AFFINITY LABELLING OF YEAST HEXOKINASE WITH BENZYLAMIDE DERIVATIVES OF ADENOSINE MONO- AND TRIPHOSPHATES BEARING AN ALKYLATING GROUP

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1. Introduction

Yeast hexokinase (hexose:ATP-6-phosphotransferase, EC 2.7.1.1) exists as two isoenzymes PI and PII. Both isoenzymes were found to dimerize at neutral and slightly acid pH [1]. It was shown that dimerization results in the decrease of specific activity. Slow-burst-type kinetics were demonstrated to be typical of the dimeric enzyme, which was interpreted as resulting from some conformational change accompanying the catalytic action of the enzyme [2]. These differences between the behaviour of dimer and monomer were suggested to be due to some conformational transition taking place in the course of association–dissociation [3].

The affinity labelling kinetics were expected in some cases to be highly sensitive to the local change of conformation of a biopolymer in the vicinity of the reactive group of the label within the specific complex [4]. Therefore, we have studied the reaction of PI isoenzyme of yeast hexokinase with two alkylating reagents: 4-(*N*-2-chloroethyl-*N*-methylamino)-benzylamides of ATP; and AMP ($ClRCH_2NHpppA$ and $ClRCH_2NHpA$) in the conditions favouring either the monomeric or dimeric form of hexokinase. We found that the dimer and monomer exhibit sharp difference towards the alkylation with these reagents. The monomer is efficiently inactivated with $ClRCH_2$-NHpA and is completely insensitive towards the ATP derivative and, vice versa, the dimer is readily inactivated by $ClRCH_2NHpppA$ and does not react with the AMP derivative.

2. Materials and methods

Crude hexokinase from NPO Biokhimreactiv (Latvian SSR) or Fluka pract. (FRG) was purified by DE 52 chromatography and G-100 gel filtration according to [1]. The purified enzyme was 150–250 units/mg (1 unit of enzyme activity is defined as the amount of enzyme which catalyses the conversion of 1 μmol ATP/min at 30°C). The ratio of the rates of phosphorylation of D-fructose and D-glucose was 2.7 thus indicating that PI isoenzyme was mainly present in commercial samples of the enzyme [5].

4-(*N*-2-chloroethyl-*N*-methylamino)-Benzylamide of ATP was prepared as in [6] and AMP 4-(*N*-2-chloroethyl-*N*-methylamino)-benzylamide was prepared as in [7]. These compounds contained 80–85% of the reactive Cl atoms [8]. Hydroxyanalogs of 4-(*N*-2-chloroethyl-*N*-methylamino)-benzylamides of AMP and of ATP were prepared by hydrolysis of respective chloroanalogs as in [9].

The enzyme activity was measured potentiometrically [10] using pH-meter OP-205 (Radelkis, Hungary), or pH-262 (USSR). The incubation was performed in a thermostatted cell with 2.4 ml solution 5 mM $ATPNa_2$, 10 mM $MgCl_2$, 20 mM D-glucose, 0.2 M NaCl at either pH 6.5 or 8.5. The kinetic curves were linear over <5–20 min. The slope of the linear part was taken as a measure of the hexokinase activity.

The inhibitory action of hydroxyanalogs of the reagents was measured using the same technique in the presence of inhibitors in the amounts indicated in the legends to figures or in the appropriate parts of the text.

The alkylation of enzyme was performed using

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10^{-7} M enzyme, 0.7 mM $MgCl_2$, 1.3 mM D-glucose by incubation of the reaction mixture with analog. Aliquots taken at various time intervals were poured into standard mixture for the activity assay. The zero point of the same kinetic curve was used as a control experiment. The apparent rate constants were determined as the initial slopes (b) of the semilogarithmic plot of the time course of inactivation using a least squares approximation of the initial parts of the kinetic curves (usually up to 50% of conversion) in the form:

$$\ln \alpha = bt + ct^2$$

(α = the ratio of the activities at the time t and time zero)

3. Results

At pH 8.5 the unreactive analog of the ATP derivative $HORCH_2NHpppA$ does not inhibit the phosphate transfer catalysed by yeast hexokinase PI at 10^{-4} M of the competitor. K_m for ATP measured with our enzyme in the same condition was 1.5×10^{-4} M in agreement with [11,12]. Therefore, we may conclude that introduction of a bulky substituent in the γ-position of ATP results in significant decrease of the affinity of the ATP moiety towards monomeric enzyme. In accordance with this observation the incubation of monomeric enzyme with the reactive derivative $ClRCH_2NHpppA$ did not lead to the enzyme inactivation.

