermodynamic state functions. Although the denaturation temperatures are nearly the same when the pH values of the solutions containing only sodium phosphate are changed, it may be seen that the T_p value at pH 7.99 is less than those at pH 7.51 and 8.41 by about 2 K if the data in Table 1 are compared more meticulously. Skerker *et al*. [6] studied the HLADH from the Sigma Chemical Company and indicated that 96% of the enzyme is E type of isoenzymes and the isoelectric point of the major component of the E type enzyme is 8.0. Therefore it may be said that the total number of positively-charged side chains is not equal to that of negatively-charged side chains in most enzyme molecules when the pH value is larger or less than 8.0, and that the molecules with non-zero net charge are slightly more stable than the molecules with zero net charge. This phenomenon, the stability before and after the isoelectric point being different from the stability at the isoelectric point, was recorded for the gelatin and casein [8]. The sol of gelatin is more stable at its isoelectric point, but the sol of casein is less stable at its isoelectric point. Therefore the thermostability of HLADH before and after the isoelectric point of its major component is slightly higher, this behaviour is similar to that of casein, which is a weakly hydrophilic protein. On either side of the isoelectric point of casein the zeta-potential, which exists in the electric double layers on the surface of the casein molecules with non-zero net charge, and the full hydration of casein molecules caused by the

zeta-potential are the cause of casein sol being more stable than at its isoelectric point. It appears that this explanation is also suitable to HLADH.

As far as the metalloenzymes are concerned, anion-binding is significant in biological functions. Andersson *et al*. [9] reported that binding of Cl^- with HLADH, at pH 8.4 and the concentration of 0.5 mol dm^{-3} Cl^-, results in the variation of ^{35}Cl NMR spectrum. Recently, Przybycien *et al*. [10] found the perturbations of the secondary structure of HLADH induced by KSCN. In the present work, the influences of 0.5 mol dm^{-3} NaCl and 0.2 and 0.5 mol dm^{-3} NaSCN on the denaturation curve of HLADH were studied at pH 8.41. It can be seen from the curves 4 and 5 in Fig. 1 and the relevant data in Table 1 that the shape, position and area of denaturation peak in the 0.5 mol dm^{-3} NaCl solution are nearly the same as those in the solution without NaCl. This fact indicates that the chloride ions almost do not influence the thermostability of HLADH, although they can be bound into the coenzyme-binding domain of HLADH [9]. Interestingly, the T_p value of HLADH obtained by Koch-Schmidt [3] was 82.5°C in 0.1 mol dm^{-3} NaCl solution at pH 8.1. Their T_p value and ours are very close when the T_p value at pH 7.99 is interpolated to that at pH 8.1. This fact shows that in the presence of 0.1 and 0.5 mol dm^{-3} NaCl the ionic strength is changed considerably, but the influence on the thermostability of HLADH is slight. This also means that the electrostatic interaction is not a dominant factor for the thermostability of HLADH. In addition, the binding of Cl^- with the positively charged Arg-47 and Arg-271 positioned in the coenzyme-binding domain of HLADH [9] almost does not influence the thermodynamic parameters of heat denaturation of HLADH in this work. The curves 4 and 6 in Fig. 1 and the relevant data in Table 1 show that the heat denaturation peak of HLADH shifts drastically towards the lower temperature side and the peak area decreases considerably in the presence of 0.2 or 0.5 mol dm^{-3} NaSCN. Przybycien *et al.* [10] found that β sheet content in HLADH precipitated by KSCN is larger than that in the native structure of HLADH and α helix content is less than that in the native structure. Therefore it may be concluded that the presence of SCN^- caused the drastic change of HLADH molecular conformation, and this influence increases with the increase of SCN^- concentration. The anion SCN^- is an anion capable of depressing drastically the thermostability of HLADH. This effect of SCN^- is completely different from that of Cl^-. This is in agreement with the position order in the well-known Hofmeister series of anions. Cl^- is a kosmotrope, SCN^- is a chaotrope. In aqueous solutions, the protein conformation is dependent on the water structure. In Hofmeister series, the ability of SCN^- to break water structure is much higher than that of Cl^- [11]. The effects of the two anions on the thermostability of HLADH are different, mainly because their influences on the water structure are different. On the other hand, Syvertsen *et al*. [12] stated that the anion SCN^- could bind with HLADH and the binding site was not the catalytic zinc ion, but rather was the anion-binding site containing Arg-47. On the basis of our results, it can be known that the influence of the binding of SCN^- on the thermostability of HLADH is neither to be ruled out nor to be negligible.

