Inactivation of yeast hexokinase by 2-aminothiophenol

Evidence for a 'half-of-the-sites' mechanism

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Yeast hexokinase is a homodimer consisting of two identical subunits. Yeast hexokinase was inactivated by 2-aminothiophenol at 25 °C (pH 9.1). The reaction followed pseudo-first-order kinetics until about 70 % of the phosphotransferase activity was lost. About 0.65 mol of 2-aminothiophenol/mol of hexokinase was found to be bound after the 70 % loss of the enzyme activity. Completely inactivated hexokinase showed a stoichiometry of about 1 mol of 2-aminothiophenol bound/mol of the enzyme. The evidence obtained from kinetic experiments, stoichiometry of the inactivation reaction and fluorescence emission measurements suggested site-site interaction (weak negative co-operativity) during the inactivation reaction. The approximate rate constants for the reversible binding of 2-aminothiophenol to the first subunit (K_1) and for the rate of covalent bond formation with only one site occupied (k_2) were 150 μM and 0.046 min^{-1} respectively. The inactivation reaction was pH-dependent. Dithiothreitol, 2-mercaptoethanol and cysteine restored the phosphotransferase activity of the hexokinase after inactivation by 2-aminothiophenol. Sugar substrates protected the enzyme from inactivation more than did the nucleotides. Thus it is concluded that the inactivation of the hexokinase by 2-aminothiophenol was a consequence of a covalent disulphide bond formation between the aminothiols and thiol function at or near the active site of the enzyme. Hexokinase that had been completely inactivated by 2-aminothiophenol reacted with *o*-phthalaldehyde. Fluorescence emission intensity of the incubation mixture containing 2-aminothiophenol-modified hexokinase and *o*-phthalaldehyde was one-half of that obtained from an incubation mixture containing hexokinase and *o*-phthalaldehyde under similar experimental conditions. The intensity and position of the fluorescence emission maximum of the 2-aminothiophenol-modified hexokinase were different from those of the native enzyme, indicating conformational change following modification. Whereas aliphatic aminothiols were completely ineffective, aromatic aminothiols were good inhibitors of the hexokinase. Cyclohexyl mercaptan weakly inhibited the enzyme. Inhibition of the hexokinase by heteroaromatic thiols was dependent on the nature of the heterocyclic ring and position of the thiol-thione equilibrium. The inhibitory function of a thiol is associated with the following structural characteristics: (a) the presence of an aromatic ring, (b) the presence of a free thiol function and (c) the presence of a free amino function in the close proximity of the thiol function. Non-polar interactions between 2-aminothiophenol and the hydrophobic environment of the active site of the hexokinase are, in part, responsible for inhibition of the enzyme. An important finding of the reaction between yeast hexokinase and 2-aminothiophenol is the fact that the inactivation reaction followed a 'half-of-the-sites' mechanism.

INTRODUCTION

Previous reports from our laboratory described the inactivation of the catalytic subunit of cyclic AMP-dependent protein kinase (Puri *et al.*, 1985a), cyclic GMP-dependent protein kinase (Puri *et al.*, 1985b) and yeast hexokinase (Puri & Roskoski, 1984; Puri *et al.*, 1985c) by *o*-phthalaldehyde. The important finding in all these cases was the fact that *o*-phthalaldehyde inactivated these enzymes by cross-linking closely spaced cysteine and lysine residues at or near the regulatory and/or catalytic site(s) in the hydrophobic environment. The affinity of *o*-phthalaldehyde was superior to that of pyridoxal 5'-phosphate and [5'-(*p*-fluorosulphonyl)]-benzoyladenine for the lysine residue(s) buried in the hydrophobic surroundings of the active site of yeast

hexokinase (homodimer) (Puri *et al.*, 1985c). Otieno *et al.* (1975, 1977) showed that the essential cysteine residue in yeast hexokinase was not readily available for covalent modification by the alkylating reagents because of its location in the hydrophobic cleft of the hexokinase. The hydrophobicity of the microenvironment of the active site was suggested as the reason for the high $\text{p}K_{\text{app}}$ value (6.8) of the critical dicarboxylic acid residue at or near this site of the hexokinase (Bohnenstark & Hofmann, 1969; Pho *et al.*, 1977). Pho *et al.* (1977) suggested that the affinity of yeast hexokinase for aromatic compounds, e.g. tyrosine ethyl ester, nitrotyrosine ethyl ester and phenylephrine, was compatible with the presence of an essential glutamic acid residue in the non-polar surroundings of the active site. *N*-

Abbreviation used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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Aroylglucosamines were shown to inhibit yeast hexokinase better than *N*-acylglucosamines (Maley & Lardy, 1955). Bennett & Steitz (1978) suggested that hydrophobic effect alone favoured the active conformation of yeast hexokinase in the presence or in the absence of glucose.

In our continuing effort to learn more about the structural organization of the active site of yeast hexokinase, we have examined its inactivation by a variety of thiols. In the present paper we describe in detail the inactivation of yeast hexokinase by 2-aminothiophenol. The nature of the covalent adduct formed, structural requirements necessary for interaction of the inhibitor with the active site and conformational changes following inactivation of the hexokinase by thiophenols are discussed. A preliminary report of this investigation has previously appeared (Puri *et al.*, 1986).

