Enhancement by Cysteinyl Thiols of Acetyltransferase-mediated, but Not of Sulfotransferase-mediated, Binding of a Pyrolysate-derived N-Hydroxyarylamine, 2-Hydroxyamino-6-methyldipyrido[1,2- α :3',2'- d]imidazole, to DNA

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The effect of thiols on the activation of a pyrolysate-derived N-hydroxyarylamine, 2-hydroxyamino-6-methyldipyrido[1,2- α :3',2'- d]imidazole (N-hydroxy-Glu-P-1), was studied *in vitro*. In hepatic cytosol of rats, [³H]-N-hydroxy-Glu-P-1 bound covalently to calf thymus DNA in the presence of acetyl CoA or 3'-phosphoadenosine-5'-phosphosulfate (PAPS). The extent of the binding of N-hydroxy-Glu-P-1 in a PAPS-dependent system was decreased by the addition of 10 mM glutathione, N-acetyl-L-cysteine, 2-mercaptoethanol or dithiothreitol. However, acetyl CoA-dependent binding of N-hydroxy-Glu-P-1 was stimulated by the addition of 10 mM N-acetyl-L-cysteine (3 fold), L-cysteine (2 fold) or glutathione (1.2 fold), but not 10 mM 2-mercaptoethanol or L-methionine. After hydrolysis of the modified DNA, no difference was detected in the physicochemical properties of the nucleoside adduct formed in the acetyl CoA-supported system with and without thiols. These results indicate that thiols with a cysteine residue are able to affect the activation of carcinogenic heterocyclic arylamines selectively by the modulation of the acetyltransferase-mediated, but not the sulfotransferase-mediated, pathway.

Key words: Heterocyclic N-hydroxyarylamine — Pyrolysate activation — DNA binding — Cysteinyl thiol — Acetyltransferase.

Similar to the activation of homocyclic analogues of arylamines such as 2-aminofluorene, a glutamic acid pyrolysate, Glu-P-1,³ is first N-oxidized to the N-hydroxy derivative by microsomal cytochrome P-450, especially by P-448-H type isozymes in experimental animals¹⁾ and human.²⁾ It is then further converted enzymatically to the O-acetylated and O-sulfonylated forms to cause DNA damage.³⁾

Using *in vivo* and *in vitro* systems, various types of endogenous and exogenous chemicals have been shown to modulate the effect of chemical-induced toxic and carcinogenic responses.⁴⁾ Although the mechanisms of these interactions are unclear in most cases, interactions with the reactive intermediate and with activating enzymes are considered to be involved in the modulation. In arylamine activation, N-hydroxylation is known to be influenced by the presence of various chemicals.⁵⁻⁸⁾ Several authors⁹⁻¹¹⁾ have reported that such modulation also

occurred at the second step of the activation, O-esterification. Smith *et al.*¹²⁾ investigated the interaction of N-hydroxy-2-acetylaminofluorene with tRNA, polyG and N-acetylmethionine, and found differences in the reactivities of various esterified forms towards these trapping agents. Meerman *et al.*¹³⁾ also observed modulating effects of methionine and GSH on the activation of N-hydroxy-2-acetylaminofluorene. The esterification is considered to be a critical step for the manifestation of genetic damage by arylamines, but the mechanism of the modulating action of chemicals on the N-hydroxyarylamine esterification is not yet well understood.

GSH and related agents are regarded as good protecting agents against chemically induced cellular damage.¹⁴⁾ Mutagenic activities of heterocyclic arylamines, however, have been reported to be enhanced in the presence of several thiols in the Ames test system.¹⁵⁻¹⁷⁾ In our recent studies, N-hydroxy-Glu-P-1 has been shown to be activated by mammalian cytosolic acetyltransferase^{18,19)} and sulfotransferase,^{20,21)} and by acetyltransferase in *Salmonella*.^{22,23)} These results suggest the possibility that thiols modulate the enzymatic activation of N-hydroxy-Glu-P-1 to the DNA binding product by interaction with the activating enzyme in the liver. Therefore, we have studied the effect of thiols on the acetyltransferase- and sulfotransferase-mediated binding of N-hydroxy-Glu-P-1 in hepatic cytosol of rats.

