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The studies of cooperative regions in T7 RNA polymerase

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Abstract

The heat denaturation of bacteriophage T7 RNA polymerase (T7RNAP) was studied by scanning microcalorimetry. The thermodynamic parameters of the denaturation were estimated within the pH range 6–9. The analysis of the denaturation curves showed the presence of two cooperative parts of the T7RNAP molecule melting according to the 'all-or-none' principle. The molecular masses of these parts were determined as 22 and 77 kDa. These values are close to the molecular masses of protein domains obtained from X-ray diffraction and limited trypsinolysis data. The smaller N-terminal domain was shown to increase the thermostability of the 'catalytic' C-terminal domain within the intact T7RNAP molecule.

Key words: Bacteriophage T7 RNA polymerase; Heat denaturation; Scanning microcalorimetry; Domain structure

1. Introduction

Bacteriophage T7 DNA-dependent RNA polymerase (T7RNAP) is a suitable model for the studies of the physico-chemical characteristics of transcription. T7RNAP (M_w 98 kDa) is a single-subunit protein capable of carrying out the full cycle of transcription from the specific promoter without additional protein factors [1]. Biochemical [2–4] and X-ray diffraction [5] data suggest that the T7RNAP molecule consists of two structural domains: one of 80 kDa (C-terminal) that contains the catalytic center of the enzyme, and one of 20 kDa (N-terminal) that evidently participates in the template and/or RNA product binding. The polypeptide fragment corresponding to the C-terminal domain can be isolated after limited digestion of the enzyme with trypsin, while the N-terminal fragment is rapidly degraded [3]. Little is known about the interaction between the domains within the protein globule and its role in the maintaining of the T7RNAP molecular structure.

Scanning microcalorimetry investigations of several globular proteins have demonstrated the usefulness of this technique in studies of the structural organization of protein molecules and interactions between domains [6–8].

In this work the microcalorimetric study of T7RNAP and its 80 kDa proteolytic fragment was carried out at pH 6–9. The results obtained suggest that the T7RNAP molecule consists of two relatively independently melting cooperative units ('energetic domains' of our definition [7,8]) the sizes of which coincide with those of structural domains [5]. The role of the N-terminal domain in the stabilization of the C-terminal one and the whole enzyme structure is also discussed.

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Abbreviations: T7RNAP, bacteriophage T7 DNA-dependent RNA polymerase.

2. Materials and methods

T7RNAP (200,000 U/mg) was purified from an *E. coli* producent strain as described in [9]. T7RNAP activity was assayed as in [9] using plasmid pGEM-2 (Promega) as a template. The concentration of the enzyme was determined spectrophotometrically using $\varepsilon_{280} = 1.4 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ [10]. Limited digestion of T7RNAP with trypsin at pH 7.2 was carried out as in [3,11]. The 80 kDa fragment of the enzyme was purified on a Sephacryl S200 column and was 95% pure as judged by SDS gel-electrophoresis. Prior to calorimetric experiments the protein solutions were dialyzed against 150 mM potassium phosphate buffer (pH range 6–9) containing 5 mM β-mercaptoethanol.

Calorimetric measurements were carried out on a differential adiabatic scanning microcalorimeter DASM-4 (Russia) at a heating rate of 1 K/min and a T7RNAP concentration of 0.5–1.2 mg/ml. The thermodynamic parameters of thermal denaturation, namely T_d (denaturation temperature), ΔH_{cal} (calorimetric denaturation enthalpy) and ΔH_{eff} (effective or van 't Hoff denaturation enthalpy) were calculated from experimental calorimetric curves as described in [6]. The ratio $\Delta H_{cal}/\Delta H_{eff}$ (R) was used as a criterion for the heat denaturation cooperativity [6–8]. To analyze functions of excess heat capacity we used the software package THERMCALC [6], allowing deconvolution of calorimetric curves to the peaks, corresponding to 'all-or-none' transitions. The hydropathic indexes were calculated by the method of Kyte and Doolitle (computer program GRAVY) [12] that systematically evaluates the hydrophilic and hydrophobic tendencies of a polypeptide chain. This program uses a hydropathy scale in which each amino acid has been assigned a value reflecting its relative hydrophilicity and hydrophobicity.