The chelant 1,10-phenanthroline (OP) and some metalloenzymes containing zinc can form the protein–Zn–OP binary complexes and cause these enzymes to inac-

tivate. The investigation by Drum *et al*. [13] showed that at most two OP molecules bind with the two catalytic zinc ions existing in a native dimer molecule of HLADH, and thereby cause its reversible inactivation. Therefore the thermal inactivation method studying the thermostability is not applicable to the inactive binary complexes. Under the conditions similar to Drum's and reaching to the saturation binding, the influence of OP-binding on HLADH thermostability was observed by DSC in this work. Curves 1 and 2 in Fig. 1 and relevant data in Table 1 show that OP-binding causes the heat denaturation peak of HLADH to be removed towards the higher temperature side, the peak area to be decreased significantly. The heat denaturation temperature of HLADH was raised by about 10 K due to OP-binding. In contrast to this, Nath *et al*. [14] used 44.44 mass% of sucrose to raise the heat denaturation temperature of the yeast alcohol dehydrogenase from 63 only to 70°C. Therefore OP is a very effective thermostabilizing agent.

Any protein in aqueous solution exists in the hydrated state. As mentioned above the water-structure is very momentous to the thermostability of HLADH. In order to know thermodynamically the action of water as solvent, we use the following thermodynamic cycle

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

where N represents the HLADH molecules in native state; D the HLADH molecules in denatured state; ΔH_d is the denaturation enthalpy determined by calorimetry; ΔH_N^h and ΔH_D^h are the hydration enthalpies of HLADH in native and denatured states respectively; ΔH_d^c is the denaturation enthalpy of HLADH in non-hydrated state, which is calculated by the thermodynamic cycle. ΔH_d^c represents the enthalpy change caused by the change of various interactions in the interior of HLADH molecules before and after the denaturation. It is the enthalpy change stemming from the alteration of HLADH molecular conformation. Obviously, the following relationship exists

$$\Delta H_d = \Delta H_d^c + (\Delta H_D^h - \Delta H_N^h) = \Delta H_d^c + \Delta H_d^h \quad (1)$$

Therefore the denaturation enthalpy ΔH_d determined by calorimetry comprises the difference between the hydration enthalpies in native state and in denaturated state, in addition to the contribution ΔH_d^c from the conformation alteration of HLADH molecules. This difference between the hydration enthalpies represents quantitatively the influence of water molecules in the denaturation process of HLADH. At present the quantity ΔH_d^h can not be determined in experiment. This is due to that it is not practicable the enzyme conformation to be unchanged during dehydration. As mentioned above, 96% of the HLADH from the Sigma is the enzyme of E type. On the basis of the primary structure of the HLADH E chain given by Jornvall [15] and the hydration enthalpies and the increments of the hydration heat capacities

of 20 amino acid residues on unfolding in Ooi's Table 8 [16], we calculated the difference of hydration enthalpies ΔH_d^h of a HLADH E chain on unfolding at heat denaturation temperature by use of the additivity method of thermodynamic quantities of amino acid residues. Because the Ser in the N-terminal and the Phe in the C-terminal had exposed to water before unfolding [1], the contributions from the two residues were not considered when the additivity methods was used. From the data at pH 7.99 in Table 1, the heat denaturation of HLADH is at 81.5°C, the heat denaturation enthalpy ΔH_d is 303.6 kJ mol^{-1} E chain (the mole mass of one E chain, i.e. one subunit is 40000 [1]). According to the primary structure of the E chain and the thermodynamic parameters of hydration of amino acid residues, it may be obtained by the calculation that ΔH_d^h= – 4841 kJ mol^{-1} E chain at 81.5°C.