The alternative explanation of the latter could be the absence in the active site of the enzyme of any group capable of reacting with 2-chloroethylamines. However, the respective AMP analog $ClRCH_2NHpA$ irreversibly inhibits hexokinase in the same conditions (fig.1). The semilogarithmic plot of the relative residual activity α of the enzyme vs time is not linear (fig.2). Therefore, the initial slope of the plot was taken as an apparent rate constant k_{app}. The dependence of k_{app} on the reagent concentration x_o plotted as k_{app}/x_o vs k_{app} is linear (fig.2, inset) in accordance with the equation for the reaction proceeding in the complex:

$$\frac{k_{app}}{x_o} = \frac{k_o \gamma}{K_x} - \frac{k_{app}}{K_x} \qquad [13]$$

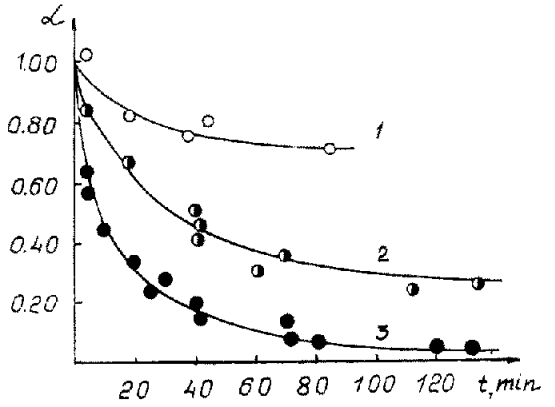


Fig.1. Kinetic curves of inactivation of hexokinase by $ClRCH_2NHpA$; (α) relative residual activity. Initial concentration of the reagent: (1) $x_o = 5 \times 10^{-5}$ M; (2) $x_o = 1 \times 10^{-4}$ M; (3) $x_o = 5 \times 10^{-4}$ M at pH 8.5, 30°C.

K_x being the dissociation constant for the enzyme–reagent complex, k_o the first order rate constant of the consumption of $ClRCH_2NHpA$ in solution, γ the ratio of the reaction rates of transformation of the reagent inside the complex and in solution [13]. The least squares values of the parameters are found to be $K_x = 1.1 \times 10^{-3}$ M, $k_o = 0.35$ min^{-1}.

In fig.3 one can see that ADP protects the enzyme against inactivation. Both results testify that the reaction between $ClRCH_2NHpA$ with hexokinase is the affinity labelling of the enzyme.

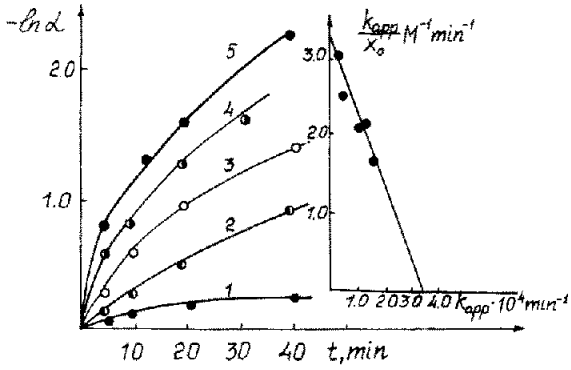


Fig.2. Semilogarithmic plot of the relative residual activity α vs time for the inactivation time course of hexokinase with $ClRCH_2NHpA$: (1) $x_o = 5 \times 10^{-5}$ M; (2) $x_o = 1 \times 10^{-4}$ M; (3) $x_o = 2 \times 10^{-4}$ M; (4) $x_o = 5 \times 10^{-4}$ M; (5) $x_o = 1 \times 10^{-3}$ M at pH 8.5, 30°C. Inset: the dependence of k_{app} on the $ClRCH_2NHpA$ concentration.

