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COVALENT BINDING OF N-HYDROXY-TRP-P-2 TO DNA BY CYTOSOLIC PROLINE-DEPENDENT SYSTEM

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SUMMARY: A new enzymatic activation system for the covalent binding of a mutagenic metabolite of a tryptophan pyrolysate, N-hydroxy-Trp-P-2, is described. The system exists in hepatic cytosolic fraction of rats, requiring ATP and some amino acids as the cofactor. Proline was the most effective among amino acids examined. These results suggest that N-hydroxy-Trp-P-2 formed by microsomal cytochrome P-450 is activated by prolyl-tRNA synthetase or related enzyme(s). Possible roles of sulfation and acetylation in the formation of the covalent adducts were also discussed.

Some arylamines and arylamides are well known carcinogens which require metabolic activations to exert their effects in mammals. N-Hydroxylation of these compounds has been established as the first step in the metabolic activations (1,2). Many studies have provided evidence that esterification of hydroxylamines with sulfate, acetate, and others is necessary to initiate neoplastic lesions through the covalent interaction with macromolecules (1,2). Most of these studies, however, have been done with substituted arylamines and arylhydroxamic acids such as N-hydroxy-2-acetylaminofluorene (3) and N-hydroxy-4-methylaminoazobenzene (4), and data with non-substituted arylamines were limited (5,6,7). A carcinogenic arylamine, Trp-P-2, which was originally detected as a potent promutagen in pyrolysates of tryptophan (8,9), has been shown to be contained in various foods and amino acid pyrolysates (10,11). We previously reported that Trp-P-2 was converted by liver microsomal cytochrome P-450 system to an N-hydroxy derivative, 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]-

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indole (N-hydroxy-Trp-P-2), which was highly mutagenic to Salmonella typhimurium TA 98 (12,13,14).

With regard to the covalent binding to DNA, we reported that N-hydroxy-Trp-P-2 reacted with calf thymus DNA without the aid of an activating enzyme system (15). Recently, we also demonstrated that seryl-tRNA synthetase purified from yeast catalyzed the covalent binding of N-hydroxy-Trp-p-2 to DNA (16). We herein report the possible role of this aminoacyl-tRNA synthetase-dependent system in rat livers for the binding of N-hydroxy-Trp-P-2 to DNA.

MATERIALS and METHODS

Male Sprague-Dawley rats were given i.p. a PCB mixture, KC-500, at a single dose of 500 mg/kg. The animals were killed 7 days after the administration. Livers were purfused in situ with 0.25 M sucrose-50 mM Tris-acetate (pH 7.4) and homogenized with 3 volumes of the same buffer. Cytosol (105,000 x g supernatant fraction) and microsomes were prepared as described (14). Cytosol was applied to a column of sephadex G-25 (3 x 40 cm). A 50 mM Tris-acetate (pH 7.4) containing 1 mM dithiothreitol (DTT) was used as the eluant. The fraction (G-25 fraction) eluted as a void peak was combined and stored at -80°C until use. 3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) acetic acid salt was kindly donated by Dr. T. Sugimura, National Cancer Research Institute, Tokyo, Japan. ^{3}H-[ring labeled]-Trp-P-2 was obtained from New England Nuclear, Boston, Mass. The specific activity was 344 mCi/m mole and the purity (ca. 99 %) was checked by TLC and HPLC. ATP, AMP, GTP, adenosine 3'5'-diphosphate (PAP), DTT, p-nitrophenylsulfate, S-acetyl coenzyme A and calf thymus DNA (type 1) were obtained from Sigma chemicals, St. Louis, Mo. N-Hydroxy-2-acetylaminofluorene (N-hydroxy-AAF) was synthesized as previously described (17). N-Hydroxy-Trp-P-2 used in this study was prepared by incubation of ^{3}H-Trp-P-2 with hepatic microsomes. Isolation of N-hydroxy-Trp-P-2 was performed as described previously (16).

An incubation mixture for the covalent binding of N-hydroxy-Trp-P-2 consisted of 3 mM ATP, 10 mM L-serine or L-proline, 3 mM magnesium acetate, 1 mM DTT, 50 mM potassium bicine (pH 8.0), 1 mg of calf thymus DNA, 2 mg protein of cytosol or G-25 fraction, and 4.12 to 7.56 nmoles of labeled N-hydroxy-Trp-P-2 in a final volume of 1 ml. The reaction was started by addition of N-hydroxy-Trp-P-2 dissolved in 0.05 ml of 20 mM sodium acetate (pH 4.5) and performed for 30 min at 37°C. A 2 ml portion of ethyl acetate and 1 ml of 1 M sodium acetate were added to terminate the reaction. After centrifugation and aspiration of the organic layer, 2 ml of phenol saturated with 0.1 M Tris-acetate (pH 7.4) was added to the aqueous layer and proteins in the mixture were denatured. A portion (1.5 ml) of the aqueous layer was re-extracted with an equal volume of the same phenol reagent. An aliquot (1.2 ml) of the resultant aqueous layer was treated with 2 volumes of ethanol and the precipitated DNA was washed with 2 ml of ethanol. A 0.5 ml solution (0.15 M NaCl-0.015 M sodium

