Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 67-74

PURIFICATION OF N-HYDROXY-2-ACETYLAMINOFLUORENE REDUCTASE FROM RABBIT LIVER CYTOSOL

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Received September 16, 1985

SUMMARY. N-Hydroxy-2-acetylaminofluorene reductase was purified from rabbit liver cytosol by fractionation with ammonium sulfate, and chromatography with DEAE-cellulose, Sephadex G-200 and hydroxylapatite. The purified enzyme was homogeneous by the criterion of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of the enzyme was estimated to be 34,000 by the electrophoresis and by gel filtration on Sephadex G-200. The enzyme required cysteine, glutathione, dithiothreitol, 2-mercaptoethanol, NADPH or NADH as an electron donor. The enzyme activity was inhibited by p-chloromercuribenzoic acid, N-ethylmaleimide, cupric sulfate or disulfiram, but little by oxygen.

N-Hydroxy-2-acetylaminofluorene (N-hydroxy-AAF), which is a proximate carcinogenic metabolite of 2-acetylaminofluorene (AAF), undergoes a variety of metabolic reactions such as conjugation (1), dehydroxylation (2-6), deacetylation (2-4,6,7), isomerization (5) and hydroxylation (8). Among these reactions, the dehydroxylation, i.e. the reduction of N-hydroxy-AAF to AAF is an important detoxication step of the hydroxamic acid. Recently, Yamazoe et al.(6) suggested that cytochrome P-450, especially cytochrome P-448 is involved in the conversion of N-hydroxy-AAF to AAF and 2-aminofluorene catalyzed by rat liver microsomes. On the other hand, an earlier work of Gutmann and Erickson (5) indicated that N-hydroxy-AAF was reduced to AAF by the cytosolic fraction at a 25-fold faster rate than by the microsomal-mitochondrial sediment from rat livers. However,

0006-291X/85 $1.50

no reports are available describing the cytosolic enzyme responsible for the reduction of N-hydroxy-AAF. Here we report the purification and some properties of N-hydroxy-AAF reductase from rabbit liver cytosol.

MATERIALS AND METHODS

AAF was purchased from Tokyo Kasei Kogyo Co.,Ltd., 4-acetylaminobiphenyl from Aldrich Chemical Co. and phenacetin from Katayama Chemical Industry Co.,Ltd., respectively. N-Hydroxy-AAF (9), N-hydroxy-4-acetylaminobiphenyl (10) and N-hydroxyphenacetin (11) were synthesized from the corresponding nitro compounds by the methods described in the references.

Male New Zealand white rabbits (2.1-2.5 kg) were used in all experiments. The animals were stunned by a blow on the head and exsanguinated. The liver was immediately perfused with 1.15 % KCl and homogenized in 4 volumes of the KCl solution. Microsomal and cytosolic fractions were obtained from the homogenate by successive centrifugation at 9,000 x g for 20 min and 105,000 x g for 60 min. The former fraction was washed once with the KCl solution.

A typical incubation mixture consisted of 0.5 μmol of a substrate, an enzyme source, 5 μmol of an electron donor and 0.1 mmol of NaF in a final volume of 1 ml of 0.1 M phosphate buffer (pH 7.4). The mixture was incubated for 10 min at 37° in an air or nitrogen atmosphere. The reaction was stopped by rapidly chilling the tubes in ice and adding 0.1 ml of 2.5 N NaOH. The mixture, after adding 20 μg of an internal standard, was extracted once with 5 ml each of ether and the extract was evaporated to dryness in vacuo. The residue was dissolved in 0.1 ml of methanol and then subjected to high pressure liquid chromatography (hplc). Hplc was performed in a Gilson 1B high pressure liquid chromatograph equipped with a M & S Variactor 311 ultraviolet absorption detector. The instrument was fitted with a 15 cm x 4.6 mm (I.D.) M & S Pack C18 column. The chromatograph was operated at a flow rate of 0.7 ml/min at ambient temperature and at a wavelength of 254 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 7.5, 10, 12.5 or 15 % gel containing 0.1 % sodium dodecyl sulfate according to the method of Weber and Osborn (12). Standard proteins used for estimation of molecular weight were bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500). The molecular weight was also estimated with a calibrated column (1.5 x 90 cm) of Sephadex G-200. Bovine serum albumin, hen egg albumin (45,000), chymotrypsinogen A (25,000) and cytochrome c (12,500) were used as standard proteins.

