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Thermal denaturation of erythrocyte carbonic anhydrase

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An experimental study on the thermal behaviour of erythrocyte carbonic anhydrase was carried out with the main aim to estimate the thermodynamic parameters that control the stability of the enzyme. The effects of thermal denaturation on the catalytic properties of the enzyme were also investigated. Below 60°C the enzyme was found to be very stable, whereas between 60 and 65°C a drastic decrease in the biological activity was observed. From the obtained results some considerations were made about the stabilization of the active form of the protein.

Carbonic Anhydrase; Thermal denaturation; Conformational stability; Bovine erythrocytes

1. INTRODUCTION

Carbonic anhydrase is a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide [1]. The enzyme has been isolated from animal and plant cells, and has been also found in some strains of the bacterial genus *Neisseria*. In erythrocytes this protein plays a key role in the transport of hydrogen and bicarbonate ions across the cell membrane.

Experimental investigations have been concerned primarily with the catalytic properties of the enzyme and in particular with the kinetic characterization of the three major isoenzyme types, known as CA-I (or B), Ca-II (or C) and CA-III (or M) [2]. Only marginal attention has been devoted to the analysis of the thermal stability of the protein and the mechanisms causing its deactivation.

An effective approach to investigate the effects of temperature on the enzyme behaviour consists of denaturating the native form of the protein and measuring some structure-related properties [3]. This methodology may provide useful suggestions on enzyme stabilization and a deeper insight into the physical principles involved in the molecular organization of proteins [4].

In the present contribution, which is included in a wider research field on the stabilization of proteins by polyhydric compounds, we analyse the thermal behaviour of erythrocyte carbonic anhydrase. Experimental work has been performed with the main aim of gaining information about the thermodynamic parameters that control the stability of the enzyme. Furthermore, in order to evaluate the effects of thermal denaturation on the catalytic activity, measurements of the residual esterase activity as a function of denaturation time have been carried out.

From the analysis of the obtained results an attempt has been made to relate the conformational stability of the protein to the observed irreversible loss of activity.

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2. MATERIALS AND METHODS

Carbonic anhydrase (EC 4.2.1.1) from bovine erythrocytes was obtained from Sigma Chemical Co. as a dialyzed and lyophilized powder. The claimed activity was 2500 W-A units per mg. All chemicals were reagent grade and utilized without further purification.

Ultraviolet difference spectroscopy was used to follow protein unfolding. In a typical experiment, the two cells of a double-beam UV-VIS spectrophotometer (Perkin Elmer Lambda 5) were filled with a solution of the enzyme in Tris buffer (0.09 M, pH 7.55) at a concentration of $1.6 \cdot 10^{-5}$ M. The protein solution was previously passed through a 0.65 μm Millipore filter. The reference cell was thermostated at the temperature of 25°C, whereas the sample cell was thermoregulated (± 0.02°C) by a digital temperature programmer (Haake PG 41). The temperature was monitored directly by a platinum resistance microprobe inserted into the cuvette. Most runs were made by continuously heating the protein solution at a rate of 0.2°C/min; lower heating rates and different thermal programs did not produce significant differences in the results obtained. Melting curves were determined at constant wavelength (291 or 300 nm) by recording the first derivative of the difference absorption spectrum as a function of temperature.

Experimental runs to investigate the kinetic behaviour of the thermally deactivated carbonic anhydrase were carried out in a jacketed pirex vessel by incubating 100 ml of an enzyme solution ($6 \cdot 10^{-6}$ M) in the absence of substrate at temperatures ranging from 40 to 70°C. Samples of the solution were periodically withdrawn, rapidly quenched and stored for activity assay.

Measurement of the esterase activity was found to be easier than the determination of the uptake of carbon dioxide. Therefore the activity was evaluated from the hydrolysis rate of the ester *p*-nitrophenyl acetate. To perform such measurements 2.3 ml of the denaturated enzyme solution were reacted at 25°C with a solution of *p*-nitrophenyl acetate in acetonitrile ($1.2 \cdot 10^{-4}$ M). The esterase activity was determined by monitoring the appearance of *p*-nitrophenolate ion at 400 nm, as reported by Pocker and Stone [5].

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3. RESULTS

Figure 1 shows a typical thermal unfolding curve. Similar plots were recorded by temperature-scanning at different wavelengths or by using different enzyme concentrations.

The reversibility of unfolding was carefully checked by cooling down some denaturated enzyme solutions to the pre-transition region and re-measuring their absorbance. For the equilibrium between native (N) and denaturated (D) carbonic anhydrase the two-state model was assumed:

$$N \rightleftarrows D$$

This transition was regarded as a monomolecular chemical reaction with an equilibrium constant equal to:

$$K = \alpha/(1 - \alpha) \quad (1)$$

wher α is the degree of conversion, i.e. the fraction of molecules present in the unfolded conformation. This variable was determined from spectrophotometric data as reported by Pace et al. [6].