3. Results and discussion

Fig. 1A shows the temperature dependence of the transitional excess heat capacity of T7RNAP at pH 7.9 (pH optimum for T7RNAP activity). The curve has an asymmetric peak with a maximum at 48.3°C. In all cases T7RNAP heat denaturation was irreversible. However, scanning calorimetry showed no sign of detectable aggregation. The absence of a heat absorption peak during T7RNAP solution reheating could result from the slow renaturation kinetics due to the high molecular mass of the protein [13]. From the calorimetric curves at pH 7.9 (five independent estimations) the thermodynamic parameters were calculated as follows:

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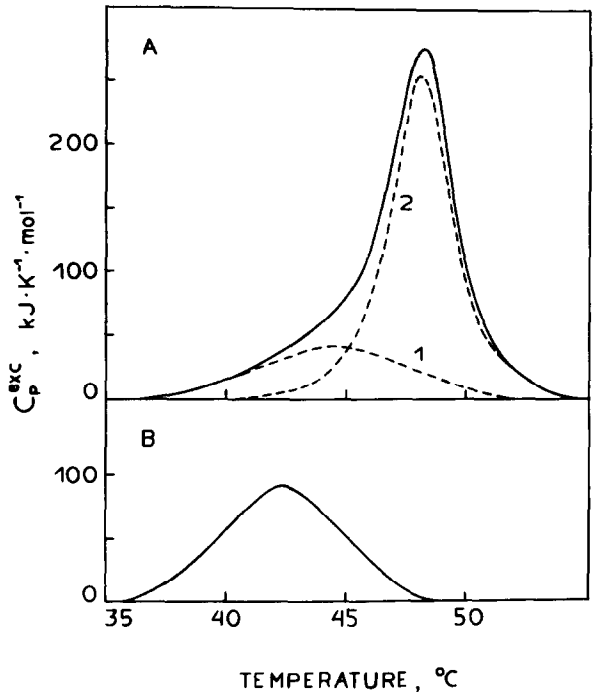

Fig. 1. The transitional excess heat capacity of T7RNAP (A) and its 80 kDa fragment (B) as a function of temperature at pH 7.9 (150 mM potassium phosphate buffer with 5 mM β-mercaptoethanol). Curves 1 and 2 (dotted lines) are the computer deconvolution of curve A.

ΔH_{cal} = 1380 ± 30 kJ/mol; ΔH_{eff} = 648 ± 16 kJ/mol and R = 2.1 ± 0.1. The latter parameter is a criterion which allows one to estimate how much the average size of a cooperative unit, i.e. the region of a protein molecule which melts more or less independently, differs from the size of the whole protein globule [6–8]. In the case of T7RNAP the value of $\Delta H_{cal}/\Delta H_{eff}$ in the pH range 6 9 indicates that at least two cooperative units differing in their thermostability exist within the enzyme molecule (Table 1).