MATERIALS AND METHODS

Hexokinase (yeast), glucose-6-phosphate dehydrogenase (yeast), sugars, nucleotides, nucleosides, thio-salicylic acid and 6-mercaptapurine monohydrate were obtained from Sigma Chemical Co. Thiophenol, cyclohexylmercaptan, cyclohexylamine, aniline, 2-, 3- and 4-aminothiophenols, 2- and 4-mercaptopyridines, 2-mercaptopyrimidine, 2-mercaptobenzimidazole, 2-mercapto-1-methylimidazole, ethyl 2-mercapto-5-methylimidazole-4-carboxylate and 2,5-dimercapto-1,3,4-thiadiazole were obtained from Aldrich Chemical Co. and used without further purification. The structures of these compounds are shown in Fig. 7.

Commercial samples of yeast hexokinase used in this work were homogeneous as judged by polyacrylamide-gel electrophoresis in the presence and in the absence of SDS and size-exclusion chromatography on a Sephacryl S-200 column. M_r values of 100000 for the dimeric (Schultze & Colowick, 1969) and 50000 for the monomeric (Derechin *et al.*, 1972) forms of yeast hexokinase were used in the calculations. Enzyme assays were performed according to the method of Schmidt & Colowick (1973). The commercial samples of yeast hexokinase had 290–400 units of activity/mg of protein. One unit of activity is defined as the amount of enzyme that catalyses the phosphorylation of 1 μ mol of glucose/min at 25 °C.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, or by using a specific absorption of 0.947 $\text{cm}^2 \cdot \text{mg}^{-1}$ (Hoggett & Kellett, 1976). Fluorescence measurements were made with an SLM 4800 spectrofluorimeter as described by Puri *et al.* (1985a).

RESULTS

Inactivation of the hexokinase by 2-aminothiophenol

The time course of inactivation of the hexokinase by 2-aminothiophenol (compound I, Fig. 7) at 25 °C (pH 9.1) is shown in Fig. 1. 2-Aminothiophenol, in the concentration range 0.05–0.08 mM, inhibited hexokinase rather slowly. For example, the hexokinase lost more than 90% of the phosphotransferase activity in 2 h when incubated with 0.8 mM-2-aminothiophenol. When the natural logarithms of the percentage residual activities were plotted as a function of time, linear relationships

Fig. 1. Time course of inactivation of yeast hexokinase by 2-aminothiophenol

A solution containing hexokinase (1.13 μ M), 2-aminothiophenol, 100 mM-Tris/HCl buffer, pH 9.1, and 0.8% (v/v) ethanol was incubated at 25 °C. Solution of 2-aminothiophenol was prepared in a 1:1 (v/v) mixture of 100 mM-Tris/HCl buffer, pH 9.1, and 95% (v/v) ethanol. At the times specified the reaction was terminated by transferring a small portion of the incubation mixture into 1 ml of the assay mixture and residual phosphotransferase activity was determined. The data are plotted as the natural logarithm of the percentage activity remaining versus time. 2-Aminothiophenol concentrations were as follows: $\blacktriangle$, 0 mM; $\triangle$, 0.05 mM; $\blacksquare$, 0.1 mM; $\square$, 0.2 mM; $\bullet$, 0.4 mM; $\circ$, 0.8 mM. The inset shows a double-reciprocal plot of the apparent first-order rate constants versus concentration of 2-aminothiophenol.

were obtained until about 70% of the phosphotransferase activity was lost. If we assume that the rate at which enzyme activity decreased was pseudo-first-order until 30% of the activity remained, the apparent first-order rate constants (k_{app}) may be calculated from the plots according to eqn. (1):

$$-\ln(E/E_0) = k_{app} \cdot t \quad (1)$$

E_0 and E represent the enzyme activities at zero and any given time t respectively. The results given in Fig. 1 suggest site-site interactions (negative co-operativity) between the subunits.

The process of inactivation of the hexokinase by 2-aminothiophenol may be represented by eqn. (2). The plots of inverse of the apparent first-order rate constants ($1/k_{app}$) versus the reciprocal of inhibitor concentration ($1/[2\text{-aminothiophenol}]$), according to eqn. (3), gave a linear relationship (Fig. 1 inset):

$$1/k_{app} = (K_1/k_3) (1/[I]) + (1/k_3) \quad (3)$$

In these equations k_1 and k_2 are the rate constants for enzyme-inhibitor complex formation and dissociation respectively. $E-I$ corresponds to the Michaelis-Menton intermediate complex between hexokinase and 2-amino-

Fig. 2. Reversal of the phosphotransferase activity of hexokinase inactivated by 2-aminothiophenol

A solution containing hexokinase ($1.13 \mu\text{M}$), 0.8 mM -2-aminothiophenol, 100 mM -Tris/HCl buffer, pH 9.1, and 0.8% (v/v) ethanol was incubated at 25°C . The residual phosphotransferase activity ($\circ$) was monitored at the specified times. After 2 h, portions of the incubation mixture were treated with 10 mM -dithiothreitol (Δ), 2-mercaptoethanol ($\square$) and cysteine ($\blacksquare$) (indicated by the arrow). The residual phosphotransferase activity was determined at the specified times. Residual phosphotransferase activity after 3.5 h in a control incubation mixture was 80% of that at zero time.

thiophenol. It is assumed that k_3 corresponds to the rate constant for covalent formation between only one subunit of hexokinase (E) and 2-aminothiophenol (I). It is also assumed that EI represents the final product of the reaction between enzyme and inhibitor even when the reaction has proceeded to 70% inactivation, i.e. EI does not yield further species such as E'I (Kitz & Wilson, 1962).