The effective enthalpy change on denaturation was calculated from the temperature dependence of K by the van't Hoff equation:

$$(\partial \ln K/\partial T)_p = \Delta H_{VH}/RT^2 \quad (2)$$

The obtained linearized plot is shown in Fig. 2.

The melting temperature, i.e. the temperature at the midpoint of the thermal transition (α=0.5), was derived by setting K=1 in the integrated form of van't Hoff equation. The following values were determined: T_m = 62.5°C, ΔH_{VH} = 257 kcal·mol^{-1}.

In order to provide a quantitative estimate of the conformational stability of the enzyme, the value of ΔG at 25°C was calculated from the equation:

$$\Delta G(T) = \Delta H_{VH} \ (1 - T/T_m) - \Delta c_p \ [(T_m - T) + T \ \ln(T/T_m)] \quad (3)$$

In the absence of reliable calorimetric data the change in heat capacity, Δc_p, was estimated by assigning a contribution of 0.015 kcal·mol^{-1}·K^{-1} to each amino acid residue [7]. We obtained: Δc_p = 3.9 kcal·mol^{-1}·K^{-1}, ΔG(25°C) = 20 kcal·mol^{-1}.

From esterase activity measurements, the fraction of activity left at different denaturation times was determined. This quantity was calculated as the ratio of the activity at a given time to the initial activity (enzyme not denaturated). It is important to point out that all measurements were made at 25°C and pH 7.55, conditions in which the enzyme is stable; therefore the corresponding values of the residual activity account only for irreversible loss of activity. Some deactivation curves are shown in Fig. 3. In Fig. 4 the fraction of activity left after a denaturation time of one hour is also reported.

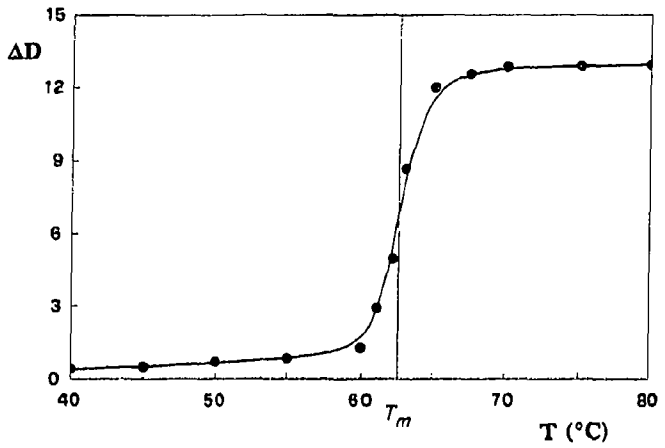

Fig. 1. Thermal unfolding curve (λ=291 nm)

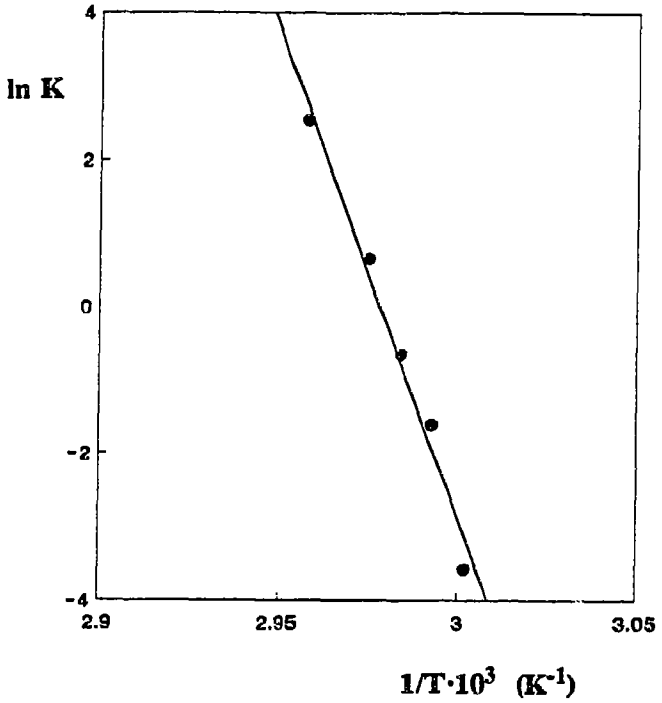

Fig. 2. Van't Hoff plot

4. DISCUSSION

From the analysis of the experimental results the following considerations can be made

(i) Thermal transition between native and partially unfolded carbonic anhydrase is a highly cooperative process, as results from the sharpness of the melting curve (Fig. 1) and from the value of ΔH_{VH}.