RESULTS AND DISCUSSION

Table I shows comparative abilities of rabbit liver fractions to reduce N-hydroxy-AAF to AAF. The liver cytosol by itself exhibited, under both aerobic and anaerobic conditions, a signi-

Table I. Reductase activity of rabbit liver microsomes and cytosol towards N-hydroxy-2-acetylaminofluorene

Fraction	2-Acetylaminofluorene formed (μmol/10 min/g liver)	
	Aerobic	Anaerobic
Microsomes	0.05	0.05
+ NADPH	0.05	0.07
+ NADH	0.05	0.06
Cytosol	1.52	1.76
+ NADPH	2.32	2.68
+ NADH	2.35	2.68
+ Cysteine	3.36	3.45
+ Glutathione	3.18	3.26
+ Dithiothreitol	3.30	3.54

The incubation mixture consisted of 0.5 μmol of N-hydroxy-AAF, microsomes equivalent to 0.2 g of liver or cytosol equivalent to 0.05 g of liver, 5 μmol of an electron donor and 0.1 mmol of NaF in a final volume of 1 ml of 0.1 M phosphate buffer (pH 7.4). This level of NaF inhibited the deacetylation of AAF by liver preparations completely. AAF formed was determined by the procedure described in Materials and Methods. In the hplc, phenacetin was used as an internal standard and acetonitrile-water (1 : 1) as a mobile phase, respectively. The elution time of authentic AAF was 9.2 min. Each value represents the mean of four rabbits.

ficant N-hydroxy-AAF reductase activity which was enhanced by NADPH, NADH, cysteine, glutathione or dithiothreitol. On the other hand, the liver microsomes exhibited only a little activity even in the presence of NADPH or NADH. This fact indicated that a major liver enzyme responsible for the reduction of N-hydroxy-AAF is localized in the cytosolic fraction, in accord with the results obserbed by Gutmann and Erickson (5).

An attempt was made to purify N-hydroxy-AAF reductase from the liver cytosol. All purification procedures were conducted at 0-4° and a typical result of the purification is summarized in Table II. The liver (86 g) was homogenized with 4 volumes of 1.15 % KCl containing 0.1 mM dithiothreitol in a glass-Teflon

Table II. Purification of N-hydroxy-2-acetylaminofluorene reductase

Step	Volume (ml)	Protein (mg)	Activity* (μmol/10 min)	Specific activity (μmol/10 min/mg protein)	Purification (-fold)	Yield (%)
1. Cytosol	370	4498	116.9	0.026	1	100
2. Ammonium sulfate fraction (40-70 %)	46	1834	82.5	0.045	1.7	71
3. DEAE-cellulose	10	99	60.1	0.607	23.3	51
4. Sephadex G-200	38	14	47.4	3.386	130.2	41
5. Hydroxylapatite	36	4	36.4	9.100	350.0	31

*The incubation mixture consisted of 0.5 μmole of N-hydroxy-AAF, N-hydroxy-AAF reductase at various stages of purification (0.03-5.7 mg of protein), 5 μmole of dothiothreitol and 0.1 mmol of NaF in a final volume of 1 ml of 0.1 M phosphate buffer (pH 7.4). The incubation was carried out under aerobic conditions. AAF formed was determined as described in Materials and Methods, and the legend of Table I.

homogenizer. The cytosol obtained by centrifugation at 105,000 x g for 60 min was fractionated with ammonium sulfate. The protein that precipitated between 40 and 70 % saturation was collected, redissolved in 37 ml of 0.01 M phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol (Buffer A) and dialyzed overnight against 100 volumes of 0.01 M phosphate buffer (pH 7.4) containing 0.05 M sodium chloride and 0.1 mM dithiothreitol (Buffer B). The dialyzate was applied to a column (2.2 x 15 cm) of DEAE-cellulose (DE-52) equilibrated with Buffer B. The column was washed with 100 ml of Buffer B, and eluted with a linear gradient consisting of 150 ml of Buffer B and 150 ml of 0.01 M phosphate buffer (pH 7.4) containing 0.25 M sodium chloride and 0.1 mM dithiothreitol. Most of the protein applied to the column was removed by the initial column washing. The major portion of activity appeared as a slightly asymmetric peak which was eluted with the 0.15 to 0.20 M sodium chloride gradient. The fractions with activity were pooled and concentrated by ultrafiltration. The concentrated solution was charged on a column (1.5 x 100 cm) of Sephadex G-200 equili-

