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Radiat Environ Biophys (1990) 29:47–56

Radiation and Environmental Biophysics

Low intensity ultrasonic effects on yeast hexokinase

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Received June 1, 1989 / Accepted in revised form September 19, 1989

Summary. The kinetics of yeast hexokinase activity exposed to 1 MHz ultrasound of therapeutic intensities 0.1–1.5 W/cm^2 was studied using traditional physico-chemical methods and by the thermoinactivation approach. Analysis of the kinetic curves and the kinetic parameters, obtained by two independent methods, suggested specific perturbation processes provoked by the ultrasonic waves, viz., the mechanical breakdown of the contact site between monomer units and the subsequent sonochemical modification of the active enzyme site. Low intensity ultrasound also caused the destabilization of the molecular structure of hexokinase as revealed by the apparent thermolability of the sonicated enzyme.

Introduction

The purpose for studying ultrasonic effects on enzymes is associated with the possibility for ultrasonic initiation of structural perturbations and related biocatalytic functions, including effects on different stages of enzymatic catalysis.

Thermal inactivation of enzymes is associated with sequential structural alterations which can be investigated by the kinetic study of protein thermostability using methods developed by Gianfreda et al. (1985) and Poltorak and Chukrai (1987). These methods are of importance specifically for the study of proteins with tertiary structure, for example, yeast hexokinase which has been investigated by Furman and Neet (1983). As was shown (Poltorak and Chukrai 1987), under the influence of temperature, this enzyme undergoes dissociative breadown followed by irreversible denaturation.

Low intensity ultrasound can produce pronounced inactivating effects on enzymes in solution due to mechanical and sonochemical mechanisms (Elpiner 1964; Macleod and Dunn 1967; Braginskaya 1975; Schmidt et al.

1988; Kashkooly et al. 1980; Dunn 1985). However, data concerning changes in tertiary protein structure, caused by ultrasound, and resulting in alterations to subsequent temperature effects, appear not to have been explored. Hexokinase is suitable for such a study as the combined possible effects on its activity and on its thermostability should provide new information on physico-chemical mechanisms of inactivation of globular enzymes having tertiary structure. The present study was undertaken to investigate the kinetics of the globular oligomeric enzyme hexokinase induced by therapeutic ultrasound at ultrasonic intensities in the range 0.1 to 1.5 W/cm^2 using two independent approaches, viz., the traditional Menten kinetics and the thermoinactivation method.

Materials and methods

Prepared materials were obtained as follow: yeast hexokinase (Fluka); glucose-6-phosphate dehydrogenase from Baker's yeast (G-6-PDG) (Ferak, Berlin, Germany); monosodium salt of NADP, disodium salt of ATP, α-D-glucose (Reanal).

Determination of hexokinase enzymatic activity

The activity was determined using the accessary enzyme G-6-PDG in accordance with the scheme illustrated in Fig. 1. As the result of reactions (1) and (2), the reduced form of NADPH is produced with absorptivity at 340 nm.

The enzymatic reaction was carried out in quartz cuvettes placed in the temperature controlled 2.5 ml chamber maintained at 20° C, in 0.2 *M* borate buffer at pH 8.0. The composition of the reaction mixture was 0.4 ml of hexokinase solution in 0.05 *M* glycine buffer, pH 8.6 and 0.5 ml of the "operating mixture" in the borate buffer, pH 8.0, which had the following composition: 1.44 10^{-3} *M* NADP; 2.7 10^{-3} *M* ATP; 2.5 10^{-3} *M* MgCl;

