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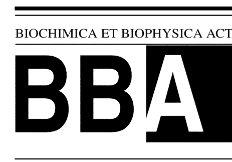

A calorimetric study of pH-dependent thermal unfolding of leghemoglobin **a** from soybean

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Abstract

The purpose of the present work is to study the pH-dependent thermal denaturation of soybean leghemoglobin fraction **a** in a cyanide complex (Lba.CN) and to compare the results with those of myoglobin (Mb), apomyoglobin (apoMb) and cyanometmyoglobin (Mb.CN) as well. Comparing measured calorimetrically change of enthalpy (ΔH_{cal}) and calculated Van't Hoff change of enthalpy (ΔH_{vh}) we have found that heat denaturation of Lba.CN can be described by the two-state transition model. The average value of the change of heat capacity (ΔC_{pd}) of Lba.CN is between such values for apoMb and Mb.CN suggesting for some stabilisation role of the cyanide ion (CN^-) on the protein molecule. The maximum change of Gibbs free energy (ΔG_{max}) of Lba.CN is between 7.0 and 11.2 kcal/mol depending on pH. The heat-denaturation of the protein occurs on heating the protein solution above 25°C while the cold-denaturation occurs on cooling the protein below 25°C. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Leghemoglobin **a**; Leghemoglobin; Soybean; Myoglobin; Differential scanning microcalorimetry

1. Introduction

The red pigment of Rhizobium-infected nitrogen-fixing legume root nodules was first characterised as an oxygen-binding hemoprotein by Kubo [1] and named leghemoglobin (Lb) by Virtanen et al. [2]. Leghemoglobin facilitates the diffusion and delivery of oxygen to the bacteroid surface at a stable low oxygen tension [3]. According to Appleby et al. [3] by an improved method for ion-exchange chromatography have been isolated and characterised six components of Lb from soybean (Lba, Lbb, Lbc_1, Lbc_2 and two Lbd components not well separated). Depending on ligand binding, the protein stability differed in the investigated pH region [4]. Cyanide ion (CN^-) makes a complex with leghemoglobin molecule, which is stable over a large pH range. Thus this complex can be investigated by means of differential scanning calorimetry. However, Myoglobin (Mb), a protein of very similar structure and function, such as their oxygen and ligand-binding properties, molecular weights (15 000–20 000) and noncovalent protein-heme linkage, has been well studied by Privalov and Kechinashvili [5]. Lb (especially Lba) has not been as thoroughly investigated by the methods of differential scanning microcalorimetry. One report

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has appeared by Makarov et al. [6], using Lb crystals from lupin.

2. Materials and methods

Leghemoglobins (Lbs) from soybean were purified by the method described by Appleby et al. [3] with some modification. The protein fraction **a** (Lba) was isolated from other Lb-isoforms using Whatman DE-52 Cellulose column (20 cm × 3 cm), equilibrated with MES buffer (10 mM, pH 5.6). All steps in leghemoglobin preparation were carried out at 4°C. A linear gradient of MES buffer (10 mM, pH 5.6 to 100 mM, pH 5.6) was applied. Lba fraction was separated between 15 mM and 27 mM of the buffer concentrations (Fig. 1A). The pooled fraction had an absorption ratio A_{560}/A_{620} of 1.7 which is characteristic of nicotinate-free ferrileghemoglobin [3]. The protein solution was concentrated in Diaflo cell over UM 10 membrane. Isoelectric focusing of chromatographic leghemoglobins demonstrated the homogeneity of Lba. For analytical IEF was used Serva Cat. No. 42919 plate with pH range 3–6 and thickness 150 μm. The plate was rolled onto cooling glass plate treated with *n*-decane as heat exchange liquid (Serva Cat. No. 18144). Anode strips were soaked in solution of 3.3 g L-aspartic acid and 3.7 g L-glutamic acid/l distilled water. Cathode strips were soaked in solution of 20 g glycine/l distilled water. The standard running conditions were applied using LKB 2117 Multiphor II unit and LKB 2197 power supply (Fig. 1B). For our calorimetric study we used Lba in water solutions of KCN and KCl in concentration 1 μM and 1 mM, respectively. The pH values of Lba.CN solutions were adjusted by adding small quantities of 0.1 M HCl or 0.1 M KOH and measured by pH-meter (Radiometer pH 83) using an INGOLD electrode. The pH region in our experiments was chosen in such a way that CN^- was bound to the Lba molecule in the pH region (pH 5.0–11.2). This was controlled by absorption spectra with respect to the absorption bands at 418 and 538 nm [7]. The protein solutions were completely equilibrated by dialysis against the same water solution during 8 h. Protein concentration (1 mg/ml) in solutions was determined by absorption measurements using the values from extinction coefficient at 418 nm ($E_{(\mathrm{mM})}$ = 119 mM^{-1}/cm). The absorption spectra were registered on SPECORD UV–VIS/C.Zeiss Jena GDR, before the calorimetric measurements.