The deconvolution of the excess heat capacity function of T7RNAP at pH 7.9 (Fig. 1A, dotted lines) clearly demonstrates that the experimental curve is the sum of two overlapping two-state transitions. To ascertain the conformity between the energetic and structural domains of T7RNAP, the calorimetric analysis of the 80 kDa proteolytic fragment (the only one available in substantial amounts [3,11]) was carried out. This fragment evidently contains all the essential determinants for promoter recognition and initiation but is able to produce only short (abortive) transcripts of 6–8 bases long [3]. The melting curve for the 80 kDa fragment has a symmetrical peak corresponding to the all-or-none transition (Fig. 1B). However, the thermodynamic parameters of the melting of the isolated 80 kDa fragment differ substantially from those of both peaks obtained as a result of the deconvolution of the intact T7RNAP melting curve (Tables 1 and 2). Nevertheless, judging from the values of ΔH_{cal} and T_d for the 80 kDa fragment denaturation it appeared reasonable to attribute the larger deconvoluted peak of Fig. 1A (curve 2, dotted line) to the melting of the C-terminal (80 kDa) domain of T7RNAP. As the conditions of calorimetric experiments (i.e. pH and ionic strength) coincided with those of the limited proteolysis [3,11], irreversible changes in the 80 kDa fragment before the temperature scan seem unlikely. Thus, the differences in the denaturation parameters apparently are due to the absence of the N-terminal domain and reflect the influence of the latter on the stability of the C-terminal domain in the intact T7RNAP molecule. To test this hypothesis and to compare the sizes of the energetic domains with those of the structural ones, the calorimetric analysis of T7RNAP melting at different pH values was carried out. The results of the deconvolution of the intact T7RNAP excess heat capacity function (Table 2) shows that in all cases the melting curve can be described as the sum of two peaks corresponding to two-state transitions differing in temperatures and enthalpies. Fig. 2 shows the dependence of the denaturation enthalpy for each deconvoluted peak on the denaturation temperature. The extrapolation of the lines obtained to the characteristic temperature near 110°C [6,14] resulted in the respective melting enthalpies ΔH_1 = 1280 kJ/mol and ΔH_2 = 4450 kJ/mol. This allows us to draw certain conclusions about the sizes of the independently melting units of the T7RNAP molecule. As was shown in [7,15], the ratio of denaturation enthalpies of independently melting domains at 110°C is equal to the ra-

Table 1
Parameters of thermal denaturation of T7RNAP and its 80 kDa fragment at different pH values (150 mM potassium phosphate buffer with 5 mM β-mercaptoethanol)

pH	Intact T7RNAP				80 kDa fragment			
	T_d (°C)	ΔH_{cal} (kJ/mol)	ΔH_{eff} (kJ/mol)	R*	T_d (°C)	ΔH_{cal} (kJ/mol)	ΔH_{eff} (kJ/mol)	R*
9.0	46.6	1217	553	2.2				
7.9	48.3	1372	646	2.1	42.3	580	572	1.0
7.0	47.0	1389	731	1.9	40.0	500	457	1.1
6.0	41.8	1120	509	2.2				

The experimental error did not exceed: ± 30 kJ/mol for ΔH_{cal}; ± 20 kJ/mol for ΔH_{eff}; ± 0.3°C for T_d; ± 0.1 for R. *$R = \Delta H_{cal}/\Delta H_{eff}$.

tio of their molecular masses. In the case of T7RNAP this ratio is 0.29, i.e. the molecular masses of the cooperative units are 22 and 77 kDa. These values are in good agreement with those (20 and 80 kDa) obtained by limited proteolysis [3,4] and from X-ray analysis [5] of T7RNAP.

Fig. 2 also demonstrates that, at the same temperatures, the values of ΔH_{cal} for the 80 kDa fragment coincide with those of the second peak in intact T7RNAP. To this end it is reasonable to attribute the second peak to the C-terminal domain of the enzyme and to suggest that the isolation of the 80 kDa fragment does not cause any significant changes in the factors responsible for stabilization of the native conformation of the C-terminal domain in the intact T7RNAP molecule.