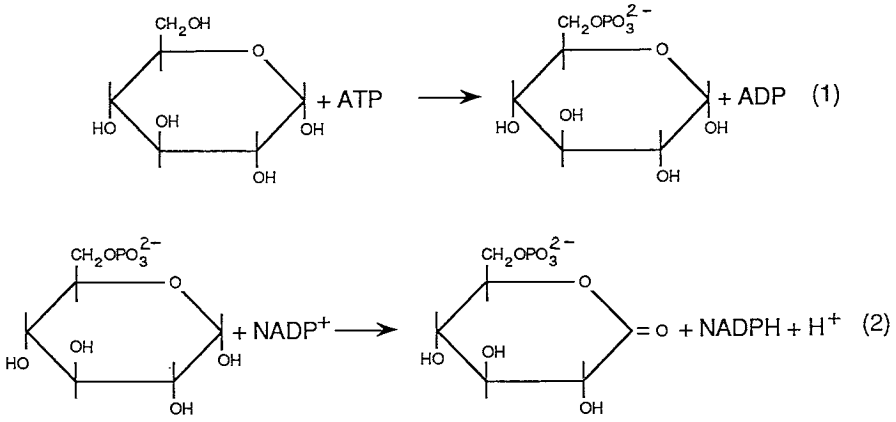

Fig. 1. Scheme describing hexokinase enzymatic activity

Statistical treatment of the experimental data

The statistical analysis was carried out using the microcomputer "Torch-725". Linear regression for the linear portions of the curves yielded mean values of dispersion of no more than 6.0×10^{-2} and correlation coefficients with minimum values of 0.98 were estimated. The accuracy for the obtained velocity of the hexokinase reaction was estimated as 3% and 10%, respectively, for the effective Michaelis constant (K_M) and for the effective velocity of the enzyme inactivation at 40° C ($K_{\text{inact.}}$).

Results and discussion

Figure 2A shows the dependence of the relative activity on the sonication time at different intensities in the range 0.2 to 1 W/cm^2 (curve 1) and at 1.5 W/cm^2 (curve 2). It was observed that ultrasound produced partial (10–12%) loss of hexokinase catalytic activity nearly independent of intensity up to approximately 1 W/cm^2 (curve 1). The ultrasonic inactivation effect is significantly greater at the 1.5 W/cm^2 exposure (curve 2). Figure 2B represents the corresponding results of sonochemical oxidation of KI, for the

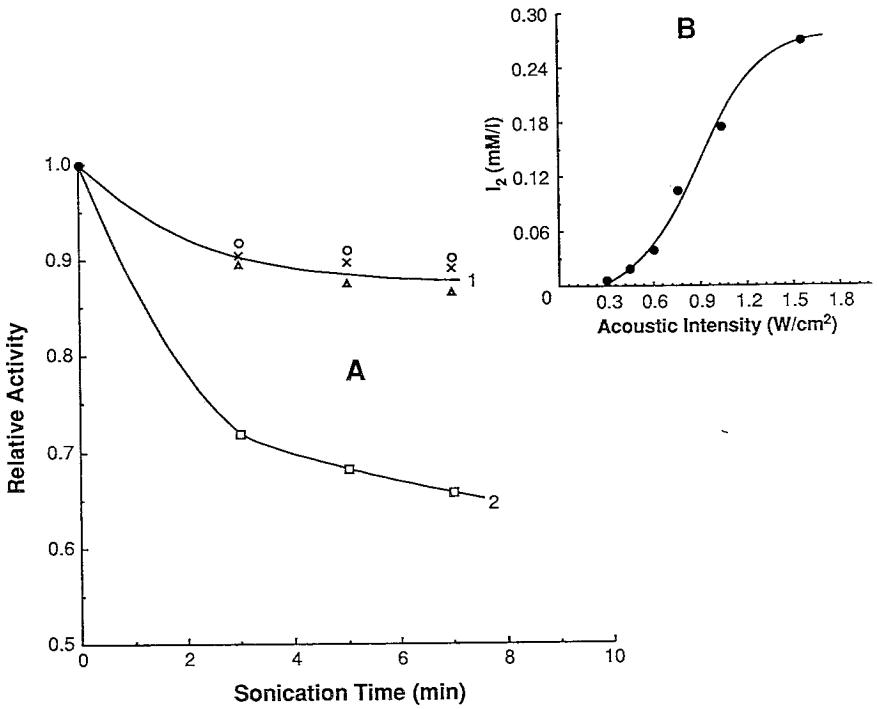

Fig. 2. A Sonication time dependence of relative hexokinase activity, A, at different intensities. curve 1: 0–0.2 W/cm^2, ×–0.4 W/cm^2, ▵–1.0 W/cm^2; curve 2–1.5 W/cm^2. E_0 0.2 mg/ml, 0.05 *M* glycine buffer, pH 8.6, 20° C. **B** Dependence of free iodine formed during the oxidation of potassium iodide, KI, on intensity of ultrasound for exposures of 5 min. KI concentration 0.5 *M*

same samples, carried out to aid in the elucidation of the mechanical or sonochemical mechanisms provoking the hexokinase results.