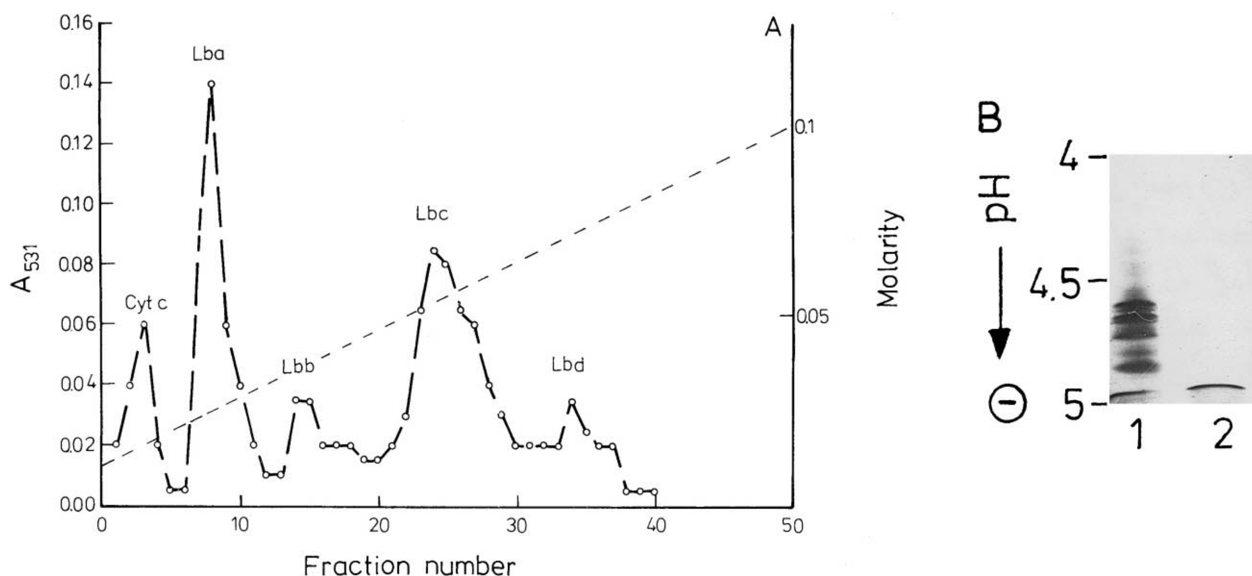

Fig. 1. (A) Elution profile, monitored at 531 nm showing the separation of soybean leghemoglobins on a DE-52-cellulose column, using a MES gradient at pH 5.6 and 4°C. (B) IEF on 150 μm Serva plate with gradient of pH 3–6. (1) Total Lb before separation on DE-52-cellulose column, (2) Lba.

Table 1
Thermodynamic characteristics of Lba.CN heat denaturation at different pH values

pH	ΔT_d (K)	ΔH_{cal} (kcal/mol)	ΔH_{vh} (kcal/mol)	$\Delta H_{vh}/\Delta H_{cal}$
5.00	342.15	109	116	1.06
5.90	350.65	130	128	0.98
7.25	352.15	136	132	0.97
8.50	352.65	132	128	0.97
9.20	354.15	133	130	0.98
9.90	342.65	109	117	1.07
11.15	338.65	104	103	1.00

ΔH_{cal} is calorimetrically measured, Van't Hoff enthalpy (ΔH_{vh}) is calculated by the sharpness of the transition ratio $\Delta H_{cal}/\Delta H_{vh}$ giving an indication for the two-state transition.