Table 1. Effect of cytosolic fraction on the covalent binding of N-hydroxy-Trp-P-2 to DNA

Preparation	Amount of N-hydroxy-Trp-P-2 bound to DNA (pmoles bound per mg DNA per 30 min)
Cytosol	99.7
G-25 fraction	80.1
None	8.0

Activities were determined in the presence of 3 mM ATP, 10 mM L-serine, 6.7 nmoles of N-hydroxy-Trp-P-2, and other necessary components as described in MATERIALS and METHODS.

citrate, pH 7.4) containing ribonuclease A (sigma, type 1-A), which had been treated in a bath at 80°C for 10 min, was added to a tube containing DNA and then the mixture was incubated for 1 hr at 37°C. Chloroform-isoamyl alcohol mixture (24:1, 1 ml) and 0.15 M NaCl-0.015 M sodium citrate (0.5 ml) containing 0.1 M EDTA and 2 % sodium dodecylsulfate were added to terminate the treatment. An aqueous layer (0.8 ml) obtained after centrifugation was treated with 2 volumes of ethanol and the mixture stood overnight. Precipitates formed were washed with ethanol and dissolved in 1 ml of 0.5 mM sodium acetate. After extraction with the same volume of ethyl acetate, the radioactivity and DNA content were measured as previously described (16).

RESULTS

We reported in our previous paper that seryl-tRNA synthetase purified from yeast catalyzed the covalent binding of N-hydroxy-Trp-P-2 to DNA (16). Thus, we wished to know whether such an amino acid dependent activation system as that in yeast exists in rats. As described in Table 1, the covalent binding of N-hydroxy-Trp-P-2 to calf thymus DNA increased by addition of cytosol in a reaction mixture fortified with serine and ATP. A similar increase was also observed with the G-25 fraction of cytosol, indicating that an enzyme(s), not a small molecule, was responsible for this increment.

Seryl-tRNA synthetase required ATP and serine for the catalysis of covalent binding of 4-hydroxyaminoquinoline-1-oxide (4-HAQO)(7) and N-hydroxy-Trp-P-2 (16). Further, proline was also reported to support the 4-HAQO binding (18). Thus we examined the effect of ATP, serine, and proline on the binding of N-hydroxy-Trp-P-2 using the G-25 fraction. Covalent binding of N-hydroxy-Trp-P-2

Table 2. Requirement for the covalent binding of N-hydroxy-Trp-P-2 to DNA

System	Amount of N-hydroxy-Trp-P-2 bound to DNA (pmoles bound per mg DNA per 30 min)	
Experiment I		
Complete	80.1	(100)
- ATP	2.5	(3.1)
- Serine	54.6	(68.2)
- Serine + proline	257.2	(321.1)
Experiment II		
Complete	230.0	(100)
- ATP	0.3	(0.1)
- ATP + AMP	0.9	(0.4)
+ AMP	153.0	(66.5)
- ATP + GTP	69.4	(30.2)

The complete system contained 3 mM ATP, 2 mg protein of G-25 fraction, 10 mM L-serine (exp. I) or L-proline (exp. II) and other necessary components as described in MATERIALS and METHODS. The concentration of GTP or AMP was 3 mM.

to DNA was almost undetectable in the absence of ATP (Table 2). Omission of serine also decreased the binding by 68%. However, addition of proline, instead of serine, resulted in the enhancement by 321%. Requirements of pyridine nucleotides were also examined in the presence of proline. AMP did not serve as a cofactor and the addition to a system containing ATP decreased the binding of N-hydroxy-Trp-P-2 to DNA. Addition of GTP, instead of ATP, also resulted in the decreased binding to DNA. Effects of 21 amino acids on the covalent binding of N-hydroxy-Trp-P-2 to DNA were compared as shown in Fig. 1. The effects varied depending on amino acids used. Among the amino acids examined, twelve amino acids increased the binding to DNA, while others diminished the binding or had no detectable effect. Proline was most effective, followed by hydroxyproline.

Several enzymes participating in the biotransformation of chemicals are contained in rat liver cytosol. Sulfotransferases, N,O-arylhydroxamic acid acyltransferase and acetyltransferase have been considered to be implicated in the covalent binding of arylamines and arylamides (1,2). Possible roles of these pathways were investigated on the covalent binding of N-hydroxy-Trp-P-2.