As can be seen from Fig. 2B, the chemical activity of cavitation is minimal at intensities of 0.2–0.4 W/cm^2. A significant increase of the radicals and sonochemical products was obtained in the sonicated media at intensities beyond 0.8–1.0 W/cm^2. Similar quadratic dependences have been observed, in addition to [I^-], also for [Cl^-] and [H_2O_2] formations under conditions of transient cavitation (Margulis 1984).

As seen from Fig. 2A, no difference was observed for the enzymatic activity decrease in the absence of sonochemical factors (0.2–0.4 W/cm^2), on one hand, and in the presence of some sonochemical activity (0.8–1.0 W/cm^2), on the other hand (curve 1). The inactivation effect became significantly greater at 1.5 W/cm^2 (curve 2) when sonochemical oxidation of potassium iodide reached critical values (Fig. 2B). The data support the view that the decrease of hexokinase activity up to 10% resulted mainly from mechanical damage of acoustic streaming, with shearing stresses of 10^3 s^{-1} and 0.5×10^2 dynes/cm^2 (Dunn 1985) and from mechanical stresses of cavitation bubbles (Elpiner 1964). At the intensity of 1.5 W/cm^2, the sonochemical effects increase sharply with a definite exponential loss of hexokinase activity (see curve 2, Fig. 2A). This is in agreement with mechanochemical inactivations of other enzymes in solution, e.g., lysozyme, malatedehydrogenase, lactatedehydrogenase, proteolytic enzymes (Macleod and Dunn 1967; Schmidt et al. 1988; Kashkooly et al. 1976; Braginskaya and Zorina 1987; Klybanov et al. 1976) and of other biological structures (Braginskaya 1975; Braginskaya and Dunn 1981).

In order to study the specific catalytic changes of sonicated hexokinase in relation to enzyme-substrate interactions, the Michaelis-Menten kinetic approach was used. The kinetic characteristics, i.e., the effective maximum rate (V_{max}) and the effective Michaelis constant (K_M) of the hexokinase reaction before and after sonication were calculated by extrapolation of the Lineweaver-Burk dependence of the log-log plot of the reciprocal values $1/V$, $1/S$ (V – rate of enzymatic reactions, S – substrate concentration). The data calculated from the corresponding experimental curves are presented in Table 1. It can be seen that sonication resulted in changes to both K_M and V_{max}.

Table 1 shows that the increase of K_M depends upon intensity of sonication, which is indicative of the decrease of enzyme-substrate affinity for

Table 1. Effective values of K_M and V_{max} for native and sonicated hexokinase. Concentration 0.2 mg/ml, time of sonication 5 min. 0.05 M glycine buffer, pH 8.6, 20° C

Intensity (W/cm^2)	$K_M \times 10^3$ (M)	$V_{max} \times 10^2$ (min^{-1})
0	3.0±0.3	4.5±0.4
0.2	3.6±0.3	4.0±0.4
0.4	5.4±0.6	3.0±0.3
1.0	5.4±0.6	3.2±0.3
1.5	6.3±0.6	2.9±0.3

soluted enzymes at the conditions of mechanochemical destruction. The simultaneous decrease of V_{max} can be indicative both of the partial diminution of active sites and of the structural alterations resulting in the decrease of the enzyme-substrate complex breakdown. As it follows from the analysis of the chemical factors prevailing (see Fig. 1 B), the first possibility can take place at the intensities greater than 0.4–0.6 W/cm^2 and the second at the conditions prior to the onset of transient cavitation, viz., 0.2–0.4 W/cm^2. The revealed effects can be related to the oxidation of SH-groups responsible for the stability and catalytic activity of native structure of hexokinase (Wilson 1983).