It is thus possible to obtain a rough estimate of the $K_1 (=k_2/k_1)$ of 2-aminothiophenol and the rate of covalent bond formation, k_3 , from the ordinate intercept and slope of the plot shown in Fig. 1 inset (cf. Kitz & Wilson, 1962; Petra, 1971). These estimated constants are for the reversible binding of 2-aminothiophenol to the first subunit (K_1) and for the rate of covalent bond formation with only one site occupied (k_3). The values for K_1 and k_3 were $150 \mu\text{M}$ and 0.042 min^{-1} respectively.

Previous work in our laboratory showed that inactivation of the hexokinase by *o*-phthalaldehyde did not involve the obligatory formation of a Michaelis-Menten complex before the isoindole derivative formation (R. N. Puri, unpublished work). The data presented in Fig. 1 inset show that inactivation of the hexokinase by 2-aminothiophenol proceeds through a reversible enzyme-inhibitor complex. This was further confirmed by the experiments showing that *N*-acetyl-D-glucosamine (K_1 1 mM), a reversible inhibitor of the hexokinase (Maley & Lardy, 1955), provided protection to the enzyme from inactivation by 2-aminothiophenol. When hexokinase was inactivated by 0.8 mM -2-aminothiophenol at 25°C for 2 h in the presence of 10 mM -, 20 mM - and 40 mM -*N*-acetyl-D-glucosamine, the residual phosphotransferase activities were 13, 19 and 24% respectively, compared with 6% in the absence of *N*-acetylglucosamine under identical experimental conditions.

Re-activation of the inactivated hexokinase by thiols

The effects of dithiothreitol, 2-mercaptoethanol and cysteine on the reversal of phosphotransferase activity of the hexokinase inactivated by 2-aminothiophenol is shown in Fig. 2. The hexokinase was first incubated for 2 h with 0.8 mM -2-aminothiophenol. It lost about 92% of the phosphotransferase activity. At this point the reaction mixture containing hexokinase inactivated by 2-aminothiophenol was further incubated with 10 mM -dithiothreitol or 2-mercaptoethanol or cysteine (indicated by a vertical arrow). Dithiothreitol restored 80% of the enzyme activity in 1.5 h and was more effective than 2-mercaptoethanol and cysteine. It should be pointed out that the phosphotransferase activity in an identical incubation mixture containing untreated hexokinase after 3.5 h was also close to 80% . The loss of phosphotransferase activity in a control experiment may be due to the presence of ethanol (used to prepare a solution of 2-aminothiophenol) in and the high pH of the incubation mixture. Thus dithiothreitol was almost completely effective in reversing the phosphotransferase activity of the enzyme inactivated by 2-aminothiophenol.

Effect of substrates and substrate analogues on inactivation of the hexokinase by 2-aminothiophenol

The results of the effect of sugars on the protection of the hexokinase from inactivation by 2-aminothiophenol are summarized in Table 1. The protection provided by the sugar substrates followed the order of their relative substrate affinities, i.e. mannose > glucose > fructose (Slein *et al.*, 1950). Galactose, 1,5-anhydroglucitol and 1,5-anhydromannitol did not provide any protection. 2-

Table 1. Effect of various sugars on the protection of the hexokinase from inactivation by 2-aminothiophenol

Solutions containing $0.57 \mu\text{M}$ -hexokinase, 100 mM -Tris/HCl buffer, pH 9.1, 0.8% (v/v) ethanol and 0.8 mM -2-aminothiophenol were incubated with the sugars (described in the Table) for 2 h at 25°C . The reactions were terminated and residual phosphotransferase activity was determined as described in the legend to Fig. 1. Appropriate controls devoid of 2-aminothiophenol were included in each case.

Addition	Concentration (mM)	Activity remaining (%)
None		7
Glucose	2	24
	10	48
Mannose	2	40
	10	97
Fructose	2	7
	10	25
Galactose	2	6
	10	4
Glucose 6-phosphate	2	9
	10	14
2-Deoxyglucose	2	12
1,5-Anhydroglucitol	15	6
1,5-Anhydromannitol	15	7

Fig. 3. pH-dependent inactivation of the hexokinase by 2-aminothiophenol

A solution containing hexokinase ($1.13 \mu\text{M}$), 100 mM-Tris/HCl buffer, 0.8% (v/v) ethanol and 0.8 mM-2-aminothiophenol was incubated at 25°C for 2 h. The pH of the incubation mixture was as follows: $\bullet$, 6.8; $\square$, 7.6; $\triangle$, 8.2; $\circ$, 9.1. The solution of 2-aminothiophenol was prepared in a 1:1 (v/v) mixture of 100 mM-Tris/HCl buffer of the appropriate pH and 95% (v/v) ethanol. Residual phosphotransferase activity was determined at the specified times. Residual phosphotransferase activity in the control incubation mixtures was 92, 92, 92 and 88% at pH 6.8, 7.6, 8.2 and 9.1 respectively.

Deoxyglucose and glucose 6-phosphate provided some protection to the hexokinase from inactivation. These results are similar to those previously obtained during chemical modification of the hexokinase by *o*-phthalaldehyde (R. N. Puri, unpublished work) and 2-(*N*-bromoacetyl)-D-galactosamine (Otieno *et al.*, 1977).

Effect of pH on the rate of inactivation of the hexokinase by 2-aminothiophenol

The results presented in Fig. 3 show that increasing the pH of the incubation mixture from 6.8 to 9.1 progressively increased the rate of inactivation of the hexokinase by 2-aminothiophenol. The results point to the fact that the ionization of a functional group with a high pK_a in the hexokinase in some way contributed to the increase in rate of inactivation of the hexokinase. The thiol function of 2-aminothiophenol is likely to be ionized (*a*) because of the higher pH and (*b*) because the interaction of the lone pair of electrons on the S atom of the 2-aminothiophenol with π -electron manifold of the aromatic ring would weaken the S-H bond and increase its pK_a from 6.50 for thiophenol to a higher value (Kreevoy *et al.*, 1964).