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³ Abbreviations: Glu-P-1, 2-amino-6-methyldipyrido[1,2- α :3',2'- d]imidazole; N-hydroxy-Glu-P-1, 2-hydroxyamino-6-methyldipyrido[1,2- α :3',2'- d]imidazole; GSH, glutathione; PCB, polychlorinated biphenyls; BSA, bovine serum albumin; BHT, butylated hydroxytoluene; DTT, dithiothreitol; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; HPLC, high-performance liquid chromatography.

MATERIALS AND METHODS

Chemicals Glu-P-1 was supplied under the Research Resources Program for Cancer Research of the Ministry of Education, Science and Culture, Japan. [^3H]-N-Hydroxy-Glu-P-1 (30–50 mCi/mmol) was prepared by the incubation of [^3H]Glu-P-1 with hepatic microsomes of PCB-treated rats and was isolated by HPLC as reported previously.¹⁾ Non-radioactive N-hydroxy-Glu-P-1 was synthesized from the nitro derivative as described.²⁴⁾ PAPS, acetyl CoA, GSH, L-cysteine, L-cystine, N-acetyl-L-cysteine, BSA, methionine, BHT, DTT and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, Mo.). All chemicals used were of analytical grade. Deoxyribonuclease I (DNase I), phosphodiesterase I, nuclease P1, and alkaline phosphatase were products of Takara Chemical Co., Tokyo. Sprague-Dawley rats were obtained from Clea Japan, Tokyo. Livers were homogenized in 1.15% (w/v) potassium chloride solution containing 100 mM potassium phosphate (pH 7.4). The cytosol (105,000g supernatant) was prepared as described²⁰⁾ and kept at -80°C after the addition of 1 mM DTT. Protein concentration was determined by the method of Lowry *et al.*²⁵⁾

Measurement of DNA binding A reaction mixture for the binding assay of N-hydroxy-Glu-P-1 consisted of 50 mM potassium bicine buffer (pH 7.8), 1.25 mg of calf thymus DNA, 3 mM magnesium acetate, 0.25 mg of cytosolic protein, 0.25 mM acetyl CoA or PAPS, and 20 μM [^3H]-N-hydroxy-Glu-P-1 in a final volume of 0.5 ml. The effect of thiols was examined by the addition of 1 to 20 mM to a reaction mixture without 1 mM DTT. The reaction was started by the addition of [^3H]-N-hydroxy-Glu-P-1 and terminated by mixing with 6 volumes of chilled ethanol. Precipitated DNA was recovered by centrifugation at 2,000g, re-dissolved in 3 ml of 0.5 M NaCl, and then extracted twice with *n*-butanol saturated with distilled water. DNA in the aqueous layer was again extracted (to separate it from proteins) twice with a phenol reagent including 8-hydroxyquinoline [1 g per liter of phenol saturated with 50 mM Tris acetate (pH 7.4)]. The modified DNA was precipitated with 2 to 3 volumes of chilled ethanol, and then treated with 200 mg of activated charcoal to remove trace amounts of non-covalently associated radioactivity after redissolving in 800 μl of 5 mM bis-Tris buffer (pH 7.1).²⁶⁾ The amount of DNA was measured by a diphenylamine method²⁷⁾ using a Beckman DU-65 spectrophotometer. Roughly 70% of DNA added initially in each reaction mixture was recovered. The radioactivity associated with DNA was measured using a Beckman LS-3800 liquid scintillation counter. Experiments were done in triplicate, and no appreciable difference was observed between the results of two separate experiments.