It was shown earlier that the temperature coefficient of calorimetric denaturation enthalpy ($d(\Delta H)/dT$) practically coincides with the difference between the heat capacities of native and denatured protein states ($\Delta_d C_p$) which can be determined directly from the experimental calorimetric curve [6]. These parameters were calculated from Fig. 2 as $\Delta_d C_p^1 = 14$ kJ/K·mol and $\Delta_d C_p^2 = 56$ kJ/K·mol for the first and second deconvoluted peaks, respectively. As has been shown in [16], the heat capacity increment of the unfolding of globular proteins is mainly due to the hydration of groups exposed to water upon unfolding and correlates with the change in the water-accessible surface area of the non-polar groups ($\Delta_N^D ASA^{np}$) screened from water in the protein globule. Using the $\Delta_N^D ASA^{np}$ plot vs. $\Delta_d C_p$ obtained for a number of proteins tested by the calorimetric method in [16] the values of $\Delta_N^D ASA^{np}$ for T7RNAP (per one amino acid residue) were estimated: 45 Å^2 and 75 Å^2 for the N- and C-terminal domains, respectively. Following [16], one can suggest that the C-terminal domain contains more hydrophobic contacts and can form a compact globular structure whereas the N-terminal one accepts a looser conformation. These data correlate with the values of hydropathic indexes calculated by us for each domain on the basis of its primary structure according to [12]: −3.47 (more hydrophilic) and −2.51 (more hydrophobic) for the N- and C-terminal domains, respectively.

It follows from Tables 1 and 2 that both the temperature and enthalpy of the denaturation for the 80 kDa fragment are substantially lower than those obtained as a result of deconvolution for the C-terminal domain denaturation in intact T7RNAP at the same pH values. It is known that short-time proteolytic digestion of T7RNAP between amino acid residues 172–179 does not cause the dissociation of the nicked enzyme and that the 20 kDa and 80 kDa fragments are tightly bound to each other due to hydrophobic interactions [3]. The melting of the N-terminal domain upon heat denaturation of intact T7RNAP precedes that of the C-terminal one, whereas the T_d of the isolated 80 kDa fragment is less than the T_d of the N-terminal domain at the same pH values (Tables 1 and 2). It seems likely that the region of initial unfolding of the N-terminal domain is beyond the zone of interdomain contact within the T7RNAP molecule, while the corresponding region of the C-terminal domain is located inside such a zone. From this standpoint the increase in the T7RNAP C-terminal domain T_d as compared with 80 kDa fragment becomes apparent. Thus, the decrease in the thermal stability of the C-terminal domain in the absence of the N-terminal one suggests that the functional role of the latter, besides the stabilization of the complex of T7RNAP with the template and/or RNA product [3,4], is also connected with the stabilization of the C-terminal domain within the enzyme's structure.

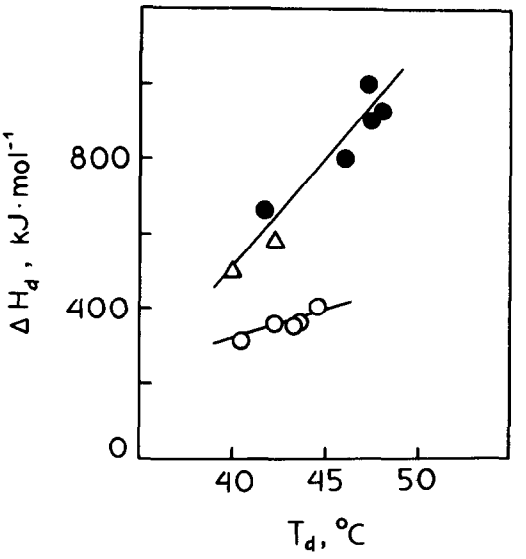

Fig. 2. The dependence of denaturation enthalpy on denaturation temperature for the first deconvoluted peak of the calorimetric melting curve of T7RNAP (○), for the second deconvoluted peak of the calorimetric melting curve of T7RNAP (●), and for the 80 kDa fragment of T7RNAP (△) (150 mM potassium phosphate buffer with 5 mM β-mercaptoethanol).

Table 2
Thermodynamic parameters of transitions obtained by deconvolution of excess heat capacity function for T7RNAP at different pH values (150 mM potassium phosphate buffer with 5 mM β-mercaptoethanol)

pH	T_d^1 (°C)	ΔH_d^1 (kJ/mol)	T_d^2 (°C)	ΔH_d^2 (kJ/mol)
9.0	42.3	357	46.0	800
7.9	44.6	411	48.0	920
7.0	40.5	310	47.1	1010
6.0	41.1	359	41.1	660

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