Stoichiometry of the reaction between the hexokinase and 2-aminothiophenol

In an effort to ascertain further the mechanism by which 2-aminothiophenol inactivated yeast hexokinase, the kinetic order of the enzyme-inhibitor reaction was determined by the method previously described (Levy *et al.*, 1963; Borchardt *et al.*, 1978). The magnitude of the pseudo-first-order rate constant, k_{app} , depends on the concentration of inhibitor as described in eqn. (4), where

Fig. 4. Stoichiometry of the reaction between hexokinase and 2-aminothiophenol

The apparent first-order rate constants at various concentrations of 2-aminothiophenol were calculated from the linear portion of the plots shown in Fig. 1 and plotted against the logarithm of the concentration of 2-aminothiophenol. Average kinetic order of the inactivation reaction was calculated from the slope of the plot.

k' is a first-order rate constant and n is a number equal to the average order of the reaction with respect to the concentration of the inhibitor (I), i.e. 2-aminothiophenol. By using eqn. (5), the order of the reaction was estimated experimentally by plotting the $\log k_{app}$ versus $\log[I]$ with the slope equal to n , the kinetic order of the reaction:

$$k_{app} = k'[I]^n \quad (4)$$

$$\log k_{app} = \log k' + n \cdot \log[I] \quad (5)$$

Fig. 4 shows the plot of $\log k_{app}$ versus $\log[I]$ for the inactivation of the hexokinase by 2-aminothiophenol. The data show that approx. 0.65 mol of 2-aminothiophenol was bound to the hexokinase after 70% loss of the phosphotransferase activity.

Treatment of the hexokinase with DTNB at or below 30°C for 2 h brought about complete inactivation of the enzyme with concomitant modification of two thiol groups (Expt. A in Table 2). When the hexokinase was incubated with DTNB at 35°C for 20 h, 8 mol of thiol groups/mol of the enzyme was modified (Expt. B in Table 2). These results are in accord with the findings that yeast hexokinase contains 8 mol of thiol groups/mol of the enzyme (Jones *et al.*, 1975; Otieno *et al.*, 1975, 1977; R. N. Puri, unpublished work). When the hexokinase was completely inactivated by 2-aminothiophenol, about 1 mol of thiol groups/mol of the enzyme was modified (Expt. C in Table 2). Chemical modification of the hexokinase by iodoacetamide in the presence of mannose resulted in the modification of about 6 mol of thiol groups/mol of the enzyme without significant loss of the enzyme activity (Expt. D in Table 2). Subsequent treatment of the iodoacetamide-modified enzyme with 2-aminothiophenol resulted in complete loss of the phosphotransferase activity with modification of an additional 1 mol of thiol groups/mol of the enzyme (Expt. E in Table 2). The data presented in Fig. 4 and Table 3 are consistent with the fact that modification of about 1 mol of thiol groups/mol of the hexokinase by 2-aminothiophenol led to complete inactivation of the enzyme.

Table 2. Thiol groups modified in the reaction between the hexokinase and various reagents

In Expts. A–C a solution containing hexokinase (1 mg/500 μ l), 100 mM-Tris/HCl buffer, pH 9.1, 0.8% (v/v) ethanol and 0.8 mM-2-aminothiophenol was incubated at 25 °C for 2 h. The incubation mixture was subjected to gel filtration on a Sephadex G-25 column (0.45 mm $\times$ 18.5 mm) equilibrated and eluted with 100 mM-Tris/HCl buffer, pH 8.6, at a flow rate of 1 ml/3 min. A solution containing hexokinase was incubated in the absence of 2-aminothiophenol under identical conditions and carried through the gel-filtration step. The protein-containing fractions were collected. In a subsequent step, a solution containing the modified or unmodified hexokinase (120 μ g/1 ml), 100 mM-Tris/HCl buffer, pH 8.6, and 100 mM-NaCl was incubated with 1 mM-DTNB as described in the Table. In Expts. A and B the numbers of thiol groups modified were determined as described by Ellman (1959). In Expt. C the number of thiol groups modified by 2-aminothiophenol was determined by subtracting the value obtained by titration with DTNB from the total number of thiol groups in the hexokinase. In Expt. D hexokinase was incubated with iodoacetamide in the presence of mannose and then subjected to gel filtration as described above. In Expt. E the iodoacetamide-modified hexokinase was incubated with 2-aminothiophenol under the conditions described in the Table and then gel-filtered on a Sephadex G-25 column before titrating with DTNB.

Expt.	First treatment	Second treatment	Reaction conditions	Activity remaining (%)	SH groups modified (mol/mol of hexokinase)
A	Hexokinase + DTNB (1 mM)	–	30 °C, 2 h	0	2.03
B	Hexokinase + DTNB (1 mM)	–	35 °C, 20 h	0	8.10
C	Hexokinase + 2-aminothiophenol	–	25 °C, 2 h	6	0.92
D	Hexokinase + mannose (7 mM)	–	35 °C, 2 h	100	–
		Iodoacetamide (6 mM)	35 °C, 20 h	3	5.90
E	Hexokinase + mannose (7 mM) + iodoacetamide (6 mM)	–	35 °C, 20 h	95	5.85
		2-Aminothiophenol (0.8 mM)	25 °C, 2 h	0	6.90

Table 3. Inhibition of phosphotransferase activity of the hexokinase by thiols derived from various heterocyclic systems

Solutions containing the hexokinase (1.13 μ M), 100 mM-Tris/HCl buffer, pH 9.1, 0.8% (v/v) ethanol and 0.8 mM-thiol (described in the Table) were incubated at 25 °C for 2 h. Residual phosphotransferase activities were determined as described in the legend to Fig. 1. Solutions of the thiols, except ethyl 2-mercapto-5-methylimidazole-4-carboxylate and 6-mercaptopurine, were prepared in Tris/HCl/ethanol mixture as described in the legend to Fig. 1. Solutions of ethyl 2-mercapto-5-methylimidazole-4-carboxylate and 6-mercaptopurine were prepared in 1 M-NaOH and 1 M-HCl respectively. The structures of the thiols are described in the legend to Fig. 7.