Esterification of N-hydroxy-Glu-P-1 The extents of O-acetylation and O-sulfonylation were measured as reported previously.^{20, 28)} The measurement is based on the reductive formation of Glu-P-1 from the O-acetoxy- or O-sulfonyloxy derivative formed by cytosolic O-acetylation and O-sulfonylation of N-hydroxy-Glu-P-1, respectively, in the presence of thiols. The typical reaction mixture consisted of 50 mM bicine-KOH (pH 7.8), 0.5 mg/ml of hepatic cytosol protein, 0.25 mM acetyl CoA or PAPS, 1 mM DTT and 20 μM N-hydroxy-Glu-P-1 in a final volume of 0.2 ml. The effect of thiols was examined by the addition of 1 to 20 mM of either one of the thiols. The reaction was done for 10 min at 37°C and terminated by the addition of 0.6 ml of methanol. The fluorescence in the supernatant was measured after centrifugation at 2,500g for 5 min. Emission and excitation wavelengths were 445 and 376 nm, respectively.

Analysis of nucleoside adduct N-Hydroxy-Glu-P-1-modified DNA (25 mg/50 ml) was enzymatically hydrolyzed into nucleosides as reported previously.²⁹⁾ The radiolabeled adduct was recovered by extraction with water-saturated *n*-butanol. The extent of hydrolysis as estimated by taking account of the extraction efficiency was 72–82%. The organic extract was evaporated under reduced pressure and the residue was subjected to HPLC after redissolving it in 30% methanol-water. HPLC was conducted with a JASCO Tri-Rotar VI system connected with a Nucleosil 7C18 column (4 $\times$ 300 mm). The nucleosides were eluted with a gradient of distilled water-methanol at a flow rate of 1.5 ml/min. The radioactivity in each collected fraction (1.5 ml) was determined by liquid scintillation counting. The pH/solvent partitioning of the modified nucleoside was performed by the method of Moore and Koreeda³⁰⁾ using a diethyl ether/*n*-butanol (85:15) mixture.

RESULTS

Cytosolic activation of N-hydroxy-Glu-P-1 In the system containing hepatic cytosols from rats, N-hydroxy-Glu-P-1 was activated to a reactive intermediate, which react readily with calf thymus DNA (Table I). The extent of the covalent binding of [^3H]-N-hydroxy-Glu-P-1 to DNA was increased effectively by the addition of cofactors of cytosolic acetyltransferase and sulfotransferase, acetyl CoA and PAPS, respectively. These results were consistent with our previous results on the enzymatic activation^{18, 21)} and confirmed the role of cytosolic acetyltransferase and sulfotransferase in the activation of N-hydroxy-Glu-P-1. Using a similar system, the effect of ATP on the extent of the binding of N-hydroxy-Glu-P-1 was also examined, but no appreciable increase in the binding was detected, suggesting a negligible contribution of the amino-tRNA synthetase system^{31, 32)} to

the hepatic activation of N-hydroxy-Glu-P-1 in rats (data not shown).

Modulation of enzymatic activation of N-hydroxy-Glu-P-1 To understand the mechanism of the interaction of chemicals with cytosolic enzyme-mediated activation of

Table I. Requirements for the Acetyltransferase- and Sulfotransferase-mediated Binding of N-Hydroxy-Glu-P-1

System	Amounts bound to DNA (pmol/mg of DNA/mg of protein/30 min)	(%) ^{a)}
Sulfation		
Complete	90 ± 18	(100)
minus PAPS	6 ± 2	(6.6)
minus cytosol	2 ± 1	(2.2)
minus PAPS and cytosol	2 ± 1	(2.2)
Acetylation		
Complete	472 ± 11	(100)
minus acetyl CoA	13 ± 3	(2.7)
minus cytosol	4 ± 1	(0.8)
minus acetyl CoA and cytosol	2 ± 1	(0.4)

a) Relative values compared to the respective controls (complete system) are shown as percentages. The data are mean values ± SD of triplicate determinations in three different experiments. The complete system consisted of 50 mM potassium bicine (pH 7.8), 1 mM DTT, 1.25 mg of calf thymus DNA, 3 mM magnesium acetate, 0.25 mg of cytosolic protein, 0.25 mM acetyl CoA or PAPS, and 20 μM N-hydroxy-Glu-P-1 in a final volume of 0.5 ml. Other experimental details are described in "Materials and Methods."

