THE EFFECT OF ULTRASOUND AS A NEW METHOD OF STUDYING CONFORMATIONAL TRANSITIONS IN ENZYME ACTIVE SITES

pH- and temperature-induced conformational transitions in the active centre of penicillin amidase

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1. Introduction

In [1] we offered a new method of studying the conformational changes in the active centres of enzymes. The essence of the method can be summed up as follows. Ultrasonic cavitation has been shown to produce hydroxyl and hydroperoxyl radicals in water solution [2]. The penetration of these radicals in an enzyme active centre results in the enzyme being inactivated due to destruction of certain functional group important for catalytic activity (in the case of α-chymotrypsin the loss of the catalytic activity is due to destruction of the tryptophan-215 residue of the enzyme active centre [1]). It has been shown that the first-order rate constant for the inactivation of enzymes by ultrasonic cavitation* is a parameter sensitive to the conformational state of the enzyme active centre. This is due to the fact that the accessibility of the above mentioned functional groups for the free radicals, generally speaking, changes with conformational changes in the enzyme active centre (induced by pH, temperature, added effectors etc.). Thus, the kinetic study of enzyme inactivation by ultrasonic cavitation allows us to determine the parameters of the conformational changes in the enzyme active centre. It should be pointed out that the advantage of the 'ultrasound method' over the well-known 'reporter group method' is that the former does not demand any 'reporters' (chromophores, as well as fluorescent, paramagnetic or other probes) in an enzyme active centre, which may themselves perturb the conformation of the centre. Finally, with the aid of this method one can investigate changes occurring just in the active centre of an enzyme which is the most interesting for mechanism of catalysis.

The present work is concerned with the study of penicillin amidase (penicillin amidohydrolase, EC 3.5.1.11) from *E. coli*. This enzyme catalyzes hydrolysis of various penicillins to yield carboxylic acid and 6-aminopenicillanic acid which is used as the nucleus for semisynthetic penicillins [3,4]. Here we report the results of investigation of pH and temperature influence on the properties of penicillin amidase by means of both kinetic and ultrasound methods.

2. Materials and methods

Penicillin amidase from *E. coli* was isolated and purified as described previously [5]. Benzylpenicillin and potassium chloride were special grade reagents from commercial sources. The kinetics of penicillin amidase-catalyzed hydrolysis of benzylpenicillin was studied using a Radiometer pH-Stat (TTT-1c, Denmark) by titrating the acid product with potassium hydroxide (0.01 N). The reaction was carried out in thermostated cells at an ionic strength of 0.1 M (KCl), no buffer was added. In all the experiments the control for spontaneous hydrolysis of benzylpenicillin was carried out, and correction in some cases (e.g. at the extreme pH values) was introduced.

Ultrasound was generated by a UTS-1M 880 kHz generator. The working intensity was 2 W/cm^2. The

* Below by the effect of ultrasonic cavitation on the enzyme we shall mean the action of ultrasound induced free radicals on the enzyme.

sonication vessel containing 10 ml of the enzyme solution (1×10^{-7}M) was carefully thermostated, during sonication the temperature did not rise by more than 1°C. The activity of penicillin amidase during the ultrasound treatment was determined by aliquots being withdrawn and introduced into the pH-Stat cell containing benzylpenicillin solution (4.4×10^{-3}M) at pH 7.5, 25°C, 0.1 M KCl. Under these conditions the concentration of the substrate is much higher than the Michaelis constant for the reaction (*vide infra*), so the enzyme is practically saturated with the substrate.

In the present work we were mostly interested in the pH and temperature action which produce a specific effect on the ultra sound inactivation of penicillin amidase. This was exemplified in our work by sonication of proflavine solution [1]. Thereby the factors were considered as specific when they did not affect the process of decoloration of proflavine solution [6], but did affect the first-order rate constant for the ultrasound inactivation of penicillin amidase.

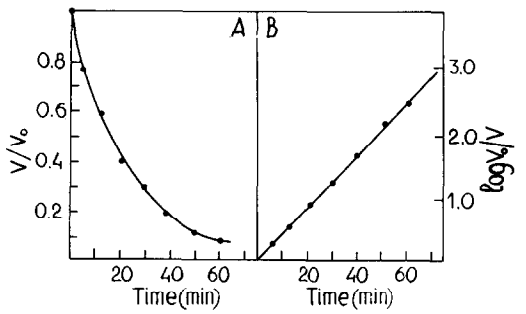

Fig. 1. A) Kinetics of penicillin amidase inactivation under ultrasonic cavitation. B) Semilogarithmic plot of curve A. 45°C, 0.1 M KCl, 0.012 M phosphate buffer, pH 7.5, $[E]_0 = 1 \times 10^{-7}$ M.