Calorimetric studies were performed with the differential scanning microcalorimeter DASM-4 (Biopribor, Puschino, Russia) equipped with two equivalent 0.4 ml platinum cells, at a scanning rate of 1°C/min. Before the experimental study of Lba.CN, a thermally induced unfolding of lysozyme was used to calibrate the instrument. The calibration results appeared to be in a good agreement to the same values reported by Ginsburg and Zolkeiwski [8].

3. Results and discussion

The thermal unfolding of Lba.CN at several different pH values between 5.0 and 11.15 was investigated by DSC. Denaturation temperature (ΔT_d), denaturation enthalpy (ΔH_{cal}) and Gibbs free energy (ΔG) were determined at various pH values. Experimental measurements were performed at low ionic strength. ΔH_{cal} values and temperature positions at ΔT_d at different pH are presented in Table 1. The temperature of denaturation changes with pH increasing up to pH 7.2 and decreasing at higher pH values (Fig. 2). The changes of the enthalpy (ΔH_{cal}) of thermal denaturation depends on pH and this relationship is shown in Fig. 3. The maximum of this curve occurs at pH 7.2. In the extreme acidic and the more alkaline regions, ΔH_{cal} decreases significantly. The coincidence obtained in the whole investigated region for calorimetrically measured ΔH_{cal} and Van't Hoff enthalpy (ΔH_{vh}) calculated by the sharpness of the transition shows that Lba.CN thermal denaturation is a two-state process which appears to be common property of small one-domain globular proteins (Table 1). Using the linear type of the enthalpy dependence (ΔH_{cal}) on the temperature of denaturation (ΔT_d) we obtain the average value $\Delta C_{pd} = 2.19$

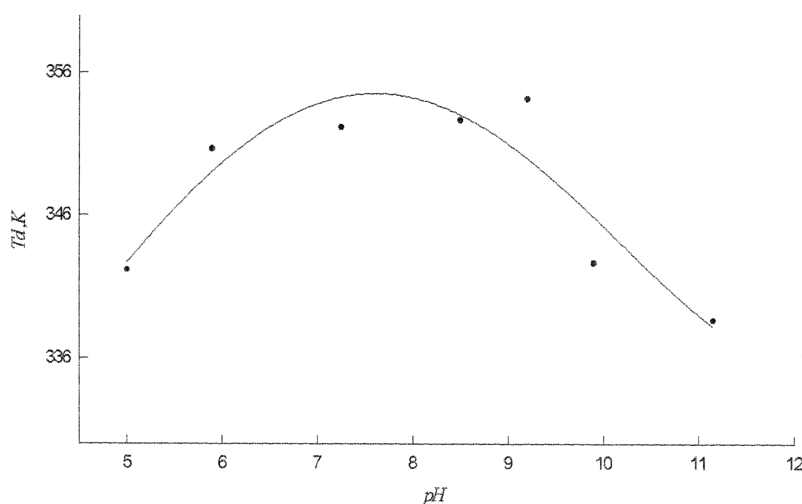

Fig. 2. pH dependence of the thermal stability (ΔT_d) of Lba.CN determined by DSC in a water solutions of 1 μM KCN and 1 mM KCl. The smooth curve through the data was obtained by non-linear regression.