N-hydroxyarylamines, the effects of thiols and other chemicals on the binding of N-hydroxy-Glu-P-1 were studied. As described in Table II, sulfotransferase-mediated binding of N-hydroxy-Glu-P-1 was decreased in the presence of 10 mM GSH. The extent of the binding was also lowered by the addition of 10 mM N-acetyl-L-cysteine or L-cysteine, although an oxidized form of L-cysteine, L-cystine, had no inhibitory effect on the covalent binding. Other thiols, 2-mercaptoethanol and DTT, also showed inhibitory effects on the DNA binding. Furthermore, the addition of 0.1 mM *tert*-butylated hydroxytoluene caused an effective decrease of the DNA binding to 13.5% of the control. Although L-methionine and BSA are known to decrease the binding of N-hydroxy-2-acetylaminofluorene to macromolecules in mammalian cytosols by trapping the reactive intermediate to form the respective stable methionine and protein adducts,^{13, 33, 34)} the sulfotransferase-mediated binding of N-hydroxy-Glu-P-1 was not reduced by the addition of 10 mM methionine or 10 mg/ml of BSA in the present study.

Acetyl CoA-dependent binding of N-hydroxy-Glu-P-1 was also influenced by several chemicals. Similar to the case with sulfotransferase-mediated activation, addition of 10 mM 2-mercaptoethanol or DTT showed strong inhibitory effects on acetyltransferase-mediated binding of N-hydroxy-Glu-P-1 to DNA. However, the acetyltransferase-mediated binding was much increased in the presence of 10 mM N-acetyl-L-cysteine or L-cysteine. GSH also showed a similar increase, although the oxidized form of L-cysteine, L-cystine, had no stimulatory effect on the binding. An antioxidant, butylated hydroxy-

Table II. Effects of Chemicals on the Cytosolic Acetyl CoA- and PAPS-dependent Binding of N-Hydroxy-Glu-P-1 to DNA

Chemical added	Concentration (mM)	Amounts bound to DNA (pmol/mg of DNA/mg of protein/30 min)	
		PAPS-dependent	Acetyl CoA-dependent
None		140 ± 7 (100) ^{a)}	472 ± 11 (100)
GSH	10	36 ± 5 (25.7)	581 ± 8 (123.1)
N-Acetyl-L-cysteine	10	96 ± 9 (68.6)	1,400 ± 123 (296.6)
L-Cysteine	10	113 ± 14 (80.7)	969 ± 54 (205.3)
L-Cystine	10	161 ± 8 (115.0)	451 ± 20 (95.6)
2-Mercaptoethanol	10	65 ± 3 (46.4)	2 ± 1 (0.4)
DTT	10	84 ± 2 (60.0)	68 ± 3 (14.4)
Methionine	10	174 ± 7 (124.2)	282 ± 7 (59.7)
BHT ^{b)}	1	19 ± 2 (13.5)	52 ± 4 (11.0)
BSA ^{c)}		130 ± 6 (92.8)	315 ± 9 (66.7)

a) Relative values as compared to the respective control are shown as percent.

b) Butylated hydroxytoluene.

c) Bovine serum albumin was added at the concentration of 10 mg/ml.

The data are shown as mean ± SD of three different determinations. Other experimental conditions are described in "Materials and Methods."

toluene, decreased the extent of the acetyltransferase-mediated binding of N-hydroxy-Glu-P-1 to DNA with similar efficiency to that in the case of the sulfotransferase-mediated reaction.

