Cold denaturation of yeast phosphoglycerate kinase: which domain is more stable?

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Abstract Under destabilising conditions both heat and cold denaturation of yeast phosphoglycerate kinase (PGK) can be observed. According to previous interpretation of experimental data and theoretical calculations, the C-terminal domain should be more stable than the N-terminal domain at all temperatures. We report on thermal unfolding experiments with PGK and its isolated domains, which give rise to a revision of this view. While the C-terminal domain is indeed the more stable one on heating, it reveals lower stability in the cold. These findings are of importance, because PGK has been frequently used as a model for protein folding and mutual domain interactions.

Key words: Protein folding; Cold denaturation; Domain interaction; Circular dichroism; Differential scanning calorimetry

1. Introduction

Phosphoglycerate kinase (PGK) from yeast represents a suitable model for studying certain aspects of protein folding. Consisting of two structural domains of approximately equal size [1], PGK is useful for investigations of the role and mutual interaction of domains. Under destabilising conditions, both heat and cold denaturation can be observed. A problem, which is closely related to protein folding, is the prediction of the stability of a protein from its three-dimensional structure. Advances in this field, often termed structural thermodynamics, have been achieved during the last few years [2–4]. The adequate prediction of the stability of proteins on the basis of the three-dimensional structure is an important check of the present level of our understanding of the interactions and forces which are important in protein folding.

In aqueous solution, only heat denaturation of PGK is observable [5–7]. Under destabilising conditions, such as 0.7 M guanidinium chloride (GdmCl), both heat and cold denaturation occur above 0°C [8,9]. A remarkable feature of the thermal unfolding of PGK is: while upon heat denaturation both domains unfold co-operatively, the two structural domains unfold as separate units when the temperature is lowered. The distinct unfolding of both domains in the temperature range between 20°C and 0°C has been well demonstrated with different physical methods [8,9]. However, we have good evidence that experimentally observed unfolding transitions have been assigned to the wrong domains. Cold denaturation experiments with the isolated domains and a careful re-evaluation of data of the entire protein molecule demonstrate that in contrast to the previous interpretation of data the N-terminal domain is more stable than the C-terminal domain in the cold.

2. Materials and methods

Recombinant phosphoglycerate kinase (EC 2.7.2.3) and the individual domains were prepared according to the procedures described previously [10]. Purified proteins were precipitated in ammonium sulfate and stored at 4°C. Before use, protein pellets obtained by centrifugation were dissolved in 20 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA, 1 mM DTT, and different amounts of GdmCl. In order to remove remaining ammonium sulfate, the protein solutions were desalted using an FPLC fast desalting column HR 10/10 (Pharmacia). Protein concentrations were determined spectrophotometrically taking $A^{0.1\%}_{1\,cm}$ (280) = 0.495 for the wild-type protein [11], $A^{0.1\%}_{1\,cm}$ (280) = 0.32 for the C-terminal domain, and $A^{0.1\%}_{1\,cm}$ (280) = 0.629 for the N-terminal domain [10].

Circular dichroism (CD) spectra were obtained on a J-720 spectrometer (JASCO, Japan) equipped with a temperature control system (NESLAB, USA) using 10-mm and 1-mm cells and protein concentrations of about 2 g/l and 0.25 g/l for the near- and far-UV regions, respectively.

Differential scanning calorimetric (DSC) measurements were performed on an MC-2D microcalorimeter (MicroCal Inc., Northampton, MA) equipped with the DA-2 data acquisition system. With respect to the scan rate dependence reported in [9], cold denaturation was studied at 20 K/h. Heat induced unfolding was measured at a scan rate of 60 K/h. The protein concentration was about 2.5 g/l.

3. Results

3.1. Circular dichroism

The temperature dependences of the far-UV ellipticities of PGK and the isolated N- and C-terminal domains under destabilising conditions are shown in Fig. 1. Because PGK and the isolated domains exhibit somewhat different stabilities in GdmCl-induced unfolding experiments [12], it is necessary to monitor the unfolding in the cold at slightly different GdmCl concentrations, particularly at 0.5 M, 0.6 M, and 0.7 M GdmCl for the C-terminal domain, the N-terminal domain, and the entire PGK molecule, respectively. At these concentrations the

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Abbreviations: PGK, phosphoglycerate kinase; GdmCl, guanidinium chloride; CD, circular dichroism; DSC, differential scanning calorimetry.

