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Kinetic study on the irreversible thermal denaturation of *Schistosoma japonicum* glutathione s-transferase

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Abstract

The thermal unfolding pathway of the *Schistosoma japonicum* glutathione S-transferase (Sj26GST) was previously interpreted by applying equilibrium thermodynamics and a reversible two-state model (Kaplan et al., (1997) *Protein Science*, 6, 399–406), though weak support for this interpretation was provided. In our study, thermal denaturation of Sj26GST has been re-examined by differential scanning calorimetry in the pH range of 6.5–8.5 and in the presence of the substrate and S-hexylglutathione. Calorimetric traces were found to be irreversible and highly scan-rate dependent. Thermogram shapes, as well as their scan-rate dependence, can be globally explained by assuming that thermal denaturation takes place according to one irreversible step described by a first-order kinetic constant that changes with temperature, as given by an Arrhenius equation. On the basis of this model, values for the rate constant as a function of temperature and the activation energy have been determined. Data also indicate that binding of GSH or S-hexylglutathione just exert a very little stabilising effect on the dimeric structure of the molecule.

Keywords: Differential scanning calorimetry; Thermal denaturation; Glutathione S-transferase; Irreversible

1. Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) make up a family of multifunctional dimeric enzymes that have been involved in the detoxification of harmful physiological and xenobiotic compounds [1,2]. The dimeric structure of the molecule is important regarding the formation of a non-substrate ligand binding region in the amphipathic cleft at the dimer interface, as well as in the building of fully functional catalytic sites near the subunit interface. GSTs have been classified into a variety of species-independent gene classes, and high-resolution crystal structures have been elucidated for a number of proteins [1–3]. Dimerization is highly specific and only occurs between subunits within the same gene class. Structural features at the dimer interfaces of the GSTs suggest two major groupings of subunit interfaces, the α/μ/π/Sj26 subunit type and the θ/σ subunit type [4]. The interface for the former group is curve, hydrophobic, and involves a prominent hydrophobic lock-and-key inter-subunit interaction motif whereas in the latter group it is flatter, hydrophilic, and lacks the lock-and-key feature.

Equilibrium unfolding studies with representatives of the α/π/Sj26 subunit grouping [hGSTA1-1 [5]); πGSTP1-1 [6,7] and Sj26GST [8]] indicate that there is a tight coupling between dissociation/association and unfolding/refolding for that proteins. These findings suggest that dimeric quaternary structure is essential and that interactions at the dimer interface are the main contributions to protein folding and stability. However, all these unfolding/folding studies were performed using chemical denaturants as urea and guanidinium chloride while thermal denaturation mechanisms for GSTs have not been sufficiently examined to date.

Schistosoma japonicum glutathione S-transferase (Sj26GST) displays a two-state unfolding/refolding process which is highly populated with folded dimer and unfolded monomer at equilibrium. Notwithstanding, this process is reversible only when unfolding is solvent-induced as it occurs when urea is used [8–10].

In the present report, thermal stability of Sj26GST was investigated by differential scanning calorimetry (DSC) at

Abbreviations: Sj26GST, glutathione S-transferase from *Schistosoma japonicum*; GSH, reduced glutathione; DSC, differential scanning calorimetry

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several pH values ranging from 6.5 to 8.5, at different scan rates, and in the presence of ligands in order to reassess the pathway of thermal unfolding for this dimeric enzyme.

2. Materials and methods

Recombinant Sj26GST was expressed [11] and purified by affinity chromatography on immobilized glutathione [12]. After affinity purification, the enzyme was homogeneous as judged by SDS-PAGE [13]. GSH and S-hexylglutathione were purchased from Merck and Sigma, respectively.

Calorimetric measurements were performed on a MicroCal VP-DSC (Northampton, MA) with cell volumes of 0.5 mL at the indicated scan rates. All enzyme solutions were buffer exchanged on a PD-10 column (Amersham Biosciences). Protein concentrations were determined from absorbance measurements at 278 nm using an extinction coefficient of $7.01 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for the dimer. Prior to scanning, all solutions were degassed under vacuum. Calorimetric experiments were carried out in 10 mM Hepes at pH 7.5 or 10 mM sodium phosphate at pH 6.5, or 10 mM Bicine at pH 8.5. Calorimetric cells were kept under an excess pressure of 30 psi to prevent degassing during the scan. Protein concentrations were about 10–15 μM per GST dimer in all experiments, unless otherwise is indicated. DSC curves were obtained by scanning buffered solutions of Sj26GST at temperatures from 10 °C to 80 °C.

Reversibility of the thermal transitions was checked by reheating the samples after fast cooling from the previous protein solution scan. Since thermal transitions were always found to be completely irreversible, the calorimetric traces were baseline-corrected by subtracting the sample reheating scans.