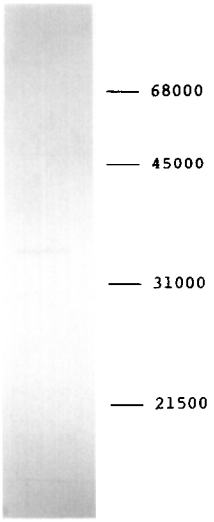

Fig.1. Electrophoresis of N-hydroxy-2-acetylamino-fluorene reductase at the final stage of purification

brated with Buffer A and elution was conducted with the same buffer. The activity appeared as a symmetric peak at fractions which corresponded to the elution volumes of 104.5 to 148.5 ml. The active fractions were combined and applied to a column (1.5 x 5 cm) of hydroxylapatite equilibrated with Buffer A. After washed with 50 ml of Buffer A, the column was eluted with a linear gradient established with 100 ml of Buffer A and 100 ml of 0.05 M phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol. The activity emerged as a sharp symmetric peak which was eluted at the buffer concentration between 0.01 and 0.022 M.

N-Hydroxy-AAF reductase obtained here was examined in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It exhibited a single band with a molecular weight of 34,000 in either case of a gel concentration of 7.5, 10, 12.5 or 15 %, indicating that it was homogeneous. Fig.1 shows the result obtained with 10 % polyacrylamide gel. The molecular weight for the enzyme was also determined to be 34,000 by gel filtration on Sephadex G-200.

Table III. Electron donor requirement of N-hydroxy-2-acetylaminofluorene reductase

Addition	2-Acetylaminofluorene formed (μmol/10 min/mg protein)	
	Aerobic	Anaerobic
None	0.01	0.03
Cysteine	1.29	1.37
Glutathione	0.86	0.91
Dithiothreitol	1.35	1.46
2-Mercaptoethanol	0.58	0.64
NADPH	0.34	0.37
NADH	0.33	0.38

In this experiment, purified N-hydroxy-AAF reductase was dialyzed overnight against 0.01 M phosphate buffer (pH 7.4) prior to use. The incubation mixture consisted of 0.5 μmol of N-hydroxy-AAF, the dialyzed enzyme (0.12 mg of protein) and 5 μmol of an electron donor in a final volume of 1 ml of 0.1 M phosphate buffer (pH 7.4). AAF formed was determined as described in Materials and Methods, and the legend of Table I. Each value represents the mean of three experiments.

The purified enzyme which had been dialyzed against 0.01 M phosphate buffer (pH 7.4) required a thiol compound or a reduced pyridine nucleotide as an electron donor (Table III). The enzyme activity was inhibited by p-chloromercuribenzoic acid, N-ethylmaleimide, cupric sulfate or disulfiram (Table IV). N-Hydroxy-4-acetylaminobiphenyl was also reduced to 4-acetylaminobiphenyl by the purified enzyme, but the reaction rate was much lower than that of N-hydroxy-AAF. With N-hydroxyphenacetin, no detectable reduction was observed (Table V).

Generally, drug-reducing activities of liver enzymes are sensitive to oxygen with exception of nitroreductase (13,14) and azoreductase (15,16) activities of DT-diaphorase. For example, reductase activities of aldehyde oxidase towards sulfoxides (17), nitrosamines (18), N-oxides (19,20), azo dyes (21), hydroxamic acids (22) and nitro compounds (23,24) are markedly inhibited by

Table IV. Effect of inhibitors on N-hydroxy-2-acetylaminofluorene reductase

Addition	Concentration (M)	Inhibition (%)
p-Chloromercuribenzoic acid	2×10^{-4}	81
N-Ethylmaleimide	1×10^{-3}	86
Cupric sulfate	1×10^{-3}	52
Disulfiram	1×10^{-4}	56
Potassium cyanide	1×10^{-3}	0
Sodium arsenite	1×10^{-3}	0

The incubation mixture consisted of 0.5 μmol of N-hydroxy-AAF, purified N-hydroxy-AAF reductase (0.05 mg of protein), an inhibitor and 5 μmol of dithiothreitol in a final volume of 1 ml of 0.1 M phosphate buffer (pH 7.4). The incubation was carried out under aerobic conditions. AAF formed was determined as described in Materials and Methods, and the legend of Table I. Each value represents the mean of three experiments.

oxygen. Therefore, the nature of N-hydroxy-AAF reductase to be resistant to oxygen is noteworthy as well as its unique electron donor requirement and substrate specificity.