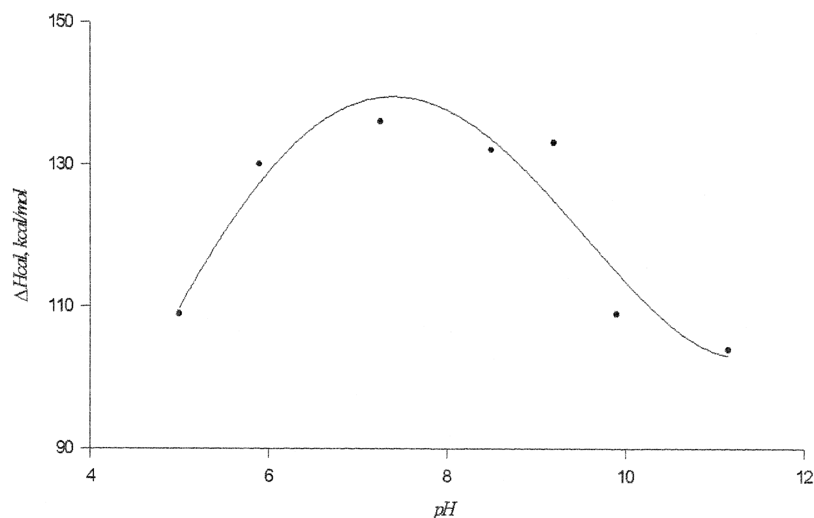

Fig. 3. pH dependence of direct measured enthalpy (ΔH_{cal}) of thermal denaturation of Lba.CN. For a single DSC data the standard deviation is 0.1 kcal/mol.

kcal/mol K for Lba.CN solution that is little lower with respect to the same value for Mb determined by Privalov et al. [9]. $\Delta C_{pd} = 2.34$ kcal/mol K for metmyoglobin (metMb) and 1.13 kcal/mol K for apoMb and $\Delta C_{pd} = 2.7$ kcal/mol K for Mb.CN according to Privalov et al. [4]. The average ΔC_{pd} value of Lba.CN as limited by those of apoMb and Mb.CN suggests for more open(water exposed) structure of Lba.CN than Mb.CN. The protein stability strongly depends on pH in the acidic region. That is due to the ionisation of the acidic groups at pH values below the isoelectric point (p$I = 4.4$). The same happens in case of metMb [9], and that probably results from their similar structures. The obtained values for ΔH_{cal} and ΔC_{pd} can be used to calculate the Gibbs energy (ΔG) as a function of temperature (T) [9].

$$\Delta G(T) = \Delta H_{cal}(T_d - T)/T_d - (T_d - T)\Delta C_{pd} + T\Delta C_{pd}\ln(T_d/T)$$

Gibbs energy change (ΔG) as a function of temperature (T) at several pH values is shown in Fig. 4. It can be seen that all the curves have a similar shape, the curve maximum is at neutral pH while at low and high pH values, ΔG values decrease. We should add

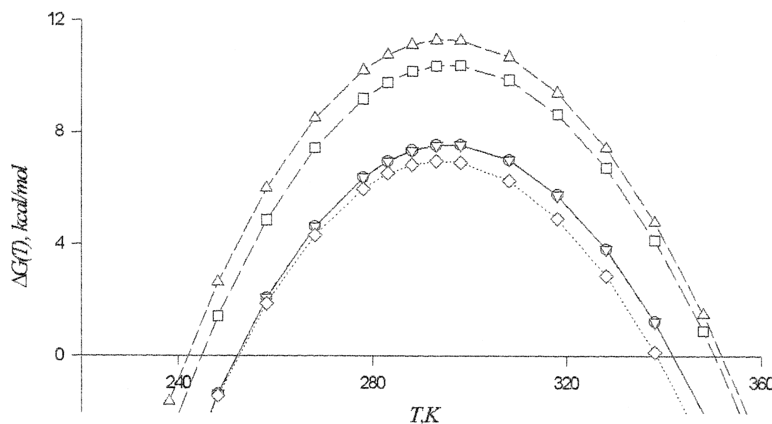

Fig. 4. Temperature dependence of the native and denatured Lba.CN Gibbs free energy difference (ΔG) in water solutions of 1 μM KCN and 1 mM KCl at various pH values: △ pH 7.25, □ pH 5.90, ○ pH 5.00, ▽ pH 9.90 and ◇ pH 11.15.