Thiol	Activity remaining (%)
None	88
2-Aminothiophenol	6
2-Mercaptopyridine	62
4-Mercaptopyridine	50
2-Mercaptopyrimidine	72
2-Mercapto-1-methylimidazole	86
Ethyl 2-mercapto-5-methylimidazole-4-carboxylate	78
2-Mercaptobenzimidazole	80
6-Mercaptopurine	80
2,5-Dimercapto-1,3,4-thiadiazole	30

Reaction between 2-aminothiophenol-modified hexokinase and *o*-phthalaldehyde

Previous work from our laboratory showed that inactivation of the hexokinase by *o*-phthalaldehyde was the result of formation of an isoindole derivative between

the cysteine and lysine residues at or near the active site of the enzyme subunit (R. N. Puri, unpublished work). When the hexokinase, completely inactivated (Expt. C in Table 2) by 2-aminothiophenol, was treated with *o*-phthalaldehyde, the incubation mixture exhibited a fluorescence emission maximum at 405 nm characteristic of the hexokinase-*o*-phthalaldehyde adduct (Puri & Roskoski, 1984). The intensity of the fluorescence emission maximum was about one-half of that obtained from an incubation mixture containing untreated hexokinase and *o*-phthalaldehyde under identical conditions (Fig. 5). These results are consistent with the data presented above that showed that complete inactivation of the hexokinase by 2-aminothiophenol was the consequence of a modification of about 1 mol of thiol groups/mol of the enzyme.

Comparison of the rates of inactivation of the hexokinase by aliphatic, alicyclic and aromatic thiols

Results of the relative rates of inactivation of the hexokinase by various thiols are presented in Fig. 6. Whereas 0.8 mM-2-aminothiophenol inactivated the hexokinase almost completely in 2 h, cysteine and GSH at the same concentration had no effect on the phosphotransferase activity of the enzyme. Instead, cysteine was shown above (Fig. 2) to have a beneficial effect on the restoration of the activity of the hexokinase inactivated by 2-aminothiophenol. Cyclohexylmercaptan (compound IV, Fig. 7), an alicyclic thiol, brought about only 20% inactivation of the hexokinase in 2 h. Cyclohexylamine did not inactivate the hexokinase. Addition of cyclohexylamine to incubation mixture containing the hexokinase and cyclohexylmercaptan did not influence the inactivation of the enzyme by the mercaptan. Thiophenol (compound V, Fig. 7) brought about 70% inactivation of the enzyme in 2 h. The inactivation by

Fig. 5. Fluorescence emission spectrum of the incubation mixture containing 2-aminothiophenol-modified hexokinase and *o*-phthalaldehyde

The hexokinase (500 $\mu\text{g/ml}$) was incubated with 0.8 mM-2-aminothiophenol for 2 h as described in the legend to Fig. 1. The modified enzyme was isolated by gel filtration on a Sephadex G-25 column as described previously (Puri *et al.*, 1985a). A control incubation mixture was carried through the same sequence. The 2-aminothiophenol-modified or unmodified hexokinase (obtained from the control experiment) was treated with 1.2 mM-*o*-phthalaldehyde at 25 $^{\circ}\text{C}$ for 1.5 min. Protein concentration in both cases was 22 $\mu\text{g}/150\ \mu\text{l}$. Fluorescence emission spectra were recorded as described by Puri *et al.* (1985a). Tracing 1, 2-aminothiophenol-modified hexokinase; tracing 2, 2-aminothiophenol-modified hexokinase and *o*-phthalaldehyde; tracing 3, hexokinase and *o*-phthalaldehyde.

Fig. 6. Inactivation of hexokinase by various thiols

A solution containing hexokinase (1.3 μM), 100 mM-Tris/HCl buffer, pH 9.1, 0.8% (v/v) ethanol and 0.8 mM-thiol was incubated at 25 $^{\circ}\text{C}$ for 2 h. Solutions of 2-, 3- and 4-aminothiophenol were prepared in a 1:1 (v/v) mixture of 100 mM-Tris/HCl buffer, pH 9.1, and 95% (v/v) ethanol. Solutions of thiophenol and cyclohexylmercaptan were prepared in 95% (v/v) ethanol. Residual phosphotransferase activities were determined as described in the legend to Fig. 1. $\square$, Control; $\blacktriangle$, cyclohexylmercaptan; $\bullet$, 3-aminothiophenol; $\blacksquare$, 4-aminothiophenol; $\triangle$, thiophenol; $\circ$, 2-aminothiophenol.