Properties of thiol enhancement of the covalent binding

The effect of changes in the concentration of several thiols on the acetyltransferase-mediated binding of N-hydroxy-Glu-P-1 is shown in Fig. 1. The binding of N-hydroxy-Glu-P-1 was decreased by the addition of a

Fig. 1. Extents of acetyltransferase-mediated binding of N-hydroxy-Glu-P-1 to DNA in the presence of various concentration of thiols. ▲; N-acetyl-L-cysteine, △; L-cysteine, ◆; GSH, ◇; methionine, ●; L-cystine, ○; 2-mercaptoethanol. The reaction mixture contained 0.25 mM acetyl CoA or PAPS, 20 μ M N-hydroxy-Glu-P-1, 0.25 mg of hepatic cytosol, 1.25 mg of calf thymus DNA, 0 to 20 mM each thiol and other necessary components as described in "Materials and Methods."

low concentration (1 mM) of each thiol examined. The extent of the binding was, however, increased with increase in the concentration of N-acetyl-L-cysteine, L-cysteine or GSH up to 10 mM, and then diminished by

Fig. 2. Relative rates of the O-esterification of N-hydroxy-Glu-P-1. The rate of the O-esterification was measured in terms of the reductive formation of Glu-P-1. ◆; GSH, ▲; N-acetyl-L-cysteine, △; L-cysteine, ●; L-cystine. In this experiment, the rate of the non-enzymatic formation of Glu-P-1 was subtracted from each experimental value. The rate of non-enzymatic reduction in the system without cytosol was not increased, but rather decreased slightly at high concentrations of thiols. For example, the rates of the non-enzymatic reduction in the acetyl CoA- and PAPS-system (without cytosol) were 19.6% and 6.8% of those of their complete systems (with cytosol), respectively, and the rates were decreased to 13.6% and 4.1%, respectively, in the presence of 20 mM N-acetyl-L-cysteine.

Fig. 3. HPLC profile of an N-hydroxy-Glu-P-1-modified nucleoside formed in the acetyltransferase-mediated activating system. The modified nucleosides formed by enzymatic hydrolysis of N-acetoxy-Glu-P-1-modified DNA were separated by reversed-phase chromatography. The radioactivity associated with a [3 H]Glu-P-1-bound nucleoside is shown as open (from the mixture including N-acetyl-L-cysteine) and closed bars (from the mixture without N-acetyl-L-cysteine). Other experimental details are described in "Materials and Methods."

further increase in their concentrations. L-Methionine and L-cystine had no appreciable effect throughout the ranges examined, and 2-mercaptoethanol showed a clear dose-dependent inhibition of the covalent binding.

After enzymatic O-esterification of N-hydroxyarylamines, considerable portions of the esterified forms are converted to the corresponding arylamines by reductive cleavage.^{12, 20, 28, 35} To assess the effects of thiols on the rate of the esterification of N-hydroxy-Glu-P-1, reductive formation of Glu-P-1 was quantitated fluorometrically. As shown in Fig. 2, the formation of Glu-P-1 was decreased by thiols in the acetyl CoA-supported system. No correlation was observed between the extent of the enhancement in the covalent binding and rate of the esterification. In this experiment, the formation of Glu-P-1 was 2-fold higher in the acetyl CoA-supported than the PAPS-supported system, and was less inhibited by thiols in the acetyl CoA-supported than the PAPS-supported system, which is consistent with the results on the activation of N-hydroxy-Glu-P-1.

Cysteiny l thiols may increase the extent of the binding of N-hydroxy-Glu-P-1 by way of the formation of another adduct than that detected in the absence of cysteinyl thiols. Thus, the nucleoside adduct was analyzed by HPLC after enzymatic hydrolysis of the modified DNA. As shown in Fig. 3, the radioactivity associated with the N-hydroxy-Glu-P-1 moiety migrated as a single major radioactive peak on a reversed-phase column with a water-methanol gradient system. Retention time and absorption spectra of the adduct peak in DNA hydrolysates, obtained with and without N-acetyl-L-cysteine, were identical, and also no difference was observed between the two isolated adducts in their pH-partition profile³⁰ (data not shown).