Table V. Substrate specificity of N-hydroxy-2-acetylaminofluorene reductase

Substrate	Amide formed (μmol/10 min/mg protein)
N-Hydroxy-acetylaminofluorene	7.74
N-Hydroxy-4-acetylaminobiphenyl	1.06
N-Hydroxyphenacetin	0

The incubation mixture consisted of 0.5 μmol of a substrate, purified N-hydroxy-AAF reductase (0.05 mg of protein) and 5 μmol of dithiothreitol in a final volume of 1 ml of 0.1 M phosphate buffer (pH 7.4). The incubation was carried out under aerobic conditions. AAF formed was determined as described in Materials and Methods, and the legend of Table I. The assay of N-hydroxy-4-acetylaminobiphenyl reductase activity was performed in the same manner as that of N-hydroxy-AAF reductase activity described above. The elution time of authentic 4-acetylaminobiphenyl was 8.7 min. The reduction of N-hydroxyphenacetin was examined in a similar way, but acetanilide was used as an internal standard and acetonitrile-water (3 : 7) as a mobile phase, respectively, in the hplc. Each value represents the mean of three experiments.

REFERENCES

1. Miller,J.A. (1970) Cancer Res. 30, 559-576.
2. Irving,C.C. (1964) J.Biol.Chem. 239, 1589-1596.
3. Lotlikar,P.D., Miller,E.C., Miller,J.A. and Margreth,A. (1965) Cancer Res. 25, 1743-1752.
4. Grantham,P.H., Weisburger,E.K. and Weisburger,J.H. (1965) Biochim.Biophys.Acta 107, 412-424.
5. Gutmann,H.R. and Erickson,R.R. (1969) J.Biol.Chem. 244, 1729-1740.
6. Yamazoe,Y., Ishii,K., Yamaguchi,N., Kamataki,T. and Kato,R. (1980) Biochem.Pharmacol. 29, 2183-2188.
7. Irving,C.C. (1966) Cancer Res. 26, 1390-1396.
8. Razzouk,C., Batardy-Grégoire,M. and Roberfroid,M. (1982) Cancer Res. 42, 4712-4718.
9. Cramer,J.W., Miller,J.A. and Miller,E.C. (1960) J.Biol.Chem. 235, 885-888.
10. Miller,J.A., Wyatt,C.S., Miller,E.C. and Hartmann,H.A. (1961) Cancer Res. 21, 1465-1473.
11. Hinson,J.A. and Mitchell,J.R. (1976) Drug Metab.Dispos. 4, 430-435.
12. Weber,K. and Osborn,M. (1969) J.Biol.Chem. 244, 4406-4412.
13. Sugimura,T., Okabe,K. and Nagao,M. (1966) Cancer Res. 26, 1717-1721.
14. Kato,R., Takahashi,A. and Oshima,T. (1970) Biochem.Pharmacol. 19, 45-55.
15. Huang,M.-T., Miwa,G.T. and Lu,A.Y.H. (1979) J.Biol.Chem. 254, 3930-3934.
16. Huang,M.-T., Miwa,G.T., Cronheim,N. and Lu,A.Y.H. (1979) J.Biol.Chem. 254, 11223-11227.
17. Tatsumi,K., Kitamura,S. and Yamada,H. (1983) Biochim.Biophys. Acta 747, 86-92.
18. Tatsumi,K., Yamada,H. and Kitamura,S. (1983) Arch.Biochem. Biophys. 226, 174-181.
19. Kitamura,S. and Tatsumi,K. (1984) Biochem.Biophys.Res.Commun. 120, 602-606.
20. Kitamura,S. and Tatsumi,K. (1984) Biochem.Biophys.Res.Commun. 121, 749-754.
21. Kitamura,S. and Tatsumi,K. (1983) Chem.Pharm.Bull.(Tokyo) 31, 3334-3337.
22. Sugihara,K., Kitamura,S. and Tatsumi,K. (1983) Chem.Pharm.Bull. (Tokyo) 31, 3366-3369.
23. Wolpert,M.K., Althaus,J.R. and Johns,D.G. (1973) J.Pharmacol. Exptl.Therap. 185, 202-213.
24. Kitamura,S., Narai,N., Hayashi,M. and Tatsumi,K. (1983) Chem. Pharm.Bull.(Tokyo) 31, 776-779.