Fig. 7. Structures of the thiols used

(I), 2-Aminothiophenol; (II), 3-aminothiophenol; (III), 4-aminothiophenol; (IV), cyclohexylmercaptan; (V), thiophenol; (VI), thiosalicylic acid; (VII), 2-mercaptopyridine; (VIII), 4-mercaptopyridine; (IX), 2-mercaptopyrimidine; (X), 2-mercapto-1-methylimidazole; (XI), ethyl 2-mercapto-5-methylimidazole-4-carboxylate; (XII), 2-mercaptobenzimidazole; (XIII), 6-mercaptapurine; (XIV), 2,5-dimercapto-1,3,4-thiadiazole.

thiophenol was reversed by dithiothreitol. Aniline (0.8 mM) neither inactivated nor influenced the rate of inactivation of the hexokinase by thiophenol. The rate of inactivation of the hexokinase by 3-aminothiophenol (compound II, Fig. 7) or 4-aminothiophenol (compound III, Fig. 7) was much lower compared with that of 2-aminothiophenol. These results suggest that inactivation of hexokinase by the thiols followed the order: aromatic > alicyclic > aliphatic.

Reaction of the hexokinase with heterocyclic thiols

The extent of inactivation of the hexokinase by various heterocyclic thiols (0.8 mM) over a period of 2 h was investigated and the results are summarized in Table 3. The 2- or 4-mercaptopyridine (compound VII or compound VIII respectively, Fig. 7) moderately inhibited hexokinase. The thiols derived from the imidazole ring system were practically ineffective in inhibiting the hexokinase. 6-Mercaptopurine (compound XIII, Fig. 7) did not inhibit the enzyme. The extent of inhibition by the thiol derived from the 1,3,4-thiadiazole ring system (compound XIV, Fig. 7), to our surprise, was comparable with that of the thiophenol (Fig. 4). In general, the extent of inhibition by heterocyclic thiols followed the order: pyridine > pyrimidine > imidazole = purine.

DISCUSSION

The inactivation of hexokinase by 2-aminothiophenol exhibited a stoichiometry of about 1 mol of ligand covalently bound/mol of the holoenzyme (dimer) (Fig. 4 and Table 3). This suggests that the above inactivation reaction followed a 'half-of-the-sites' mechanism. The first criterion for enzymes following a 'half-of-the-sites' mechanism is that the enzyme be an oligomer containing equivalent subunits (Levitzki *et al.*, 1971; Lazdunski, 1974). Available evidence indicates that the yeast hexokinase is a homodimer (Rustum *et al.*, 1971; Derechin *et al.*, 1972; Hoggett & Kellett, 1976; Steitz *et al.*, 1981). The second important criterion for the enzymes following a 'half-of-the-sites' mechanism is that there be clear evidence of site-site interaction(s). Since hexokinase is a homodimer, the data presented in Fig. 1 suggest that during the inactivation process the binding of 2-aminothiophenol to one subunit diminished its affinity for the second subunit (cf. Clements *et al.*, 1979). The inactivation reaction showed weak negative co-operativity between the two identical subunits of the hexokinase. The third feature of the enzymes following a 'half-of-the-sites' inactivation mechanism is that there should be ligand-induced conformational change during the inactivation process (cf. Levitzki *et al.*, 1971; Clements *et al.*, 1979). We found that the fluorescence maximum of yeast hexokinase inactivated by 2-aminothiophenol was shifted towards a higher wavelength by about 10 nm and its intensity was decreased by 58 % (results not shown) compared with those of the native enzyme ($\lambda_{\text{exc.}}$ 290 nm, $\lambda_{\text{em.}}$ 340 nm; cf. Menezes *et al.*, 1972). We therefore believe that negative co-operativity shown by hexokinase in solution results from ligand-induced conformational change and does not appear to be the result of heterologous interactions between the two subunits (cf. Peters & Neet, 1977). It is thus possible to say that inactivation of yeast hexokinase by 2-aminothiophenol followed a 'half-of-the-sites' mechanism.

The reversal by various thiols at the same concentration of inactivation by 2-aminothiophenol followed the order: dithiothreitol > 2-mercaptoethanol > cysteine (Fig. 2). These experiments demonstrated that (a) the inactivation of hexokinase by 2-aminothiophenol involved disulphide bond formation between thiol groups of the enzyme and inhibitor, and (b) the thiol group of the enzyme is most probably located at or near the active site of the enzyme.

The results of the protection studies with sugars (Table 1) show that those sugars that are good substrates for hexokinase provided protection to it from inactivation by 2-aminothiophenol. The results also emphasize the

importance of the presence and proper orientation of the hydroxy groups at positions 1, 3, 4 and 6 in the sugars necessary for interaction with the modification site of hexokinase (Steitz *et al.*, 1977; Anderson *et al.*, 1978). Protection provided by glucose 6-phosphate may be attributed to conformational changes in the hexokinase induced by the interaction of the sugar phosphate with the enzyme (McDonald *et al.*, 1979; Jarori *et al.*, 1981). We also examined the effect of ATP, ADP, AMP, 2'-AMP, 3'-AMP and (9- β -D-arabinofuranosyl)adenine 5'-triphosphate on the inactivation of hexokinase by 2-aminothiophenol and found that only 10 mM-ATP provided some beneficial effect (results not shown). The presence or absence of Mg^{2+} did not affect the extent of protection provided by the nucleotides. Similar results have been obtained by other investigators (Jones *et al.*, 1975; Peters & Neet, 1978). The results of the protection studies lend support to the view that the thiol group of hexokinase that is modified during inactivation is more likely to be located at or near the active site of the enzyme.