The excess molar heat capacity was calculated using a molecular mass of 25483 Da and a partial volume for the protein equal to 0.728 ml/g, as calculated from the amino acid sequence [14]. The chemical baseline was subtracted according to the procedure of Takahashi and Sturtevant [15] using the Origin v7.0 software. After baseline subtraction the excess molar heat capacity and the temperature were included as input data for calculations using the "*Scientist*" (MicroMath Scientific Software, St. Louis, USA) software. The correlation coefficient r used as a criterion for the accuracy of fitting was determined by the equation,

$$r = \sqrt{\left(\sum_{i=1}^{n}(y_i - y_i^m)^2 - \sum_{i=1}^{n}(y_i - y_i^{calc})^2\right) / \sum_{i=1}^{n}(y_i - y_i^m)^2} \quad (1)$$

and it is calculated automatically by the 'Scientist' software. In Eq. (1), y_i and y_i^{calc} are the experimental and calculated values of the function, respectively; y_i^m is the mean of experimental values of the function, and n is the number of points.

The validity of the one step irreversible model was established using the methods described by Sánchez-Ruiz et al. [16].

Method A. The first-order kinetic constant at each temperature is given by

$$k = \frac{\nu C_p^{exc}(T)}{\Delta H_{cal} - \Delta H(T)} \quad (2)$$

where C^{exc}_p and ΔH are the excess heat capacity and the enthalpy change evolved up to a given temperature T, respectively, and ΔH_{cal} is the total enthalpy change of the process. The activation energy can be obtained from the k values by using the Arrhenius equation $k = A \cdot \exp(-E_a/RT)$, where A stands for the pre-exponential factor. For each scan rate, k was evaluated for all data in the interval $(T_m - 3) \leq T \leq (T_m + 3)$.

Method B. The temperature dependence of heat evolution is given by the equation:

$$\ln\left(\ln\left\{\frac{\Delta H_{cal}}{\Delta H_{cal} - \Delta H(T)}\right\}\right) = \frac{E_a}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right). \quad (3)$$

Plotting the left side of this equation *vs.* 1/T gives rise to a straight line with a slope of $-E_a/R$, for each heating rate.

Method C. The effect of the scan rate on T_m is given by the equation:

$$\ln\left(\frac{\nu}{T_m^2}\right) = C - \frac{E_a}{RT_m} \quad (4)$$

where ν (°C/min) stands for the scan rate, and C is a constant equal to $A \cdot R/E_a$.

Method D. Finally, the activation energy can be also calculated using the equation:

$$E_a = \frac{eRT_m^2 C_p^{exc}(T_m)}{\Delta H_{cal}} \quad (5)$$

where C^{exc}_p (T_m) is the heat capacity at T_m, and e stands for the base of the natural logarithm.

3. Results and discussion

We have investigated the thermal unfolding of Sj26GST at several pH values in the range of 6.5 to 8.5 using different buffer solutions. Fig. 1A shows the DSC thermogram of Sj26GST obtained at a scanning rate of 1.17 K/min in Hepes at pH 7.5. The excess heat capacity *vs.* temperature profile contains a single and asymmetric peak (with a maximum at 56.5 °C for this scanning rate). The thermal denaturation of Sj26GST was always irreversible (either in the absence or in the presence of ligand), as no transition could be detected in reheating runs (even when the first run had been stopped immediately after the end of transition) (Fig. 1B). No aggregation was observed in the samples extracted from the calorimetric cell at pH 7.5 and pH 8.5. However, after DSC experiments in phosphate buffer at pH 6.5 aggregation was evident both in the calorimetric traces and samples withdrawn from the calorimetric cell.

3.1. Irreversibility of the DSC transitions

Due to irreversibility of the thermal denaturation of Sj26GST thermodynamic parameters cannot be obtained. Nervertheless, it is possible to obtain some information from the analysis of the irreversible process. Irreversible processes associated with thermal denaturation of proteins are generally sensitive to the scan rate because they are under kinetic control. However, when thermal transition is previous to the irreversible step,

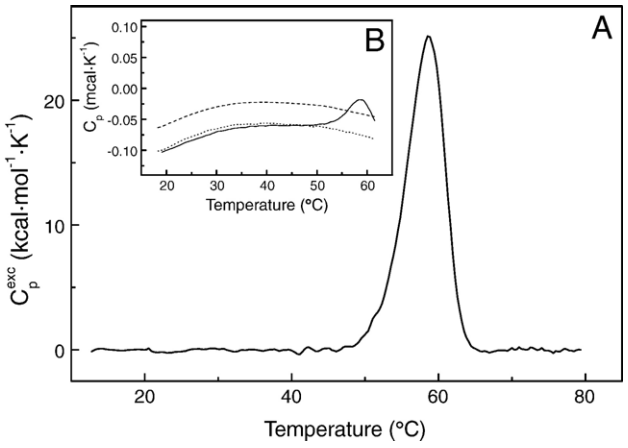

Fig. 1. Differential scanning calorimetry transitions. A. Sj26GST DSC scan data at 1.17 K/h and pH 7.5. B (inset) DSC original thermogram for Sj26GST denaturation at a concentration of 12.5 μM in 10 mM Hepes, pH 7.5. The buffer-buffer scan is shown as a dashed line. The thermogram shown as a solid line was stopped at 62 °C. The scan displayed by a dot line represents the reheating experiment after cooling of a previous scan. The experiments were performed at a scan rate of 1.5 K/min.