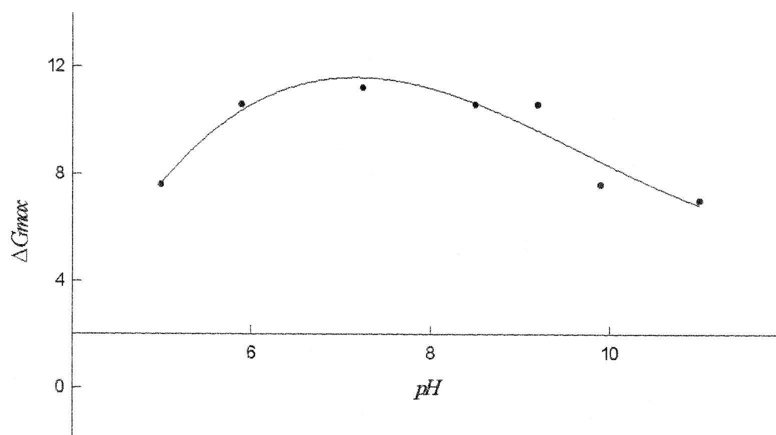

Fig. 5. pH dependence of Gibbs free energy difference (ΔG_{max}) of Lba.CN at 25°C as an indication of maximal protein stability.

here that at neutral pH ΔG is positive for a broad temperature range. The maximal value of this function is reached at temperature T_{max} where the first derivative of Gibbs energy difference with respect to the temperature is equal to zero. At temperatures above and below T_{max} the Gibbs energy should decrease and the stability of the native state which is determined by this function also decreases. According to Privalov et al. [9], the protein native state is stable in a limited temperature range, where hydrophobic interactions are strong enough and dissipative forces are too weak. Comparing with the results of Privalov et al., where $\Delta G_{max} = 11.9$ kcal/mol for metMb at pH 10.00 in glycine buffer [5], our values for ΔG_{max} are lower (between 7.0 and 11.2 kcal/mol depending on pH). For all pH values, ΔG_{max} is located between 20°C and 30°C. The highest value of 11.2 kcal/mol appears to be at pH 7.25. At pH 5.9 and 8.5 and 9.2 ΔG_{max} is approximately the same: 10.5–10.7 kcal/mol. At pH 5.0 and 9.9 the maximal value of ΔG is 7.6 and also coincides. The lowest value of ΔG_{max} is 7.0 at pH 11.15. This is noteworthy that in alkaline range of pH the stability of Lba.CN is lower than that near the pI point. This may be a factor for a conformational transition in alkaline range. The curves indicate that at temperatures above 25°C the usual heat denaturation of the protein takes place, while the other which occurs on cooling the protein solution below 25°C appears to be its cold denaturation. This differs from Mb for which the heat denaturation occurs above 30°C and cold denaturation below 30°C [9]. From this results we can suggest that maximal protein stability is at the pH range between 5.9–9.2 (Figs. 4 and 5).

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References

[1] H. Kubo, Acta Phytochim. 11 (1939) 195–200.
[2] A.I. Virtanen, J. Jorma, T. Laine, Suom. Kemistil. B 18 (1945) 49–50.
[3] C.A. Appleby, N.A. Nicola, J.G.R. Hurrel, S.J. Leach, Biochemistry 20 (1975) 4444–4450.
[4] P.L. Privalov, N.N. Kechinashvili, B.A. Atanassov, Biopolymers 10 (1971) 1865–1890.
[5] P.L. Privalov, N.N. Kechinashvili, J. Mol. Biol. 86 (1974) 665–684.
[6] A.A. Makarov, G.N. Mgeladze, A.I. Bojanov, I.P. Kuranova, A.I. Grebencko, N.G. Esipova, Biophizika 24 (1979) 977–984.
[7] C.A. Appleby, Biochim. Biophys. Acta 189 (1969) 267–279.
[8] A. Ginsburg, M. Zolkeiwski, Biochemistry 30 (1991) 9421–9429.
[9] P.L. Privalov, Y.V. Griko, S.Y. Veniaminov, V.P. Kutushenko, J. Mol. Biol. 190 (1986) 487–498.