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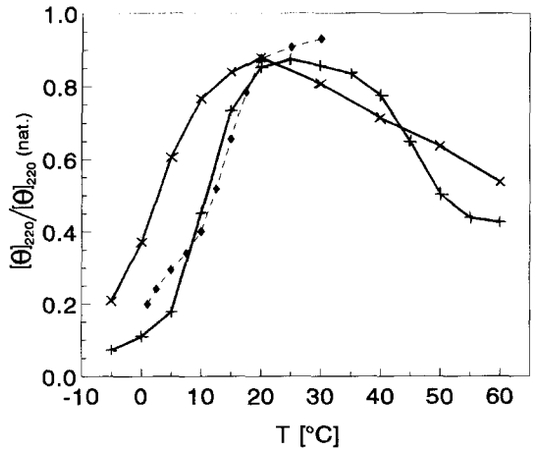

Fig. 1. Temperature dependence of the ellipticities at 220 nm of the C-terminal domain (+), the N-terminal domain (×), and PGK (♦) in the presence of 0.5 M, 0.6 M, and 0.7 M GdmCl, respectively. The ellipticities are normalized to the corresponding values at 25°C in the absence of GdmCl.

beginning of the transition curves is observed in GdmCl-induced unfolding experiments at room temperature. Unfolding and refolding of PGK is a slow process, particularly at low temperatures. Thus, special care was taken to reach the equilibrium state at each chosen temperature. Fig. 1 clearly indicates that the isolated C-terminal domain is less stable than the isolated N-terminal domain upon cold denaturation. The difference in the transition temperatures is about 7°C. This difference increases to about 9°C when cold denaturation of both domains is performed at identical GdmCl concentrations (data not shown). The cold denaturation transition of PGK proceeds in two steps (Fig. 1). If the isolated C-terminal domain is less stable than the isolated N-terminal domain then this order of stability should be retained within the entire PGK molecule. Thus, the shoulder in the transition curve of PGK below 10°C could be attributed to the unfolding of the N-terminal domain. However, a more direct assignment is possible on the basis of the near-UV CD spectra shown in Fig. 2A. The CD spectrum of PGK at 277 nm results mainly from contributions of the N-terminal domain which contains 5 of the 7 tyrosines, while the CD spectrum at 295 nm originates essentially from the two tryptophans located within the C-terminal domain. At 25°C, the spectra in the absence and the presence of 0.7 M GdmCl are almost identical [9]. Thus, the temperature-induced changes of the near-UV CD spectrum at 277 nm and 295 nm of PGK (Fig. 2B) clearly reflect the distinct unfolding of the N-terminal and C-terminal domains, respectively. The changes of the ellipticity at 295 nm occur mainly above 10°C, while the ellipticity at 277 nm decreases essentially below 10°C. This univocally shows that the N-terminal domain is more stable on cooling also within the entire PGK molecule. The unfolding on heating of the isolated domains, which is also shown in Fig. 1, will be discussed below.

3.2 Differential scanning calorimetry

The molar excess heat capacities in the region of cold denaturation of PGK and the isolated C-terminal and N-terminal domains are shown in Fig. 3. The corresponding peak maximum temperatures are listed in Table 1. The destabilising conditions are the same as in the case of the CD measurements. The temperatures of the peak maxima are 6.2°C and 11.9°C for the N-terminal and the C-terminal domains, respectively. This is in good agreement with the transition curves monitored by CD. The DSC transition curve of PGK shows two partially resolved peaks at 6.8°C and 17.4°C. The differences in the positions of the peak maxima of the isolated domains compared to PGK may be partly due to the different GdmCl concentrations used for destabilisation. Therefore, the shifts of the peaks caused by mutual interactions of the domains within the PGK molecule cannot be estimated exactly. A quantitative estimation of ΔH and ΔC_p is connected with slight problems because the native reference state of the proteins is not fully populated at the individual temperatures of maximum stability and corrections with respect to the influence of GdmCl have to be taken into consideration. The analysis of calorimetric data in such cases has been discussed recently by Haynie and Freire [13]. The two domains do not show marked melting peaks on heat denaturation in the presence of GdmCl. The peak maximum tempera-

Table 1
Peak maximum temperature (T_m in °C) and related thermodynamic quantities at cold and heat denaturation of PGK and its isolated domains

GdmCl conc. (M)	T_m cold denat.	T_m heat denat.	ΔH (kJ/mol)	ΔCp (kJ/mol/K)	Ref.
PGK:					
0		57.5 ± 0.2	>800*		This work
0		56.2	845 ± 11	6.8 ± 0.6	[5]
0.7		40.8 ± 0.3	570 ± 25	36 ± 9	[9]
0.7	6.8 ± 0.3 (trs. 1)				[9]
0.7	17.4 ± 0.3 (trs. 2)				[9]
0.7		40	586		[8]
0.7	~8 (trs. 1)				[8]
0.7	~20 (trs. 2)				[8]
0.7		~40	586	31	[4]
Isol. N-term. domain					
0		42 ± 1	167 ± 23	4.5 ± 2.0	This work
0.6	6.2 ± 0.5				This work
Isol. C-term. domain					
0		59.1 ± 0.2	>430 ± 30*		This work
0.5	11.9 ± 0.5	~43**			This work

*Due to aggregation, underestimated value. **Based on additional data, not shown in Fig. 3.