In order to examine the ultrasonic effect on the tertiary structure of hexokinase by means of oligomer association-dissociation processes, the kinetics of the dependence of the specific activity of the enzyme on its initial concentration E_0 was used by the approach proposed by Poltorak and Chukrai (1987). For the hexokinase solutions, the association-dissociation equilibrium exists depending on concentrations:

$$\underset{\text{associated form}}{E_2} \underset{}{\overset{K_{ass}}{\rightleftharpoons}} \underset{\text{monomer}}{2E_1}$$

Figure 3 shows the dependence of the specific activity A_{spec} on initial enzyme concentration. It is seen that for the native enzyme (curve 1), increase of concentration is accompanied by the decrease of A_{spec} which is related to the increase of the associated form which has lesser activity

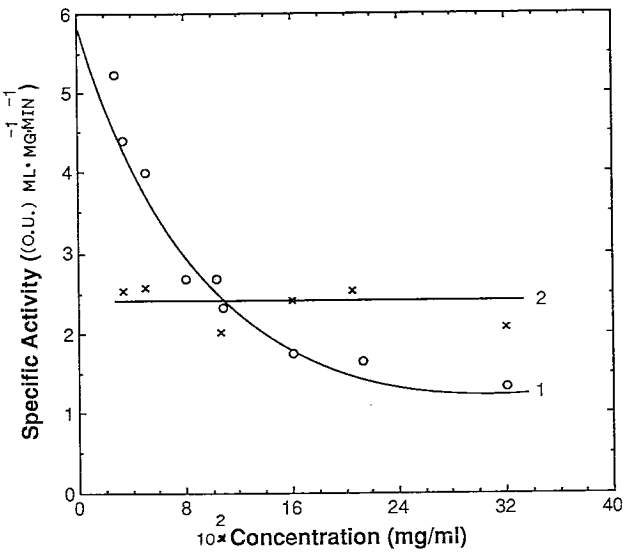

Fig. 3. Dependence of specific hexokinase activity. A_{spec}, on initial enzyme concentration: curve 1 – control solution; curve 2 – sonicated 5 min at intensity 0.4 W/cm^2; the same conditions as in Fig. 2A

(Furman and Neet 1983). Sonication of hexokinase resulted in no dependence of A_{spec} with increasing concentration (curve 2).

The decrease of A_{spec} of the sonicated hexokinase at low concentration, compared to the native enzyme, can be related to the partial diminution of active sites or to structural alterations in protein globula, which was also estimated by the above analysis of Michaelis-Menten kinetics (see Table 1).

The increase of A_{spec} at high concentrations of hexokinase (>0.1 mg/ml) resulted from the loss of capacity of active monomers to form the inactive enzyme associates. This shows that breakdown of the associated form of the protein occurs in the ultrasonic field, i.e., destruction of the contact sites between subunits occurs, which disturbs association-dissociation equilibrium.

In order to demonstrate ultrasonic effects on association equilibria, the thermoinactivation method (Poltorak and Chukrai 1987) was used. The thermoinactivation process at 40° C occurs in two stages. The first stage corresponds to the reversible breakdown of hexokinase associates to subunits and the latter, during the second stage, undergo irreversible alterations accompanied by loss of enzymatic activity (Fig. 4).

Figure 5 shows the experimental data expressed as the logarithm of the ratio of reaction speeds vs first-order coordinates for the kinetics of thermoinactivation time of exposure to 40° C for different preceding 5 min ultrasound exposures. The native hexokinase exhibits the two-stage reversible character of thermoinactivation, as seen by the break following the plateau of curve 1. No breaks occur for the sonicated enzyme solutions at all intensities of exposure. These results support the view expressed above that sonication perturbs the equilibrium between the associated and monomeric forms of hexokinase, at constant concentration, which resulted from possible ultrasonic breakdown of the contact site between monomer subunits. Therefore, the curves of thermoinactivation of the sonicated protein obey the single-stage inactivation mechanism, with no breaks appearing (Fig. 5). The rate of thermoinactivation, calculated from the slopes of the experimental curves 2–4, depends upon the intensity at which hexokinase was treated prior to the thermoinactivation procedure. The effective constants K_{inact} of thermoinactivation, which characterize enzyme thermostability after sonication at different intensities are shown in Table 2.

