Differential-scanning-calorimetric study of the irreversible thermal denaturation of 8 kDa cytotoxin from the sea anemone *Radianthus macrodactylus*

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A differential-scanning-calorimetric study of the thermal denaturation of a sea-anemone (*Radianthus macrodactylus*) 8 kDa cytolytic toxin was carried out. The calorimetric traces were found to be irreversible and scan-rate-dependent under the experimental conditions employed. Scan-rate-dependent

thermograms were explained in terms of a two-state kinetic model $N \xrightarrow{k} D$, where k is a first-order kinetic constant that changes with temperature as given by the Arrhenius equation, N is the native state of the toxin, and D the denatured one.

INTRODUCTION

Differential scanning calorimetry (d.s.c.) has been extensively used to study protein thermal denaturation [for a review, see Privalov (1989)]. Under certain conditions the thermal denaturation of some small globular proteins (Privalov, 1979) and some complex proteins (Privalov, 1982) is reversible, therefore the corresponding d.s.c. transitions are interpreted on the basis of equilibrium thermodynamics. However, the thermal denaturation of many proteins is irreversible, and therefore thermodynamic functions for the process, such as changes in entropy and Gibbs function, cannot be extracted from the d.s.c. traces simply (Edge et al., 1985; Manly et al., 1985; Hu and Sturtevant, 1987). In this case it is necessary to use other methods of analysis of the calorimetric results, with the purpose of quantitative comparison of the different experimental data. One approach may be the analysis of the d.s.c. transitions considering the kinetics of formation of the irreversibly formed protein state (see, for instance, Sánchez-Ruiz, 1992). Although several reports demonstrate the usefulness of kinetic analysis for the study of the irreversible denaturation of proteins (Sánchez-Ruiz et al., 1988a,b; Guzmán-Casado et al., 1990; Lepock et al., 1990; Conejero-Lara et al., 1991; Galisteo et al., 1991; Arriaga et al., 1992; Le Bihan and Gicquaud, 1993), some researchers still assume that equilibrium thermodynamic analysis of the transitions is permissible (Hu and Sturtevant, 1989; Bertazzon et al., 1990). Therefore additional experimental data are needed for developing experimental approaches that will allow the investigator to determine which type of information can be derived from irreversible calorimetric transitions.

There is steady opinion about reversible unfolding for small globular proteins. In this work we have found the d.s.c. traces corresponding to the thermal denaturation of a sea-anemone (*Radianthus macrodactylus*) cytolytic toxin (8 kDa; RTX-8) in 20 mM sodium phosphate, pH 7.4, to be irreversible and strongly scan-rate-dependent. Therefore the analysis of these calorimetric traces have been carried out using an approach which takes into account the possible kinetic character of the irreversible thermal

denaturation of proteins (Sánchez-Ruiz, 1992; Lepock et al., 1992). Finally, it is noteworthy that cytolytic toxins from sea anemone could become important biotechnological materials [see, for instance, Turk (1991)]. Therefore investigation of thermal stability of the RTX-8 has not only academic, but also applied, interest.

MATERIALS AND METHODS

RTX-8 isolated from sea anemone *Radianthus macrodactylus* (Monastyrnaya et al., 1990) was generously provided by Dr. Margaret M. Monastyrnaya from Pacific Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Vladivostok, Russia. The material was stored as a freeze-dried powder at -20°C . For experimental use, protein solutions were prepared by dissolving dry samples in 20 mM sodium phosphate buffer, pH 7.4. Before the experiments the sample solutions were dialysed for 12–14 h at 4°C against two changes of a large volume of the dialysis buffer. Similarly prepared samples were kept at 0°C not more than 3 h before the experiment. Protein concentration was determined according to the method of Lowry et al. (1951). The purity of RTX-8 was documented by SDS/PAGE on a 5–25 % gradient gel as previously reported (Shnyrov et al., 1990).

D.s.c. was performed in a DASM-4 instrument (Privalov and Plotnikov, 1989) with a cell volume of 0.47 ml under an extra air pressure of 152 kPa (1.5 atm) over the liquids in the cells throughout the scans to prevent any degassing during heating. Several scan rates in the range of 0.25–2.0 K/min were employed. The reversibility of the thermally induced transitions was checked by reheating the sample solution in the calorimetric cell after the cooling from the first scan. The calorimetric traces were corrected for the calorimetric baseline and for determination of enthalpy of the transitions by using a sigmoidal baseline as described by Takahashi and Sturtevant (1981). The calorimetric baseline was determined prior to scanning each sample with both cells filled with dialysis residue which also reserved in the reference cell during scanning of the toxin. The effect of the finite time response of the instrument was taken into account by carrying out the

Abbreviation used: d.s.c., differential scanning calorimetry.

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dynamic correction of the thermograms reported by López-Mayorga and Freire (1987). The enthalpy of the transitions was determined from the areas under the heat-capacity curves by means of a 25 μ W electric calibration mark.

Most experiments were carried out with a protein concentration of about 2 mg/ml; control experiments with lower (about 0.3 mg/ml) or higher (about 4 mg/ml) protein concentration showed that the kinetics and extent of the irreversibility do not change significantly with protein concentration.