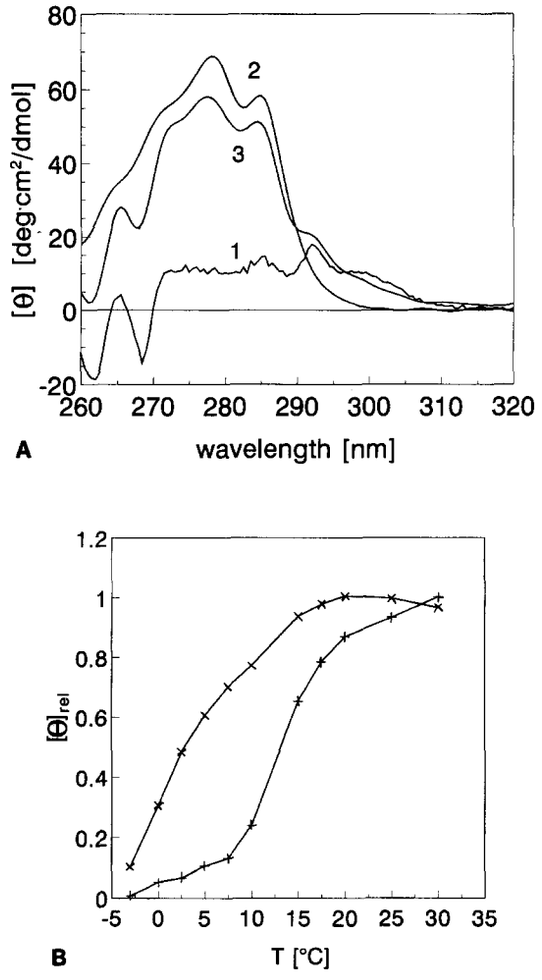

Fig. 2. (A) Near-UV CD spectra of the C-terminal domain (1), the N-terminal domain (2), and PGK (3) at 25°C in the absence of GdmCl. (B) Temperature dependence of the ellipticities at 277 nm (×) and 295 nm (+) of PGK in the presence of 0.7 M GdmCl.

ture for thermal unfolding of the C-terminal domain amounts to about 43°C, and approaches the value for intact PGK.

The scanning calorimetric curves for the heat denaturation of the isolated domains in the absence of GdmCl are presented in Fig. 4. The insert in Fig. 4 shows the scanning calorimetric recordings for PGK in the absence and in the presence of 0.7 M GdmCl. Related thermodynamic quantities are listed in Table 1.

Remarkably, the peak maximum temperature T_m of the isolated C-terminal domain is slightly increased by about 2°C compared with intact PGK. On the contrary, T_m of the N-terminal domain is drastically reduced by about 16°C. Thermal denaturation of the N-terminal domain is a two-state transition with ΔH = 167 kJ/mol and ΔC_p = 4.5 kJ/mol. Thus, the N-terminal domain is less stable upon heating than the C-terminal domain (T_m = 59.1°C and ΔH ≙ 430 kJ/mol) as originally proposed in [4].

PGK and the C-terminal domain show a strong tendency to aggregate when the thermal transition is almost completed. This complicates the precise estimation of thermodynamic quantities. Nevertheless, a crude estimation of the contribution of the domains to the overall heat denaturational effect of intact PGK can be made. The sum of ΔH of the C-terminal domain (430 kJ/mol) and of the corresponding value of the N-terminal domain (ΔH = 240 kJ/mol at 57.5°C, calculated using ΔC_p from Tab. 1) amounts to 670 kJ/mol. This value is lower than $\Delta H \geq$ 800 kJ/mol determined for PGK in this paper, and the more reliable value of ΔH = 854 kJ/mol from [5]. The data suggest a significant enthalpic contribution of the domain-domain interaction.

4. Discussion

Combining the results of the CD and the DSC investigations, the following conclusions regarding the temperature-induced unfolding of PGK and of the isolated domains can be drawn. As for the whole protein, only the heat denaturation transitions can be observed with both isolated domains in the absence of destabilising agents. The C-terminal domain shows a transition rather similar to that of PGK having a transition temperature, T_m, 2°C higher. However, thermal unfolding of the isolated N-terminal domain proceeds at a temperature 16°C lower than the co-operative unfolding of both domains within the entire PGK molecule. Taking into consideration the results of the investigations of some mutants of yeast PGK [6], the lower T_m value in the case of the N-terminal domain is not an unexpected result. For example, substituting alanine-183 by proline lowers the interdomain interactions in such a way that two partly resolved transitions differing in T_m by 10°C can be observed [6,14]. According to the results of ligand binding experiments, the low temperature transition was attributed to the unfolding of the N-terminal domain [6,14].