3. Results

3.1. The kinetics of penicillin amidase-catalyzed hydrolysis of benzylpenicillin

The investigation of the kinetics of benzylpenicillin hydrolysis catalyzed by penicillin amidase was carried out by means of both the integrated method of analysis of progress curves and the analysis of initial rates of the reaction and will be reported elsewhere [5]. In contrast to the published data [7,8], we found a very low value of the Michaelis constant for this reaction ($K_m = 3.1 \times 10^{-6}$M at pH 7.5 and 25°C). It appears that previous workers did not take into consideration the strong competitive inhibition of the penicillin amidase by the product of the hydrolysis, phenylacetic acid ($K_i \simeq 2.8 \times 10^{-5}$M) which is effective even at the initial period of the enzymic hydrolysis (cf. [5]). The catalytic constant for the penicillin amidase-catalyzed hydrolysis of benzylpenicillin is equal to 16.4 sec^{-1} (pH 7.5, 25°C).

3.2. The kinetics of penicillin amidase inactivation under ultrasound action

Ultrasonic cavitation induces the irreversible inactivation of penicillin amidase (fig. 1A), through the destruction of some functional group which is indispensable for the catalytic activity of the enzyme. For these reactions, first-order rate constants (k_{in}) were obtained from semilogarithmic plots of the relative velocity against time in the usual way (fig. 1B). It was shown in separate experiments that the k_{in} values obtained in this way do not depend on the initial concentration of the enzyme.

3.3. The pH-dependence of catalytic activity and of the ultrasound inactivation rate for penicillin amidase

Fig. 2A shows the pH-dependence of the maximum velocity (V_{max}) for the penicillin amidase-catalyzed hydrolysis of benzylpenicillin. The pH-rate profile is a bell-shaped curve with a maximum at pH 8.15. These

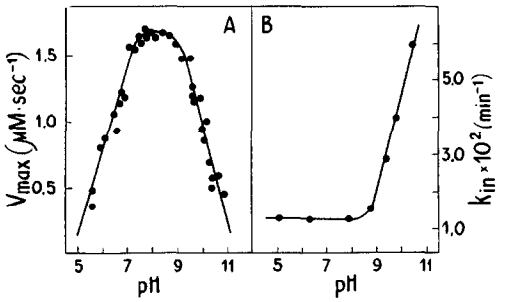

Fig. 2. A) pH-dependence of maximum velocity for the penicillin amidase-catalyzed hydrolysis of benzylpenicillin. 25°C, 0.1 M KCl, $[S]_0 = 4.4 \times 10^{-3}$ M, $[E]_0 = 1 \times 10^{-7}$ M. B) pH-dependence of the rate constants for inactivation of penicillin amidase by ultrasonic cavitation. 25°C, 0.1 M KCl, 0.012 M combined buffer (acetate phosphate and borate).

data agree reasonably well with the kinetic scheme according to which there are three equilibrium forms of the enzyme, 'acid', 'neutral', and 'base' of which only the 'neutral' form is active towards the substrate. The reversible transition between the 'acid' form and 'neutral' one of penicillin amidase is controlled by an ionogenic group of pK= 6.1. The transition between 'neutral' and 'base' forms depends on an ionogenic group of the enzyme–substrate complex with pK = 10.2.

Fig. 2B shows the pH dependence of k_{in}. It is seen the k_{in} values are practically independent of pH between pH 5 and 8.5, but sharply increase above pH 9. These data can be described by a kinetic scheme according to which there are two equilibrium states of penicillin amidase, 'neutral' and 'base' ones, the latter being inactivated by ultrasonic cavitation much faster than the former. The reversible transition between 'neutral' and 'base' forms of penicillin amidase is controlled by an ionogenic group of the free enzyme of pK= 9.9.

3.4. Temperature dependence of catalytic activity and of the ultrasound inactivation rate for penicillin amidase

As seen in fig. 3A, the logarithmic dependence of a maximum velocity for the enzyme reaction on reciprocal temperature consists of two straight lines merging into each other. Such a 'break' in the Arrhenius co-ordinates, is usually interpreted as meaning two equilibrium forms of an enzyme – 'low temperature' and 'high temperature', the activation energy of corresponding enzymatic reaction being different. Basing on the data of fig. 3A it may be concluded that the reversible transition between the enzyme forms occurs in the temperature interval between 43° and 48°C.