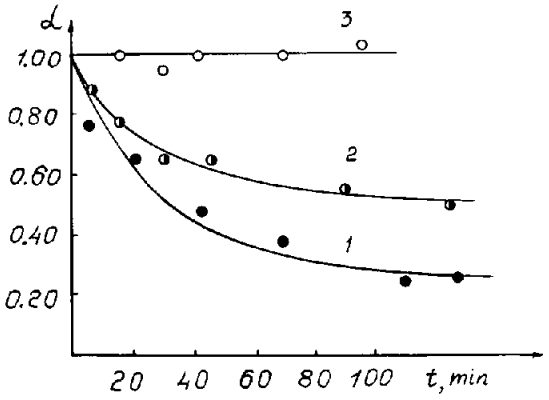


Fig.3. Kinetic curves of inactivation of hexokinase at pH 8.5, 30°C by $ClRCH_2NHpA$ (1×10^{-4} M) in the absence of ADP (1) and in the presence of 1×10^{-4} M (2) and 5×10^{-4} M (3) ADP.

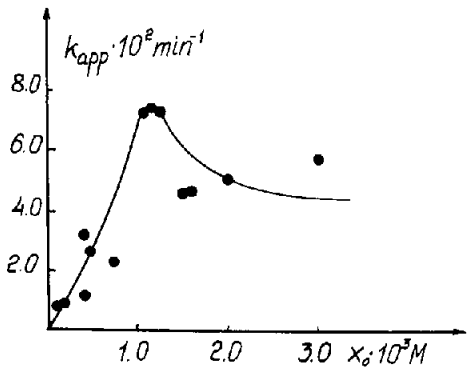


Fig.5. The dependence of k_{app} of the inactivation of hexokinase at pH 6.5, 30°C on the $ClRCH_2NHpppA$ concentration.

The K_x value is significantly higher than K_m for ATP thus indicating that the affinity of the reagent towards hexokinase is significantly lower than that of the substrate. In qualitative agreement with this result the unreactive analog of the reagent $HORCH_2NHpA$ does not reveal inhibitory action on the hexokinase reaction up to 5×10^{-4} M.

Contrary to the above data $ClRCH_2NHpppA$ readily inactivates hexokinase at pH 6.5 where dimeric enzyme predominates and ADP protects hexokinase against inactivation (fig.4). In accordance with this result the unreactive γ-substituted ATP derivative

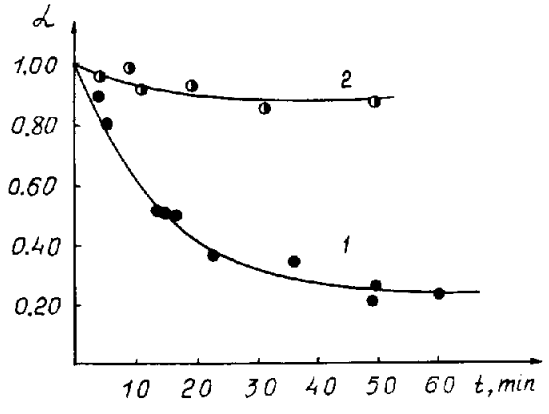


Fig.4. Kinetic curves of inactivation of hexokinase at pH 6.5, 30°C by $ClRCH_2NHpppA$ (7.5×10^{-4} M) in the absence of ADP (1) and in the presence of 1.5×10^{-3} M ADP (2).

(γ-anilide) of ATP behaves as a competitive inhibitor of hexokinase with $K_i = 1.4 \times 10^{-4}$ M.

The dependence of k_{app} on the initial concentration of the reagent x_0 is more complicated than in the former case and after reaching the maximal value 7.3×10^{-2} min^{-1} (3-fold reproduced) has a tendency to fall (fig.5). Vice versa, in the case of the dimeric enzyme, $ClRCH_2NHpA$ does not reveal any inhibitory action up to 1.6×10^{-3} M.

