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MECHANISM OF ACTIVATION OF PROXIMATE MUTAGENS IN AMES' TESTER STRAINS: THE ACETYL-CoA DEPENDENT ENZYME IN SALMONELLA TYPHIMURIUM TA98 DEFICIENT IN TA98/1,8-DNP$_6$ CATALYZES DNA-BINDING AS THE CAUSE OF MUTAGENICITY

Kazuki Saito, Yasushi Yamazoe, Tetsuya Kamataki and Ryuichi Kato

Department of Pharmacology, School of Medicine, Keio University
Shinanomachi, Shinjuku-ku, Tokyo 160, Japan

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Summary: The mechanism of activation of proximate mutagens in Ames' tester strains was described. 2-Hydroxyamino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (N-OH-Glu-P-1) and 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole (N-OH-Trp-P-2) were activated to DNA-binding species in the presence of acetyl-CoA by the enzyme(s) in Salmonella typhimurium TA98, and this enzyme was deficient in TA98/1,8-DNP$_6$. Mutagenicity of N-OH-Glu-P-1 to TA98/1,8-DNP$_6$ was much lower than that to TA98. Therefore, it was demonstrated that the acetyl-CoA dependent enzyme(s) activated N-OH-Glu-P-1 to the active form which could covalently bind to DNA and subsequently caused mutagenicity.

The Ames' bacterial mutation assay is widely used for the detection of mutagens-carcinogens by using several strains of Salmonella typhimurium (1). In this assay, mammalian liver homogenates and NADPH-generating system are added in order to activate promutagens to so-called direct mutagens. However, these activated mutagens are suggested to be still proximate mutagens which should be further activated by the enzymes of bacteria to ultimate forms responsible for covalent binding to DNA. Few investigations of the mechanisms of this activation in Salmonella tester strains have been reported. N-OH-Trp-P-2 and N-OH-Glu-P-1, heterocyclic hydroxylamines, are metabolites formed by the

Abbreviations: N-OH-Glu-P-1, 2-hydroxyamino-6-methyldipyrido[1,2-a:3',2'-d]imidazole; N-OH-Trp-P-2, 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole; Glu-P-1, 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole

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cytochrome P-450-dependent monooxygenase enzyme system from Trp-P-2 and Glu-P-1, which were isolated from pyrolysates of amino acids (2-4). These hydroxylamines shows so-called direct mutagenicities to S. typhimurium TA98 (2,3). No information was available concerning the biotransformation of these compounds to the reactive species responsible for covalent binding to DNA by bacterial enzyme(s), though activation by rat liver enzyme has been reported (5). S. typhimurium mutant strain TA98/1,8-DNP_6 was isolated as resistant to the mutagenicity of 1,8-dinitropyrene (6). Furthermore, it was recently reported that this strain was also non-responsive to some nitroso- and N-hydroxyaminoarenes (7). Therefore, a specific esterifying enzyme was suspected to be deficient in TA98/1,8-DNP_6. In this paper, we studied the mutagenic activities of N-OH-Glu-P-1 and N-OH-Trp-P-2 to TA98 and TA98/1,8-DNP_6, and we observed the activity of acetyl-CoA-dependent enhancement of DNA-binding in cell-free extracts from TA98 but not in those from TA98/1,8-DNP_6. We discuss the mechanism of activation of aromatic hydroxylamines by bacterial enzymes correlating with the deficiency in TA98/1,8-DNP_6.

MATERIALS AND METHODS

Chemicals --- N-OH-Glu-P-1 and N-OH-Trp-P-2 were synthesized as described previously (8). [^{3}H]N-OH-Glu-P-1 and [^{3}H]N-OH-Trp-P-2 were prepared by incubation of [^{3}H]Trp-P-2 and [^{3}H]Trp-P-2 with microsomes followed by isolation with high performance liquid chromatography (2,3). Acetyl-CoA was purchased from Sigma Chemical Co., St. Louis, MO.

Preparation of cytosol fraction of S. typhimurium --- S. typhimurium TA98/1,8-DNP_6 was kindly donated from Prof. H. S. Rosenkranz, Case Western University. S. typhimurium TA98 or TA98/1,8-DNP_6 were grown at 27°C for 15 hr with shaking in a medium containing 8g of nutrient broth (Difco) and 5g of NaCl per liter. Ten grams of bacterial cells were suspended in 30ml of buffer containing 0.25M sucrose, 50mM Tris-acetate (pH 7.4) and 1mM dithiothreitol, and disrupted by a French press operated at 10,000psi as described previously (9). Cell debris was removed by centrifugation at 9,000g for 20min and the supernatant was centrifuged again at 105,000g for 60min. The resulting supernatants were used as cytosol fraction.

Mutation assay --- Mutation assay was carried out as described previously (8).