Aromatic aminothiophenols were found to be good inhibitors of hexokinase (Fig. 6), whereas aliphatic aminothiols, e.g. cysteine and GSH, had no effect. The fact that cyclohexylmercaptan, an alicyclic thiol, weakly inhibited hexokinase may be explained by the fact that 'bent bonds' between the carbon atoms of cycloalkanes have high p character, i.e. sp^4 or sp^5 , and carbon-carbon bond orbitals on adjacent atoms can overlap, thus slightly delocalizing the electron density over the carbon-atom framework (cf. aromatic compounds; Coulson & Moffitt, 1947). That the presence of a free amino function adjacent to the thiol function increased the ability of an aromatic compound to function as a good inhibitor was confirmed by the fact that 2-aminothiophenol (compound I, Fig. 7) was a much better inhibitor of hexokinase than was thiophenol (compound IV, Fig. 7), 3-aminothiophenol (compound II, Fig. 7) and 4-aminothiophenol (compound III, Fig. 7). The presence of a free thiol function in the aromatic compound was a necessary requirement because inhibition was ascertained from experiments (Table 3) that showed that 2- and 4-mercaptopyridine (compounds VII and VIII respectively, Fig. 7) (Aksnes & Kryvi, 1972; Boulton & McKillop, 1974), 2-mercaptopyrimidine (compound IX, Fig. 7) (Albert & Barlin, 1962), 2-mercapto-1-methylimidazole (compound X, Fig. 7), 2-mercaptobenzimidazole (compound XII, Fig. 7) and 6-mercaptapurine (compound XII, Fig. 7) (Grimmett, 1984*a,b,c*), in which the thiol-thione tautomerism due to thioamide resonance (compound XVa $\rightleftharpoons$ compound XVb, Fig. 8) overwhelmingly favours the thione form (compound XVa) over the thiol

Fig. 8. Thiol-thione equilibrium

Schematic representation of thiol-thione equilibrium in 2-thiol-substituted nitrogen heterocycles.

Fig. 9. Mesomerism in aminothiols

Schematic representation of mesomeric structures corresponding to thiol (1a) and thione (1b) forms of 2-aminothiophenol.

form (compound XVC), were poor inhibitors of hexokinase (Table 3). 2,5-Dimercapto-1,3,4-thiadiazole (compound XIV, Fig. 7) moderately inhibited hexokinase because in this compound the thiol form predominates over the thione form, as confirmed by ^{15}N n.m.r. (Williamson & Roberts, 1978). On the other hand, mesomerism in aminothiophenols (e.g. compound 1a $\rightleftharpoons$ 1b, Fig. 9) leading to the thione type of structure (e.g. compound 1b) is not proven (Cutress *et al.*, 1974), and thus they exist primarily as thiols (e.g. compound 1b). Therefore aminothiophenols act as better inhibitors of hexokinase than do heteroaromatic aminothiols.

Several examples of protein thiol/disulphide exchange (eqn. 6) have been reported in the past decade (Rafter & Harmison, 1979; Buchanan *et al.*, 1979; Freedman, 1979; Aryan Namboodiri *et al.*, 1980; Bramson *et al.*, 1982):

But examples of the oxidative formation of protein-thiol disulphides (eqn. 7) are difficult to find. To our knowledge, inactivation of hexokinase by 2-aminothiophenol through protein-thiol disulphide formation constitutes the first example of its kind in the hexokinase literature. One of the mechanisms of this protein-thiol disulphide formation may involve (a) base-catalysed formation of the anions Enz-S^- and S-R^- , which in the presence of O_2 could yield thiyl radicals $\text{Enz-S}^\cdot$ and $\text{S-R}^\cdot$ respectively (cf. Wallace & Schriesheim 1962, 1965; Wallace *et al.*, 1963) and (b) the dimerization of the thiyl radicals to generate a mixed disulphide Enz-S-S-R (Enz, hexokinase; R, 2-aminothiophenol).

In summary, the inactivation reaction between hexokinase and 2-aminothiophenols is characterized by the following: (a) stoichiometry of 1:1 with respect to 2-aminothiophenol; (b) site-site co-operativity; (c) conformational change following inactivation of the enzyme. The inactivation of hexokinase by 2-aminothiophenol follows a 'half-of-the-sites' mechanism. The presence of a free thiol function and a benzenoid-type aromatic nucleus appear to be essential components of the structure of 2-aminothiophenol for it to act as an inhibitor of the hexokinase.