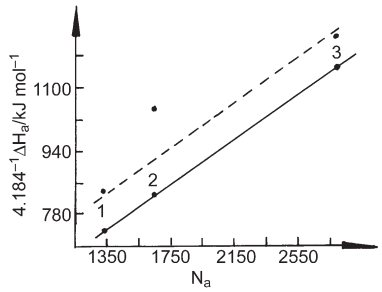

Fig. 2 Dependence of the two components ΔH_d^c and $|\Delta H_d^h|$ of heat denaturation enthalpy on the number N_a of heavy atoms

Therefore $\Delta H_d^c = \Delta H_d - \Delta H_d^h$=5145 kJ mol^{-1} E chain. There are 374 amino acid residues in one E chain of HLADH [15], and its total number of heavy atoms, C and N and O and S atoms, are 2788 (including the acetyl in N-terminal). These calculated values and the calculated results for the two globular proteins (single chain), the phage T_4 lysozyme (2LZM) and the papain (8PAP), are presented in Fig. 2. In the Figure, in the vertical direction with the same number of heavy atoms, the ordinate value of the point situated above is the numerical value of ΔH_d^c, the ordinate value of the point situated below is the absolute value of ΔH_d^h. The upper and lower points marked by 1 correspond to the data of 2LZM, the points marked by 2 the data of 8PAP, and the points marked by 3 the data of HLADH. From the solid line in Fig. 2 it may be seen that the ΔH_d^h's of the three proteins are linearly related with their numbers Na's of heavy atoms. This result shows that the influence of hydration on the heat denaturation of HLADH is similar to that of other single chain globulins and all follow the linear relationship from Ooi hydration model [16]. The $|\Delta H_d^h|$ of HLADH is larger, this is due to that the number of heavy atoms in one polypeptide chain of HLADH is more and is not a representation of the presence of other particular interactions on water molecules. The dashed line shows that the average strengths of the interactions in the interior of HLADH and 2LZM polypeptide chains are similar to each other. It may be inferred that there are more sites of strong interaction within the papain polypeptide chains and thereby the average strength of the interactions within

its polypeptide chains is larger. Therefore, the point describing the ΔH_d^c value of papain is highly over the dashed line.

The project is supported by National Natural Science Foundation of China (No 29633020).

References

1 H. Eklung, B. Nordstrom, E. Zeppezauer, G. Söderlund, I. Ohlsson, T. Boiwe, B. Söderberg, O. Tapia and C. Brändén, J. Mol. Biol., 102 (1976) 27.
2 C. Hertweck and W. Boland, J. Prakt. Chem./Chem.-Ztg., 339 (1997) 754.
3 A.-C. Koch-Schmidt and K. Mosbach, Biochemistry, 16 (1977) 2101.
4 M. Gonnelli and G. B. Strambini, Biophys. Chem., 24 (1986) 161.
5 Z. Wasylewski, P. Sucharski, A. Wolak and M. R.Eftink, Biochim. Biophys. Acta, 913 (1987) 210.
6 P. S. Skerker and D. S. Clark, Biotechnol. Bioeng., 33 (1989) 62.
7 B. Lee, Biophys. Chem., 51 (1994) 271.
8 S. Glasstone, Textbook of Physical Chemistry, Macmillan, London 1953, (2nd ed.), p. 1255.
9 I. Andersson, M. Zeppezauer, T. Bull, R. Einarsson and J.-E. Norne, Biochemistry, 18 (1979) 3407.
10 T. M. Przybycien and J. E. Bailey, Biochim. Biophys. Acta, 1076 (1991) 103.
11 P. W. Sanderson, L. J. Lis, P. J. Quinn and W. P. Williams, Biochim. Biophys. Acta, 1067 (1991) 43.
12 C. Syvertsen and J. S. McKinley-McKee, Arch. Biochem. Biophys., 228 (1984) 159.
13 D. E. Drum, J. H. Harrison, T. K. Li, J. L. Bethune and B. L. Vallee, Proc. Natl. Acad. Sci. USA, 57 (1967) 1434.
14 S. Nath, G. R. Satpathy, R. Mantri, S. Deep and J. C. Ahluwalia, Thermochim. Acta, 309 (1998) 193.
15 H. Jornvall, Eur. J. Biochem., 16 (1970) 25.
16 M. Oobatake and T. Ooi, Prog. Biophys. Molec. Biol., 59 (1993) 237.