Binding of N-OH-Glu-P-1 and N-OH-Trp-P-2 to DNA --- The incubation mixture for binding assay contained 1mM acetyl-CoA, 1mM dithiothreitol, 3mM magnesium acetate, 50mM potassium bicine (pH 8.0), 1mg of calf thymus DNA, 0.2 to 1.6mg protein of cytosol of S. typhimurium and 2.48 to 5.27μM [^{3}H]N-OH-Glu-P-1 or [^{3}H]N-OH-Trp-P-2 in a final volume of 1ml. The reaction and extraction of DNA were performed essentially as reported previously (5).

RESULTS

Mutagenicities of N-OH-Glu-P-1 and N-OH-Trp-P-2 to S. typhimurium TA98 and TA98/1,8-DNP$_6$ without external activation system are shown in Table 1. N-OH-Glu-P-1 was a potent mutagen to TA98, but showed only 24% of mutagenic activity to TA98/1,8-DNP$_6$ in comparison with TA98. On the other hand, N-OH-Trp-P-2 showed equal mutagenic activity to both TA98 and to TA98/1,8-DNP$_6$.

In the activation of N-hydroxyacetylaminofluorene, O-acetylation by acetyltransferase in rat liver has been thought to be the critical step for generating the DNA-binding species (10). We found the acetyl-CoA-dependent DNA-binding of N-OH-Glu-P-1 and N-OH-Trp-P-2 catalyzed by bacterial enzyme(s) (Table 2). Acetyl-CoA enhanced the binding of the N-hydroxylated compounds in the presence of cytosol from TA98. The binding of N-OH-Glu-P-1 was strictly dependent on acetyl-CoA and cytosol in contrast to the case with N-OH-Trp-P-2. N-OH-Trp-P-2 itself fairly bound to DNA

Table 1. Mutagenicities of N-OH-Glu-P-1 and N-OH-Trp-P-2 to S. typhimurium TA98 and TA98/1,8-DNP$_6$.

Compound	His$^+$ revertants/plate	
	TA98	TA98/1,8-DNP$_6$
N-OH-Glu-P-1 (20pmole)	170 ± 34 (100%)	41 ± 1 (24%)
N-OH-Trp-P-2 (20pmole)	1410 ± 180 (100%)	1490 ± 180 (105%)

The test compounds were dissolved in 50μl of DMSO and assayed as described in MATERIALS AND METHODS. Each value represents the mean ± S.D. of four determinations, and the number of spontaneous mutant colonies was subtracted. Data in parentheses are the percentages of numbers of his$^+$ revertants normalized with those of TA98.

Table 2. Acetyl-CoA dependent DNA-binding of N-OH-Glu-P-1 and N-OH-Trp-P-2 by cytosol fraction of S. typhimurium TA98.

System	Amounts of N-hydroxylated compounds bound to DNA (pmole/mg DNA/30min)	
	N-OH-Glu-P-1	N-OH-Trp-P-2
Complete	24.1 (100%)	19.6 (100%)
-cytosol	1.23 (5.1%)	12.9 (65.8%)
-acetyl CoA	0.48 (2.0%)	0.46 (2.3%)
-cytosol and acetyl-CoA	0.21 (0.9%)	1.94 (9.9%)

The complete system contained 1mM acetyl-CoA and 0.5mg/ml of protein of cytosol derived from TA98. The concentrations of N-hydroxylated metabolites were 2.91μM (N-OH-Glu-P-1) and 2.48μM (N-OH-Trp-P-2). Data are the means of duplicate incubations.

non-enzymatically and the binding was enhanced by the presence of acetyl-CoA.

We elucidated whether or not the enhancement of binding by acetyl-CoA was observed in TA98/1,8-DNP_6 which possessed lower sensitivity to N-OH-Glu-P-1. As depicted in Fig. 1, acetyl-CoA-dependent binding of N-OH-Glu-P-1 was remarkably stimulated by the cytosolic protein obtained from strain TA98 which was sensitive to the mutagenic action of N-OH-Glu-P-1. The cytosolic

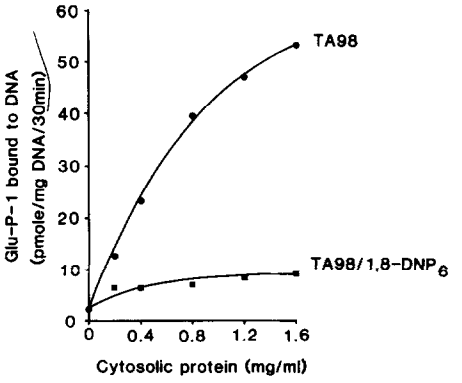

Fig. 1 Dependence of binding of N-OH-Glu-P-1 to DNA on the bacterial cytosolic protein in the presence of acetyl-CoA. The concentrations of acetyl-CoA and N-OH-Glu-P-1 were 1mM and 3.83μM, respectively. Each point represents the mean of duplicate incubations.