It is known that RTX-8 has disulphide bonds (Monastyrnaya et al., 1990). In order to check the influence of thiol-group oxidation on the irreversibility of the thermal transitions, thermal-induced transitions for RTX-8 in presence of dithiothreitol as well as 2-mercaptoethanol were investigated. Under these conditions the results showed the thermodynamical parameters for an RTX-8 solution in 20 mM sodium phosphate, pH 7.4, to be unaffected by the addition of reducing agents.

RESULTS AND DISCUSSION

Figure 1 shows the d.s.c. traces for the thermal denaturation of RTX-8 in 20 mM sodium phosphate, pH 7.4, at several scan rates. The traces were found to be calorimetrically irreversible, as no thermal effect was observed in a second heating of the RTX-8 solution. As it may be seen in this Figure the traces were scan-rate-dependent. It may be concluded, therefore, that the thermal denaturation of RTX-8 is irreversible and kinetically controlled under the conditions employed. The independence of the heat-

Figure 1 Temperature-dependence of excess heat capacity for RTX-6 in 20 mM sodium phosphate, pH 7.4

Values against the curves correspond to the different scan rates used (in K/min). The vertical bar corresponds to 2 kJ/(K·mol).

Figure 2 (a) Arrhenius plot including k data from the four scan rates used ($\circ$, 2.0; $\bullet$, 1.0; $\triangle$, 0.5; $\blacktriangledown$, 0.25 K/min), (b) plot of $\ln(v/T_m^2)$ versus $1/T_m$ (each data point corresponds to one of the four scan rates used) and (c) plot of $\ln[\ln Q/(Q-Q_i)]$ versus of $1/T$ (symbols are the same as those used in (a))

capacity increment from the scan rate testifies the absence of intermolecular aggregation at immediately after thermal transition, this being confirmed by the absence of visual changes in the sample turbidity after the d.s.c. experiment.

The simplest representation of the irreversible thermal denaturation of a protein is given by the Lumry and Eyring (1954) model, in which a reversible unfolding is followed by an irreversible process:

where N is the native species, U the reversibly unfolded intermediate and D is the irreversibly denatured form which locks the unfolded protein in a state from which it cannot refold over the time of the experiment, owing to various factors (Ahern and Klibanov, 1985; Zale and Klibanov, 1986). If we assume that all three processes are first-order ones and that $k_3 \gg k_2$, most of the U molecules will be converted into D . In this case, the concentration of U is always very low, and thus denaturation may be considered to be an irreversible first-order kinetic process $N \xrightarrow{k} D$, determined by a rate constant (k) which changes with temperature according to the Arrhenius equation.

This kind of behaviour has recently been reported for the thermal denaturation of quite a few proteins (Galisteo et al., 1992; Le Bihan and Gicquaud, 1993). The mathematical elaboration of this model (Sánchez-Ruiz et al., 1988a) led to several

methods of calculating the activation energy (E_A) of the kinetic process.

1. The first-order rate constant, k , can be obtained as a function of temperature from:

$$k = v \cdot \Delta C_p / (Q - Q_i)$$

where v (K/min) stands for the scan rate, ΔC_p for the excess heat capacity, Q for the total heat of the process, and Q_i for the heat evolved at given temperature, T . The activation energy, E_A , is calculated from an Arrhenius plot of $\ln k$ versus $1/T$. The corresponding Arrhenius plot for RTX-8, including data from the four scan rates used, is given in Figure 2(a). There is sufficiently good agreement between the rate constants calculated from the traces obtained at four different scan rates. A value of 192 ± 9 kJ/mol for the energy of activation was obtained from the slope of the Arrhenius plot in Figure 2(a).

2. The effect of scan rate of the T_m values is given by equation:

$$v/T_m^2 = (A \cdot R/E_A) e^{-E_A/R \cdot T_m}$$

where A is Arrhenius frequency factor, T_m is the temperature at the maximum heat capacity curve and R is the gas constant. E_A can be obtained from the linear fitting of $\ln(v/T_m^2)$ versus $1/T_m$ (Figure 2b), and the average activation energy thus obtained for RTX-8 is 202 ± 6 kJ/mol.

3. The dependence of the heat evolved with temperature is given by the equation:

$$\ln[\ln Q/(Q - Q_i)] = E_A/R \cdot (1/T_m - 1/T)$$

In this case a slope of the plot of $\ln[\ln Q/(Q - Q_i)]$ versus $1/T$ equal to $-E_A/R$. Corresponding plots for RTX-8 are shown in Figure 2(c) for the four scan rates involved. A value of 210 ± 8 kJ/mol of the E_A was calculated from these data.

4. The activation energy can be calculated directly from equation:

$$E_A = e \cdot R \cdot T_m^2 \cdot \Delta C_p^{\max} / Q$$

where $\Delta C_p^{\max}$ is the heat capacity at the maximum of the trace and e stands for the base of natural logarithms. The E_A value (189 ± 8 kJ/mol) obtained by using this expression is in a good agreement with those calculated above.

It is clear that the good agreement between the results obtained for RTX-8 using different methods of analysis supports the

validity of the proposed simple kinetic model for the thermal denaturation of RTX-8.

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