DISCUSSION

In our previous study,¹⁶ a heterocyclic amine intermediate, N-hydroxy-Trp-P-2, was demonstrated to form an unstable and highly mutagenic derivative in the presence of GSH with and without GSH transferase. A recent study³⁶ has also shown that a 2-nitroso derivative of Glu-P-1 reacts with GSH to yield the adducts, which bind through an N-S linkage. The adducts are formed non-enzymatically, and neither cytosol nor cofactors are required.

In the present study, thiols including a cysteine residue in their structures enhanced *in vitro* the covalent binding of a pyrolysate-derived heterocyclic N-hydroxy-arylamine to DNA. The enhancement occurred with the acetyl CoA-dependent, but not with the PAPS-dependent, binding of N-hydroxy-Glu-P-1 to DNA. The enhancement depends on the concentration of the thiols and attains the maximum at 10 mM for N-acetyl-L-

Fig. 4. A possible scheme for the formation of reactive intermediates in the presence of thiols and acetyl CoA. RSH, thiol.

cysteine, L-cysteine, and GSH. In addition, the covalent binding of N-hydroxy-Glu-P-1 to DNA was decreased in the absence of acetyl CoA or cytosol to 3% and 1.3% of that in the complete system, respectively. N-Acetyl-L-cysteine may act as an activator of acetyltransferase, but the rate of the esterification determined by the reductive formation of Glu-P-1 was not increased, but rather decreased, in the presence of N-acetyl-L-cysteine. Although thiols may react with protein and nucleophiles other than DNA and increase the extent of the DNA binding of N-acetoxy-Glu-P-1 as the result of the shift in binding counterparts, the enhancement was observed only with thiols containing an acidic moiety (cysteine group). The addition of 10 mM DTT or 2-mercaptoethanol decreased the binding. Thus the increased binding to DNA is not likely to be caused by non-specific reduction of nucleophiles other than DNA by thiols. The exact mechanism of the thiol-induced increase in the DNA binding remains uncertain, but these results may be explained by the hypothesis that the stimulation of the binding of N-hydroxy-Glu-P-1 to DNA by thiols is the result of the interaction of N-acetoxy-Glu-P-1 with thiols, but not of N-hydroxy-Glu-P-1 with thiols, analogously with the case of the interaction of N-hydroxy-Trp-P-2 and GSH.³⁷ On the analysis of the N-acetoxy-Glu-P-1-modified DNA (Fig. 3), no difference was observed in the physico-chemical properties of the modified nucleoside isolated from the reaction mixture with and without N-acetyl-L-cysteine. In addition, ³H-GSH-associated radioactivity was not incorporated into the N-acetoxy-Glu-P-1-modified nucleoside isolated in the reaction system including ³H-GSH (data not shown). Although the chemical properties of the reactive species formed in the presence of thiols are unclear, N-acetoxy-Glu-P-1 may form an unidentified complex with cysteinyl thiol, and this may serve as a slightly stabilized pool for the supply

of reactive N-acetoxy-Glu-P-1 by the spontaneous reverse reaction (Fig. 4). The possibility of the formation of other reactive species such as N(-O-SR)-Glu-P-1 is, of course, not excluded, but this remains to be clarified.

As shown in Fig. 1, the maximal enhancement effect was observed at around 10 mM N-acetyl-L-cysteine, L-cysteine or GSH, and the thiol-induced enhancement was diminished with a further increase in concentration. Although the reason for the decrease is unclear, the rate of O-acetylation of N-hydroxy-Glu-P-1, as assessed by the reductive formation, was also decreased at high concentrations of thiols. Thus, the decrease in the formation of N-acetoxy-Glu-P-1 or the increased formation of unidentified metabolites could be, in part, responsible for the

decrease in the extent of the DNA binding. In livers of mammals, GSH is present in high concentrations, 7–8 $\mu\text{mol/g}$ liver tissues.³⁸⁾ Therefore, the results obtained in this study suggest the possibility that GSH and related thiols may, in contrast to their protective role, stimulate the activation of heterocyclic arylamines in combination with cytosolic acetyltransferase in mammalian tissues.

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