Table 3. Effects of the cytosol from S. typhimurium TA98 and TA98/1,8-DNP_6 on DNA-binding of N-OH-Trp-P-2 in the presence of acetyl-CoA.

Concentration of cytosolic protein (mg/ml)	Amount of N-OH-Trp-P-2 bound to DNA (pmole/mg DNA/30min)	
	TA98	TA98/1,8-DNP_6
0	55.0	
0.2	63.7	38.5
0.4	73.8	10.2
0.8	113.7	12.8

The incubation was carried out in the presence of 1mM acetyl-CoA. The concentration of N-OH-Trp-P-2 was 5.27μM. Data are the means of duplicate incubations.

protein derived from TA98/1,8-DNP_6, on the contrary, hardly enhanced the DNA-binding in the presence of acetyl-CoA. Similar results were obtained by using N-OH-Trp-P-2 (Table 3), though the appreciable amount of non-enzymatic binding was observed in the presence of acetyl-CoA and the binding was inhibited by cytosol from TA98/1,8-DNP_6. The mechanism of this inhibition is not yet clear.

DISCUSSION

The present data indicate that N-OH-Glu-P-1 and N-OH-Trp-P-2 were activated to DNA-binding species by acetyl-CoA and enzyme(s) in S. typhimurium TA98 and this enzyme(s) was deficient in TA98/1,8-DNP_6. In the course of preparation of this manuscript, McCoy et al. (11) suggested that TA98/1,8-DNP_6 lacks an esterification enzyme which appears to be a transacetylase by experiments using intact bacterial cells. This enzyme may be identical to the enzyme which could activate N-OH-Glu-P-1 and N-OH-Trp-P-2. Mutagenic activity of N-OH-Glu-P-1 to TA98 was distinguishable from TA98/1,8-DNP_6 as shown in Table 1. These results are consistent with the results of DNA-binding. Therefore, it was deduced that N-OH-Glu-P-1 was activated by the bacterial acetyl-

CoA-dependent system to the ultimate form which could covalently bind to DNA and subsequently caused mutagenicity. The enhancement of non-enzymatic binding of N-OH-Glu-P-1 by acetylation with ketene was also reported (12). Very recently, Nagao et al. (13) suggested the involvement of sulfotransferase in activation of some hydroxylamines in TA98. On the other hand, N-OH-Trp-P-2 showed equal mutagenic activity to both TA98 and to TA98/1,8-DNp_6, though acetyl-CoA-dependent binding was not observed in TA98/1,8-DNP_6. Two explanations for these phenomena are seen to be possible. Since N-OH-Trp-P-2 can appreciably bind to DNA without any activation system (14), this degree of binding might be enough to cause mutagenicity. The other possibility was that any other mechanisms of activation operate more effectively in the bacterial cells to cause mutagenicity.

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REFERENCES

1. Ames, B. N., McCann, J. and Yamasaki, E. (1975) Mutation Res. **31**, 347-364.
2. Yamazoe, Y., Ishii, K., Kamataki, T., Kato, R. and Sugimura, T. (1980) Chem.-Biol. Interact. **30**, 125-138.
3. Ishii, K., Yamazoe, Y., Kamataki, T. and Kato, R. (1981) Chem.-Biol. Interact. **38**, 1-13.
4 Sugimura, T. and Sato, S. (1983) Cancer Res. **43**, 2415s-2421s.
5. Yamazoe, Y., Shimada, M., Kamataki, T. and Kato, R. (1982) Biochem. Biophys. Res. Commun. **107**, 165-172.
6. McCoy, E. C., Rosenkranz, H. S. and Mermelstein, R. (1981) Environ. Mutagen. **3**, 421-427.
7. McCoy, E. C., McCoy, G. D. and Rosenkranz, H. S. (1982) Biochem. Biophys. Res. Commun. **108**, 1362-1367.
8. Saito, K., Yamazoe, Y., Kamataki, T. and Kato, R. Carcinogenesis in submitted.
9. Saito, K., Kawaguchi, A., Seyama, Y., Yamakawa, T. and Okuda, S. (1981) Eur. J. Biochem. **116**, 581-586.

10. Miller, E. C. (1978) Cancer Res. **38**, 1479-1496.
11. McCoy, E. C., Anders, M. and Rosenkranz, H. S. (1983) Mutation Res. **121**, 17-23.
12. Hashimoto, Y., Shudo, K. and Okamoto, T. (1982) J. Amer. Chem. Soc. **104**, 7636-7640.
13. Nagao, M., Fugita, Y., Wakabayashi, K. and Sugimura, T. Biochem. Biophys. Res. Commun. 114, 2, 626-631.
14. Mita, S., Ishii, K., Yamazoe, Y., Kamataki, T., Kato, R. and Sugimura, T. (1981) Cancer Res. **41**, 3610-3614.