Thermodynamic studies on the hydrolysis of cytidine 2':3'-phosphate by bovine pancreatic ribonuclease A

A possible effect of the change of the structure of water

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The temperature-dependence of the ribonuclease A-catalysed hydrolysis of cytidine 2':3'-phosphate was studied in the range of temperatures 0–40°C. A break at 4°C was found both in the Arrhenius and the van't Hoff plots. It is likely that the transition observed is due to the change in the structure of water.

Bovine pancreatic ribonuclease A (EC 3.1.27.5) is one of the most studied proteins. Its chemical as well as its enzymic properties have been studied by a wide variety of techniques. Among them, the temperature-dependence of the catalysis, with cytidine 2':3'-phosphate as substrate, has been studied by several groups (Marzotto, 1970; Müller & Talsky, 1979; Matheson & Scheraga, 1979). However, these experiments were carried out at temperatures above 6°C. In this work, a study of the temperature-dependence of the ribonuclease A-catalysed hydrolysis of cytidine 2':3'-phosphate in the range 0–40°C is presented. A clear break at 4°C in the van't Hoff plot and a less defined break, at the same temperature, in the Arrhenius plot were found. This temperature suggests a possible influence of the change of structure of water on the kinetic behaviour of the enzyme.

Experimental

Materials

Bovine pancreatic ribonuclease (two times crystallized) was purchased from Cambrian Chemicals (Croydon, Surrey, U.K.). The RNAase A fraction was obtained by the method of Taborsky (1959).

2':3'-Cp was obtained from cytidine 2' (or 3')-phosphate (mixture of isomers) (Cambrian Chemicals) by the method of Szer & Shugar (1963). The purity of the product was checked by means of t.l.c. on cellulose plates with the following solvent

Abbreviations used: RNAase A, bovine pancreatic ribonuclease A; 2':3'-Cp, cytidine 2':3'-phosphate.

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systems: (i) satd. $(\text{NH}_4)_2\text{SO}_4$ solution/propan-2-ol/1M-sodium acetate (78:2:20, by vol.) and (ii) propan-2-ol/ NH_3 ($d = 0.91$)/ H_2O (14:5:1, by vol.). In both cases only a single spot corresponding to the cyclic nucleotide was found.

Methods

All enzymic assays were carried out by using the pH-stat method. An RTS-622 unit (Radiometer, Copenhagen, Denmark) was used and the jacketed reaction vessel was thermostated with circulating ethanol from an WKS cryothermostat (Colora, Lorch, Germany). The temperature was continuously measured with a thermistor (Crison, Barcelona, Spain) with the probe introduced into the reaction mixture. Nitrogen, freed of CO_2 by washing through a 10% NaOH solution, followed by a 0.2M-NaCl solution, was blown over the reaction mixture to avoid changes in the pH due to the CO_2 from the atmosphere. The washing solutions were kept at the temperature of the assay in order to avoid either evaporation or condensation of water. In order to prevent the sticking of RNAase A to the electrodes, these were washed with 1M-HCl and rinsed with large volumes of distilled water.

Solutions of substrate were prepared by dissolving 2':3'-Cp in water. Concentrations were in the range 1–20mM and NaCl was added to keep an ionic strength of 0.2. In this respect the contribution of the substrate to the total ionic strength was taken into account. The concentration of enzyme in the final reaction mixture was always 0.195 μM and was checked spectrophotometrically by using ϵ_{278} 9800 litre·mol⁻¹·cm⁻¹ (Sela & Anfinsen, 1957). The titration was carried out with NaOH prepared from a 50% (w/v) stock solution (carbonate-free) and diluted with 0.2M-NaCl to a concentration of approx.

6.3 mM. The actual concentration was obtained after each set of assays by titration with HCl, which had been previously standardized with HgO according to the method of Vogel (1963).

For the assay, 2.8 ml of the substrate solution as placed in the reaction vessel. After allowing 2 or 3 min for the equilibration of the temperature of the solution, the pH was adjusted to 7.0 by addition of NaOH with the auto-burette. The volume of alkali needed was taken into account for the calculation of the actual substrate concentration. After this, 0.5 ml of enzyme were added and the reaction was followed for 4 to 5 min. Initial velocities were obtained by drawing the tangent to the linear portion of the progress curve. Assays were carried out in duplicate or triplicate and eight different concentrations of substrate, at each temperature, were used to construct the Lineweaver-Burk graphs which showed no apparent deviations from linearity. The kinetic parameters were obtained by linear regression and in all cases the regression coefficient r^2 was higher than 0.984. The errors for both the slope and the intercept were obtained by using the formulae of Puigdomènech (1974).