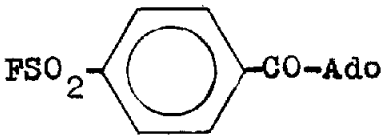
4. Discussion

The data obtained demonstrate clearly the great difference between the arrangements of the ATP binding sites of the dimeric and monomeric yeast hexokinase. The latter does not permit substitution of ATP at γ-P-position. This is the first example of the active site of such type for alkylating analogs of NTPs. $ClRCH_2NHpppA$ inactivates creatine kinase [14] and mitochondrial ATP-ase [15] and a similar dTTP derivative readily reacts with DNA polymerase I [16] and RNA-dependent DNA polymerase [17].

Dimerization of the enzyme, although necessarily introducing steric hindrance of the same part of the enzyme surface, results in the appearance of the affinity and reactivity towards $ClRCH_2NHpppA$. At the same time the reactivity towards the less bulky AMP derivative disappears. These data show that some essential rearrangement of conformation occurs in the ATP binding part of the active site of hexokinase or close to it.

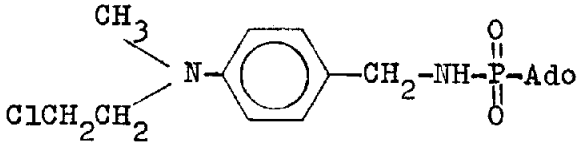
The kinetic behaviour of the dimeric enzyme seems to be more complicated as compared with monomer. This may be due to some co-operativity operating between ATP binding sites of the subunits. As shown in [18], this cooperativity may result in the extreme type of the dependence of k_{app} on x_o.

Until now, only one reactive ATP analog was used to modify hexokinase specifically, namely *p*-fluorosulfonylbenzoyl adenosine [19]. The dimension of the group attached to 5′-position of adenosine in this reagent is even smaller than that of $ClRCH_2NHpA$:



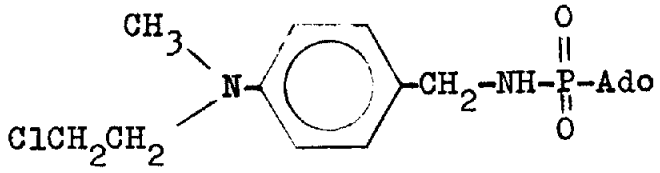
and one could expect that this reagent attacks predominantly monomeric enzyme. However, this reagent was studied at pH 7.0 where both monomer and dimer are present in comparable amounts and therefore no definite conclusion may be drawn from these data.

The reaction rate within the complex between $ClRCH_2NHpA$ and hexokinase was found to be 0.35 min^{-1}. This value may be compared with the reaction rate of hydrolysis of $ClRCH_2NHpU$ at the same temperature (30°C) found to be $k_o = 6.0 \times 10^{-3}$ min^{-1} [20]. Thus the reaction inside the complex proceeds 60-times faster than in solution. In solution aromatic 2-chloroethylamines [20,21] alkylate via intermediate formation of the respective ethyleneimmonium cation, for example:



This reaction is the rate-determining step of the overall process. It was found that the rate constant of this reaction does not significantly change when C–Cl bond-splitting proceeds inside the specific complex [22]. The increase of the hydrophobicity of the reaction medium results in the few-fold decrease of the rate constant [21].

Therefore great acceleration of the reaction inside the complex may be explained by the change of the mechanism (direct S_N2 alkylation) in the complex by analogy with [23].

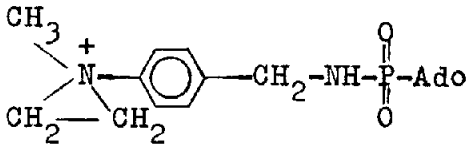


Another possible explanation may follow from the peculiarities of the mechanism of the overall process. As reactive ethylene–immonium cations form in significant amounts in solution one may suggest that these cations bearing the specific adenylate moiety may rapidly exchange with the reagent molecules in the complex thus providing a new efficient route of the modification [24,25]:

$$ClRCH_2NHpA \rightarrow \overset{+}{R}CH_2NHpA \rightleftarrows E \cdot \overset{+}{R}CH_2NHpA$$

$$\rightarrow \text{Modification products}$$

A detailed kinetic investigation is necessary to discriminate between those alternative explanations.



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