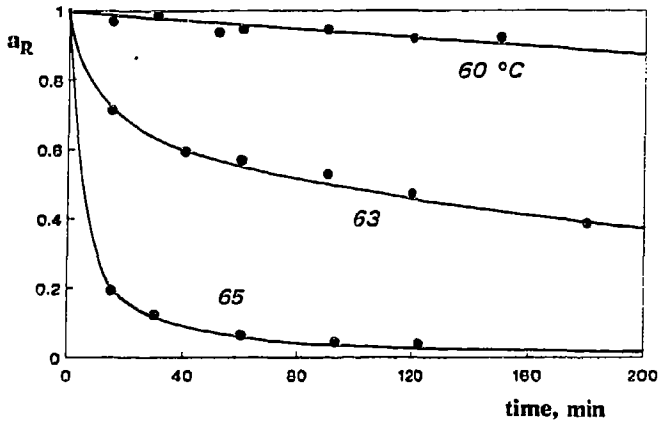

Fig. 3. Residual esterase activity vs denaturation time

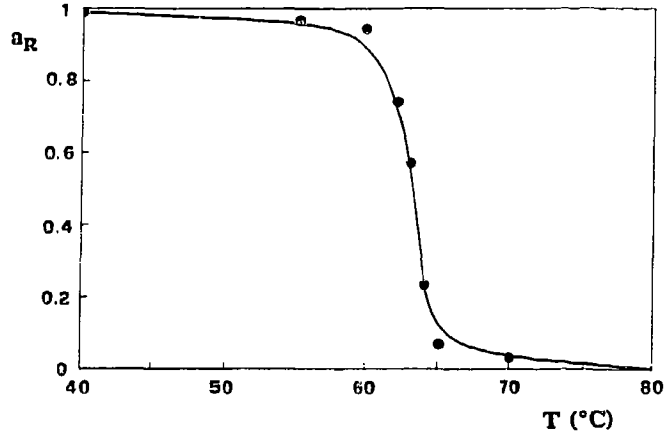

Fig. 4. Residual esterase activity after one hour

(ii) Below 60°C the enzyme recovers almost completely its biological activity, whereas in the range 60–65°C a considerable decrease in the catalytic activity takes place (Fig. 3), above 65°C the residual activity drops to zero after few minutes.

(iii) The melting temperature determined from equilibrium measurements is very close to the temperature at which a steep decrease in the residual activity occurs (see Figs 1 and 4).

As it is known, the former reflects major structural changes, whereas the latter is related to local modifications of the active site region. Therefore the observed loss of activity appears to be a consequence of large conformational changes affecting all the molecule and causing the unfolding of the protein. When the enzyme solution is cooled down to 25°C in order to measure the catalytic activity, the structural rearrangements of the polypeptide chain are likely to result in an uncorrected reconstitution of the active site region. This latter is a spherical cavity, about 15 Å in depth, where the zinc ion coordinates 3 histidinic residues and a solvent molecule in a distorted tetrahedral configuration. A strong suggestion is given from X-ray diffraction studies that an ordered network of 9 water molecules is located in the active site region. This molecular array should play a key role in the enzyme catalysis and perhaps heat-induced phenomena could have made some irreversible changes in its organization giving rise to the observed loss of activity.

Of course further experimental work is needed to elucidate the mechanism of thermal inactivation of carbonic anhydrase. A study of deactivation kinetics is in progress and preliminary results indicate that denaturation follows a complex pattern involving more structural intermediates. It seems relevant to point out that all experimental runs in the present investigation have been carried out in the absence of substrate and do not take into account any influence that substrate may have on enzyme stability. Nevertheless, the experimental evidence appears to suggest that a strengthening of the protein molecule, not specifically aimed at the active site region, could be a simple and effective strategy to minimize the irreversible loss of activity of the enzyme.

REFERENCES

[1] Pocker, Y. and Sarkanen, S. (1978) Adv. Enzymol. 47, 149–275.
[2] Deutsch, H.F. (1987) Int. J. Biochem. 19, 101–113.
[3] Lapanje, S. (1978) Physicochemical Aspects of Protein Denaturation, Wiley and Sons, New York.
[4] Robson, B. and Garnier, J. (1986) Introduction to Proteins and Protein Engineering, Elsevier, Amsterdam.
[5] Pocker, Y. and Stone, Y.T. (1967) Biochemistry 6, 668–678.
[6] Pace, C.N., Shirley, B.A. and Thomson, J.A. (1990) in: Protein Structure – A Practical Approach (Creighton, T.E. ed.) pp. 311–330, IRL Press, Oxford.
[7] Becktel, W.J. and Shellman, J.A. (1987) Biopolymers 26, 1859–1877.