Under destabilising conditions, the heat denaturation transition of the isolated C-terminal domain is shifted to a temperature slightly above 40°C. This shift of about 16°C is just the same as for PGK under comparable conditions. The results with the isolated C-terminal domain demonstrate that the heat denaturation of PGK both in the absence and the presence of GdmCl is strongly influenced by the unfolding behaviour of the C-terminal domain. Destabilisation of the N-terminal domain by 0.6 M GdmCl leads to a very broad heat denaturation

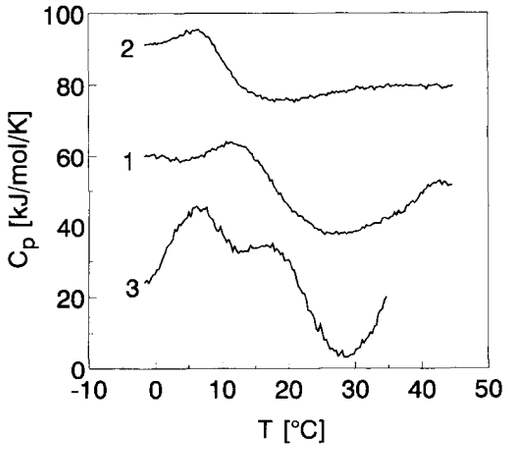

Fig. 3. Differential scanning calorimetric curves of the C-terminal domain (1), the N-terminal domain (2), and PGK (3) under destabilising conditions. The scan rate was 20 K/h. The curves have been shifted along the vertical axis for better representation.

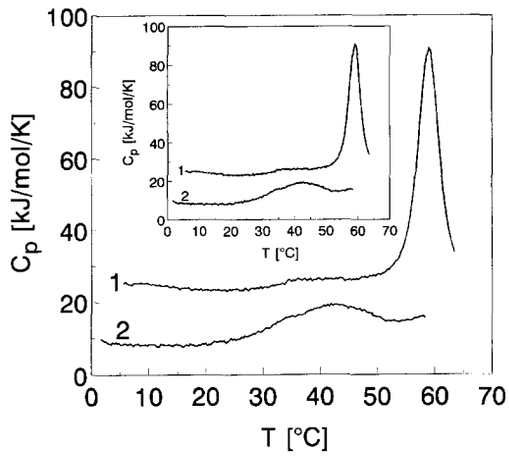

Fig. 4. Differential scanning calorimetric curves of the heat denaturation of the C-terminal domain (1) and the N-terminal domain (2) in the absence of GdmCl. The insert shows the corresponding transition curves for PGK in the absence (3A) and in the presence of 0.7 M GdmCl (3B).

transition. This is reflected in the results of both the CD (Fig. 1) and the DSC (Fig. 3) investigations. The estimation of T_m is hardly feasible under these conditions.

While our results pertaining to the heat denaturation of the isolated domains are consistent with the interpretation of previous data, this is not the case with respect to the low temperature region between −5°C and 30°C. Particularly, two results are important for the correct interpretation of the stability and the interdomain interactions at temperatures near and below room temperature. First, our CD and DSC data clearly demonstrate that the C-terminal domain is less stable on cooling. This holds for the isolated domain as well as when this domain is integrated into the entire PGK molecule. The earlier interpretation of data, assuming the N-terminal domain as the less stable domain upon cold denaturation, was mainly based on the presumption that the near-UV CD spectrum of PGK at 277 nm originates mostly from the two tryptophans of the C-terminal domain [8,9]. But, as can be clearly seen in Fig. 2A, the ellipticity at 277 nm results mainly from contributions of the N-terminal domain. Second, both domains differ also in their temperatures of maximum stability. According to the CD and DSC data, the temperature of maximum stability is about 20°C for the N-terminal domain and between 25°C and 30°C for the C-terminal domain. This means that the entire stability curve of the N-terminal domain is shifted to lower temperatures as compared to that of the C-terminal domain. The temperature of maximum stability of PGK is nearly equal to or slightly higher than that of the C-terminal domain (Fig. 3). Thus, mutual interactions between the domains should be essential also at temperatures between 25°C and 30°C. But these interactions must decrease considerably below 25°C enabling an independent unfolding of the domains in the cold. This strong temperature dependence implies that these interactions are mostly hydrophobic [8].

The findings with the isolated domains and the correct assignment of the partly resolved cold denaturation transitions of PGK improve the experimental basis for theoretical calculations to predict the stability and the folding/unfolding behaviour of multidomain proteins.

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