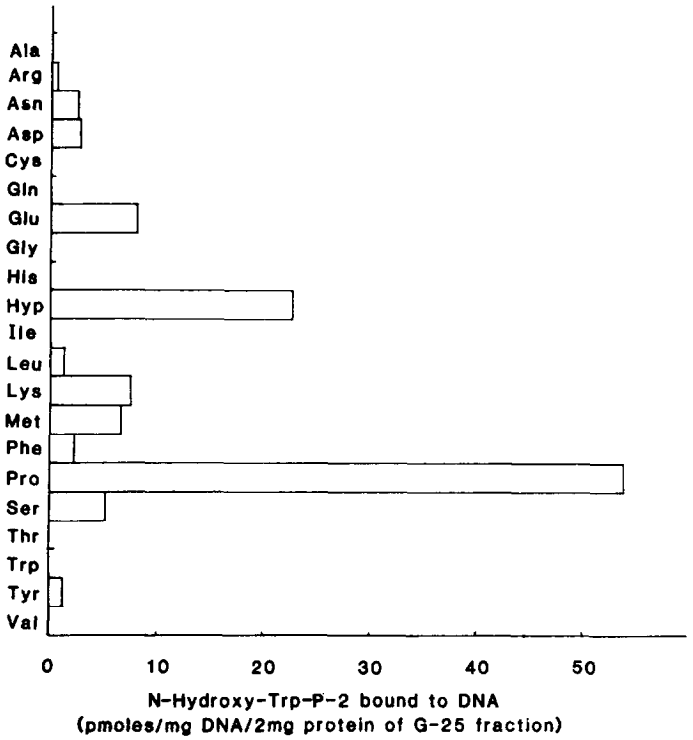

Figure 1. Rates of covalent binding of N-hydroxy-Trp-P-2 to calf thymus DNA in the presence of various amino acids
The reaction mixture contained 10 mM of each of the amino acids, except tyrosine (1 mM). Activities are represented as the corrected values obtained after subtraction of the control without amino acid (30.4 pmoles/mg DNA).

As described in Table 3, the binding was barely detectable with cytosol alone, but considerable amounts of the binding were observed in the presence of ATP. Addition of both ATP and sodium sulfate increased the binding by 137% as compared to the ATP system, but in the presence of p-nitrophenylsulfate and PAP, no binding was observed. Addition of S-acetyl coenzyme A and potassium thioacetate effectively enhanced the binding of N-hydroxy-Trp-P-2 to DNA. The increment in the binding was also observed in the presence of N-hydroxy-AAF.

DISCUSSION

Covalent interaction of chemicals with macromolecules, especially with DNA, has been considered to be a critical event for tumor

Table 3. Cytosolic activation for the covalent binding of N-hydroxy-Trp-P-2 in the presence of various cofactors

Cofactor	Binding to DNA (pmoles bound/mg DNA/30 min)
1) None	3.5 ± 2.4*
2) 3 mM ATP	32.5 ± 4.8
3) 3 mM ATP + 10 mM proline	95.5 ± 19.1
4) 3 mM ATP + 10 mM Na_2SO_4	44.6 ± 4.2
5) 0.5 mM p-Nitrophenylsulfate + 1 μM PAP	2.3 ± 0.1
6) 3 mM S-Acetyl Co A + 20 mM thioacetate	93.5 ± 14.9
7) 2 mM N-Hydroxy-AAF	59.9 ± 5.0

* Data represent the mean ± SD of 3 different determinations.
Activities were determined by addition of the corresponding cofactors in the mixture containing 2 mg protein of G-25 fraction, 7.56 nmoles of N-hydroxy-Trp-P-2, 1 mM DTT, 3 mM magresium acetate and 1 mg of calf thymus DNA.

induction. Carcinogenicities of arylamines and arylamides were also demonstrated to correlate with the reactivity to nucleic acids of their ultimate compounds such as the O-sulfate and acetate compounds (1,2).

Tada and Tada found that some of the aminoacyl-tRNA synthetases could catalyze the covalent binding of 4-HAQO to nucleic acids (7). Recently, seryl-tRNA synthetase has been shown to catalyze the covalent binding of other hydroxylamines using the preparation purified from yeast (16,19). In the present study, we showed that an enzyme system catalyzing the covalent binding of N-hydroxy-Trp-P-2 to DNA is contained in rat liver cytosol. This system required ATP and an amino acid as the cofactor. Although the activity varied to some extents, probably because of instability of the enzyme(s) during storage, proline was the most effective among the amino acids examined. These results and obsevations described above suggest that prolyl-tRNA synthetase in rat liver cytosol catalyzes the covalent event of N-hydroxy-Trp-P-2 to DNA. The binding of Trp-P-2 to DNA was reported to be enhanced by addition of ATP to a system containing microsomes and cytosol, although no mechanism was proposed (20).

In the presence of a phosphoadenosinephosphosulfate-generating system, the binding of N-hydroxy-Trp-P-2 to DNA increased to

small but significant degrees. The result suggests the sulfation of N-hydroxy-Trp-P-2. However, the binding was not detected in the system of p-nitrophenyl sulfate and PAP. In a system containing this cytosolic fraction, N-hydroxy-AAF-dependent formation of p-nitrophenol from p-nitrophenylsulfate was observed at a rate of 1.5 nmole per min, but the formation was not observed with N-hydroxy-Trp-P-2. Addition of S-acetyl coenzyme A or N-hydroxy-AAF resulted in high rates of binding to DNA. These data probably indicate that, in addition to the proline-dependent system, acetyltransferase, and arylhydroxamic acid N,O-acyltransferase are involved in the binding of N-hydroxy-Trp-P-2 to DNA.

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