Fig. 3B shows the temperature dependence of k_{in}. It can be seen that k_{in} values are practically independent of temperature between 20° and 35°C and above 52°C, but sharply (approx. 7 times) rise in a narrow temperature interval between 40° and 50°C. In this interval in absence of ultrasound treatment the enzyme activity does not change significantly at least the rate of inactivation in absence of cavitation can be neglected. These data can be explained as being a reversible conformational transition in the active centre of penicillin amidase occuring in this temperature range. If we suppose that there is only the former form of the enzyme at t<35°C, and only latter form at t>52°C, the corresponding first-order rate constants for the inactivation

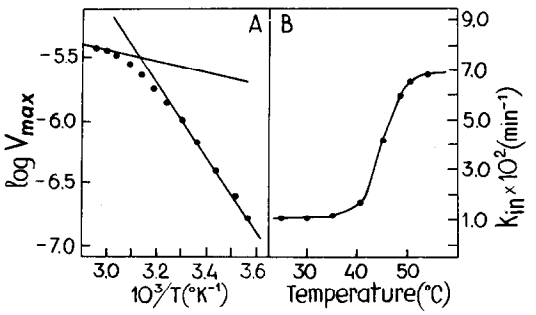

Fig. 3. A) Arrhenius plot of maximum velocity for the penicillin amidase-catalyzed hydrolysis of benzylpenicillin. pH 7.5, 0.1 M KCl, $[S]_0 = 4.4 \times 10^{-3}$ M, $[E]_0 = 4.2 \times 10^{-8}$ M. B) Temperature dependence of the rate constants for inactivation of penicillin amidase by ultrasonic cavitation 0.1 M Kcl, 0.012 M combined buffer, pH 7.5.

being not strictly temperature-dependent (see fig. 3B) we can determine the thermodynamic parameters of the conformational transition. At the temperature of conformational change $T=T_c$(i.e. temperature at which the concentrations of the conformers are equal) the following relationships valid [1]:

$$\Delta H = \frac{4RT^2}{\Delta k_{in}} \times \frac{dk_{in}}{dT} \Bigg|_{T=T_c}$$

$$\Delta S = \frac{\Delta H}{T_c}$$

where ΔH and ΔS are the enthalpy and entropy of the conformational transition, and Δk_{in} is the difference between k_{in} values corresponding to the 'high temperature' and 'low temperature' states of the enzyme, respectively. From these relationships the values ΔH = 81 kcal/mol and ΔS = 255 e.u. have been determined for T_c= 318°K (45°C) (fig. 3B).

4. Discussion

From the pH dependence of both the maximum velocity for the penicillin amidase reaction and the first-order rate constant for inactivation of the enzyme (fig. 2) it is seen that an ionogenic group of the enzyme active centre of pK~10 controls the properties of penicillin amidase (some difference between the pK

values determined from hydrolysis and inactivation data apparently arise from the fact that pK = 10.2 refers to the enzyme–substrate complex and pK = 9.9 refers to the free form of the enzyme). The sharp increase in k_{in} when that group is deprotonated can be interpreted as an 'opening' of the enzyme active centre, which becomes accessible to free radicals (for example, as a result of destroying a salt bridge which can be responsible for maintaining the catalytically active conformation of the enzyme, as it was observed with α-chymotrypsin [1]). On the contrary the ionogenic group of pK = 6.1 does not cause any change in the pH dependence of k_{in}, i.e. ionization of this group does not lead to any noticeable conformational change in the penicillin amidase active centre but renders the enzyme inactive towards benzylpenicillin. This ionogenic group may be, for exemple, a histidine imidazole residue, in analogy with α-chymotrypsin, where protonation of this group renders the enzyme catalytically inactive but does not change the conformation of the α-chymotrypsin active centre [1].

Evidently, if one proceeds from the kinetic data only (fig.3A) it is not possible to calculate ΔH and ΔS for the conformational transition in the penicillin amidase active centre or determine exactly temperature T_c for that transition. At the same time, it can be done by means of the 'ultrasound method'. The rather high ΔH and ΔS values and the narrowness of the temperature interval in which the conformational transition occurs (see above) testifies to the transition being co-operative.

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