Results and discussion

Fig. 1 shows the Arrhenius (a) and van't Hoff (b) plots for the RNAase A-catalysed hydrolysis of

2':3'-Cp at pH 7.0 and $I = 0.2$ in the range of temperatures 0–40°C.

A clear break in the van't Hoff plot is seen in the region around 4°C. A break in the Arrhenius plot also appears in the same region. The thermodynamic parameters at 1°C and 21°C (one at each side of the break) calculated according to Eyring's absolute reaction-rate theory (Dixon & Webb, 1979) are given in Table 1.

The changes found around 4°C can be interpreted as due, among other causes, to a change either of the enzyme conformation or of the enzyme environment, i.e. the solvent. This latter explanation is strongly supported by the fact that the breaks in both plots are found around 4°C. At this temperature, a transition in the structure of water occurs and, according to Eyring's significant-structure theory of liquids (Barker, 1971), an ice III-like structure appears together with clusters of ice I-like molecules and fluidized vacancies. Indeed, such a change in the solvent structure might induce a change in the protein conformation. It has been pointed out (Douzou, 1977) that at low temperatures the protein has a tighter structure because of the formation of closer intramolecular bonds. According to the results shown in Fig. 1(b), in the range 4–40°C, there is an increase in the affinity for the substrate as the temperature decreases. In the range 0–4°C, this trend is reversed.

Fig. 1. The temperature-dependence of the RNAase A-catalysed hydrolysis of 2':3'-Cp at pH 7.0, $I = 0.2$; (a) Arrhenius plot and (b) van't Hoff plot

Table 1. *Thermodynamic parameters of the RNAase A-catalysed hydrolysis of 2':3'-Cp*
The temperatures chosen are those at each side of the breaks found in Fig. 1(a,b); pH 7.0 and $I = 0.2$.

Temperature (°C)	$\Delta H^\ddagger$ (kJ·mol ⁻¹)	$\Delta G^\ddagger$ (kJ·mol ⁻¹)	$\Delta S^\ddagger$ (kJ·kg ⁻¹ ·K ⁻¹)	ΔH° (kJ·mol ⁻¹)	ΔG° (kJ·mol ⁻¹)	ΔS° (kJ·kg ⁻¹ ·K ⁻¹)
1	-9.45 ± 0.06	61.4 ± 0.1	-790 ± 2	56 ± 1	-12.1 ± 0.2	755 ± 18
21	17.6 ± 0.7	65.0 ± 0.1	-492 ± 9	-27.1 ± 0.6	-12.1 ± 0.3	-156 ± 9

The highest degree of complementarity between enzyme and substrate is found at 4°C. In addition, the change in the structure of water is likely to have a significant effect on the hydrophobic bonds (see, e.g., Scheraga, 1979). Some contribution from the changes in this type of interaction, which is known to play an important role in the binding of the substrate (Richards & Wyckoff, 1971), is to be expected.

The treatment has assumed that K_m is equivalent to K_s . Although this is so at 25°C (Hammes & Schimmel, 1965) it has not been confirmed for the lower temperatures. The change in the sign of ΔH° is most surprising and, before any other evidence is available, could be indicative that the assumption $K_m = K_s$ is not valid in the lower range of temperatures (0–4°C).

On the other hand, the values found for $\Delta H^\ddagger$ at 1°C should be taken with great care because, as they are, they would mean a negative activation energy. Although the existence of the break in the Arrhenius plot is very likely, the differences are near the margins of experimental error and, therefore, both the magnitude of the break and the actual slope of the low-temperature region are difficult to assess. It is proposed that, in the region 0–4°C, there is a qualitative decrease in the activation energy. It is likely that this phenomenon is restricted to pure aqueous solutions and thus it is not seen in some enzymic reactions carried out in mixtures of organic solvents and water (Douzou, 1977). The increase in K_{cat} in the range 0–4°C could be partially explained by the fact that at low temperatures the rate of proton transfer across the very structured water molecules is much faster than at higher temperatures (Barker, 1971). This would be important in a system subject to general acid–base catalysis (Findlay *et al.*, 1962) in which water is one of the reactants.

A possible contributing factor to the break observed in the van't Hoff plot is the temperature-dependence of the pK of some ionizable group involved in substrate binding. However, it seems

very unlikely that such a change can be the sole cause of the break and even more unlikely that it should change the sign of the slope in such an abrupt manner as is observed in Fig. 1(b). The study of the pH-independent kinetic parameters at different temperatures may be very useful in determining the nature of the observed transition.

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