It should also be noted that the kinetic study of hexokinase thermoinactivation also reveals the destabilization of hexokinase macromolecules in-

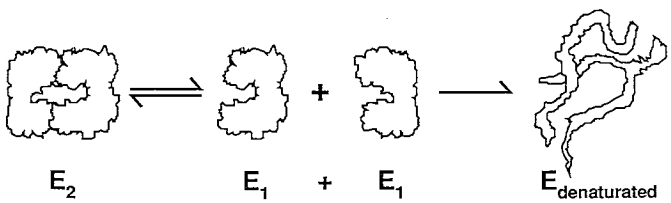

Fig. 4. Thermoinactivation. Stage 1: reversible breakdown. Stage 2: irreversible breakdown, loss of enzymatic activity

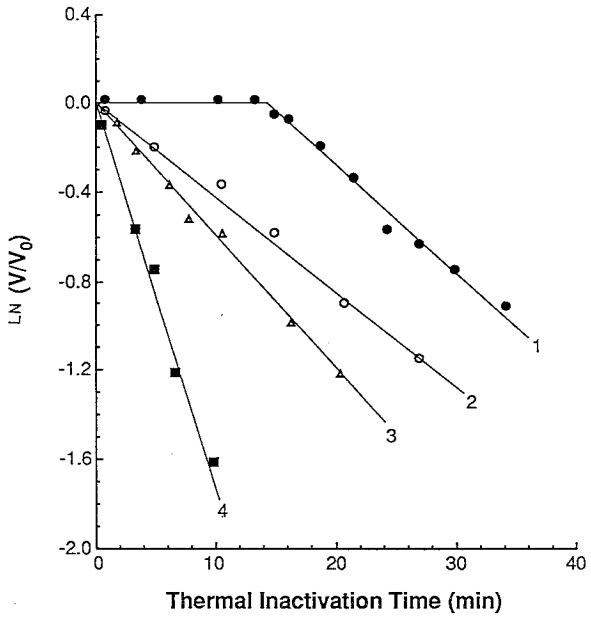

Fig. 5. Kinetics of thermoinactivation of hexokinase $E_0 = 0.2$ *mg/ml* at 40° C: curve 1 – control solution; curves 2–4 – sonicated 5 min at intensities 0.2 W/cm^2, 1.0 W/cm^2 and 1.5 W/cm^2 correspondingly at the same conditions as in Fig. 2A. The ordinate symbols are given in the Materials and methods section

Table 2. Ultrasound induced effects on hexokinase thermostability at different intensities. Concentration 0.2 mg/ml, T = 40° C

Intensity (W/cm^2)	Time of sonication (min)	$K_{inact} \times 10^2$ (min^{-1})
0.2	5	4.0
	7	4.4
0.4	5	4.1
	7	4.4
1.0	5	5.7
	7	7.2
1.5	5	10.5
	7	16.6

duced by ultrasound which was not apparent from the direct activity measurement. Thus, as was shown, there were no differences between the extent of the decrease of hexokinase activity in the solutions sonicated at intensities 0.2 and 0.4 (no cavitation present) and 1 W/cm^2 (transient cavitation; see curve 1, Fig. 2). However, at the conditions of cavitation, the different thermostabilities of the sonicated solutions were obtained (curves 2–4, Fig. 5, Table 2). It is seen that the mechanical factors of the ultrasonic field

(0.2–0.4 W/cm^2) had no influence on the thermostability of the sonicated solutions of hexokinase; there were practically no differences in K_{inact} at 40° C (Table 2). But, though the activity measurements did not reveal the differences between mechanical and relatively low sonochemical inhibiting effect (the same curve 1 for both 0.2–0.4 W/cm^2 and 1 W/cm^2, Fig. 2), the thermal stability was different in the sonicated samples by virtue of the increase in K_{inact} (Table 2). The formation of thermolabile protein structures in solution became essentially greater after the increase of sonochemical effects at 1.5 W/cm^2 (the sharp increase of thermoinactivation K_{inact}, Table 2).

Conclusion

The results presented herein yield the following for the low amplitude ultrasound induced effects on the tertiary structure of protein yeast hexokinase:

a. Partial inactivation resulted from the mechanical breakdown of the contact site between monomeric units and the perturbation of association-dissociation equilibria, confirmed by two independent methods. In the conditions of the increase of sonochemical factors, loss of capacity of enzyme-substrate complex formation occurs caused by sonochemical modifications of functional groups at the active sites of the hexokinase monomers; and

b. the specific destabilization of hexokinase molecular structure, not being revealed by direct measurements of activity, occurs as the appearance of thermolability of the sonicated solutions.

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