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REFERENCES

- Aksnes, D. W. & Kryvi, H. (1972) *Acta. Chem. Scand.* **26**, 2255-2266
- Albert, A. & Barlin, G. B. (1962) *J. Chem. Soc.* 3926-3941
- Anderson, C. M., Stenkamp, R. E., McDonald, R. C. & Steitz, T. A. (1978) *J. Mol. Biol.* **123**, 207-219
- Aryan Namboodiri, M. A., Weller, J. A. & Klein, D. C. (1980) *J. Biol. Chem.* **255**, 6032-6035
- Bennett, W. S., Jr. & Steitz, T. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4848-4852
- Bohnensack, R. & Hofmann, E. (1969) *Eur. J. Biochem.* **9**, 534-541
- Borchardt, R. T., Wu, Y. S. & Wu, B. S. (1978) *Biochemistry* **17**, 4145-4153
- Boulton, A. J. & McKillop, A. (1974) in *Comprehensive Heterocyclic Chemistry* (Katritzki, A. R. & Rees, W. A., eds.), vol. 2, pp. 58-59, Pergamon Press, New York
- Bramson, H. N., Thomas, N., Matsueda, R., Nelson, N. C., Taylor, S. S. & Kaiser, E. T. (1982) *J. Biol. Chem.* **257**, 10575-10577
- Buchanan, B. B., Wolosvik, R. A. & Schurmann, J. (1979) *Trends Biochem. Sci.* **4**, 93-96
- Clements, P. R., Barden, R. E., Ahmand, P. A. & Ahmad, F. (1979) *Biochem. Biophys. Res. Commun.* **86**, 278-284
- Coulson, C. A. & Moffitt, W. A. (1947) *J. Chem. Phys.* **15**, 151
- Cutress, N. C., Grindley, T., Katritzki, A. R. & Topsom, R. D. (1974) *J. Chem. Soc. Perkin Trans. 2*, 263-268
- Derechin, M., Rustum, Y. M. & Barnard, E. A. (1972) *Biochemistry* **11**, 1793-1797
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70-77
- Freedman, R. B. (1979) *FEBS Lett.* **97**, 201-210
- Grimmett, M. R. (1984a) in *Comprehensive Heterocyclic Chemistry* (Katritzki, A. R. & Rees, C. W., eds.), vol. 5, pp. 365-368, Pergamon Press, New York
- Grimmett, M. R. (1984b) in *Comprehensive Heterocyclic Chemistry* (Katritzki, A. R. & Rees, C. W., eds.), vol. 5, pp. 443-445, Pergamon Press, New York
- Grimmett, M. R. (1984c) in *Comprehensive Heterocyclic Chemistry* (Katritzki, A. R. & Rees, C. W., eds.), vol. 5, pp. 520-521, Pergamon Press, New York
- Hoggett, J. G. & Kellett, G. L. (1976) *Eur. J. Biochem.* **66**, 65-77
- Jarori, G. K., Kasturi, S. R. & Kenkare, V. W. (1981) *Arch. Biochem. Biophys.* **211**, 258-268
- Jones, J. G., Otieno, S., Barnard, E. A. & Bhargava, A. K. (1975) *Biochemistry* **14**, 2396-2403
- Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245-3249
- Kreevoy, M. M., Eichinger, B. E., Starry, F. E., Katz, E. A. & Sellolet, J. H. (1964) *J. Org. Chem.* **29**, 1641-1642
- Lazdunski, M. (1974) *Prog. Bio-org. Chem.* **3**, 81-139
- Levitzki, A., Stallcup, W. B. & Koshland, D. E., Jr. (1971) *Biochemistry* **10**, 3371-3378
- Levy, H. M., Leber, P. D. & Ryan, E. M. (1963) *J. Biol. Chem.* **238**, 3654-3659
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Maley, F. & Lardy, H. A. (1955) *J. Biol. Chem.* **214**, 765-772
- McDonald, R. C., Steitz, T. A. & Engelman, D. M. (1979) *Biochemistry* **18**, 338-342
- Menezes, L. C., Grouselle, M. & Pudles, J. (1972) *Eur. J. Biochem.* **30**, 81-92
- Otieno, S., Bhargava, A. K., Barnard, E. A. & Ramel, A. H. (1975) *Biochemistry* **14**, 2403-2410
- Otieno, S., Bhargava, A. K., Serelis, D. & Barnard, E. A. (1977) *Biochemistry* **19**, 4249-4255
- Peters, B. A. & Neet, K. E. (1977) *J. Biol. Chem.* **252**, 5345-5349
- Peters, B. A. & Neet, K. E. (1978) *J. Biol. Chem.* **253**, 6826-6831

- Petra, P. H. (1971) *Biochemistry* **10**, 3163–3170
- Pho, D.-B., Roustan, C., Thi Tot, A. N. & Pradel, L.-A. (1977) *Biochemistry* **16**, 4533–4537
- Puri, R. N. & Roskoski, R., Jr. (1984) Abstr. Southwest ACS Regional Meeting 40th, Lubbock, abstr. no. 32, American Chemical Society, Washington
- Puri, R. N., Bhatnagar, D. & Roskoski, R., Jr. (1985a) *Biochemistry* **24**, 6499–6508
- Puri, R. N., Bhatnagar, D., Glass, D. B. & Roskoski, R., Jr. (1985b) *Biochemistry* **24**, 6508–6514
- Puri, R. N., Bhatnagar, D. & Roskoski, R., Jr. (1985c) *Biochemistry* **24**, 3360
- Puri, R. N., Bhatnagar, D. & Roskoski, R., Jr. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 1613
- Rafter, G. W. & Harmison, G. G. (1979) *Biochim. Biophys. Acta* **567**, 18–23
- Rustum, Y. M., Massaro, E. T. & Barnard, E. A. (1971) *Biochemistry* **10**, 3509–3516
- Schmidt, J. J. & Colowick, S. P. (1973) *Arch. Biochem. Biophys.* **158**, 458–470
- Schultze, I. T. & Colowick, S. P. (1969) *J. Biol. Chem.* **244**, 2306–2316
- Slein, M. W., Cori, G. T. & Cori, C. F. (1950) *J. Biol. Chem.* **186**, 763–780
- Steitz, T. A., Anderson, W. A., Fletterick, R. J. & Anderson, C. M. (1977) *J. Biol. Chem.* **252**, 4494–4501
- Steitz, T. A., Shoham, M. & Bennett, W. S., Jr. (1981) *Philos. Trans. R. Soc. London B* **293**, 43–52
- Wallace, T. J. & Schriesheim, A. (1962) *J. Org. Chem.* **27**, 1514–1516
- Wallace, T. J. & Schriesheim, A. (1965) *Tetrahedron* **21**, 2271–2280
- Wallace, T. J., Schriesheim, A. & Bartok, W. (1963) *J. Org. Chem.* **28**, 1311–1314
- Williamson, K. L. & Roberts, J. D. (1978) *Heterocycles* **11**, 313–320

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