ISOLATION OF CARCINOGENIC AMINOAZO DYE-BINDING PROTEIN AND ITS IDENTIFICATION AS ALCOHOL DEHYDROGENASE

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Received July 19, 1973

SUMMARY: A major carcinogenic aminoazo dye-binding protein having Ip of 9.7 (isoelectric focusing) was isolated from the liver cytosol of rats given 40 mg 3'MeDAB. The protein has the molecular weight of 6.8×10^4 (gel-filtration) and two subunits of about 3.9×10^4 molecular weight (SDS-polyacrylamide gel electrophoresis). The amino acid composition was similar to that reported for liver alcohol dehydrogenase of animals. The enzymatic activity was shown to be associated consistently with the dye-binding protein fractions throughout the purification steps suggesting identity of the dye-binding protein as liver alcohol dehydrogenase.

It has been shown by a number of investigators that carcinogens interact with proteins in target organs (1-4). As to the carcinogenic aminoazo dyes the presence of some specific target proteins of both basic and nonbasic nature has been reported in the rat liver (5-8). A major dye-binding protein of basic nature which has been referred to as "slow h_2" protein was isolated and partially characterized by Sorof et al.(5,6). Another basic dye-binding protein though it may be a minor component was isolated by Ketterer et al.(7) and identified as the hepatic ligandin which may bind anionic corticoid metabolites, bilirubin and some carcinogens (9). In the preceding papers we have shown that the carcinogenic aminoazo dyes bind not only basic proteins but also nonbasic ones and the dye-binding protein pattern may undergo a tremendous change during continuous dye administration (10-12). In the present study we have isolated a dye-binding

Abbreviations: 3'-methyl-4-dimethylaminoazobenzene, 3'MeDAB; alcohol dehydrogenase, ADH; sodium dodecylsulfate, SDS; carboxymethyl cellulose, CM-cellulose.

basic protein from the liver of rats given 3'MeDAB and identified the protein as rat liver ADH.

MATERIALS AND METHODS

Male, Wistar rats weighing about 200 g received intragastric administration of 40 mg 3'MeDAB dissolved in 2 ml olive oil and were killed 40 h later. The 50% liver cytosol in 0.25M sucrose prepared by ultracentrifugation of the homogenate was subjected to a series of purification procedures as will be described later. Unless otherwise noted the entire procedures were carried out at 0°- 4°. ADH activity was assayed according to Dalziel (13). One unit of activity was defined as the enzyme amount to change 1 μmole NAD^{+} into NADH in 1 min. The molar extinction of NADH was assumed to be 6.2×10^{3} (14). ADH staining on the electrophoreograms was carried out by incubating with the reaction mixture which contains 0.1 ml of ethanol, 10 mg phenazine methosulfate, 2.5 mg Nitroblue tetrazolium in 25 ml of 0.05M Tris-HCl buffer, pH 8.5 at 37° for 15-30 min in the dark. As the control the electrophoreogram was incubated with the reaction mixture without ethanol to examine "nothing dehydrogenase" contribution (15). Protein was assayed by a micro-biuret method (16) with bovine plasma albumin as standard. For measuring protein-bound dye, proteins were treated with hot 10% CCl_3COOH (100°, 5 min), washed successively with 1M acetate buffer, pH 4.7, chloroform-methanol mixture (1:1 v/v) and methanol, and finally dissolved in an adequate amount of formic acid and subjected to spectrophotometry. Absorbance at 525 nm was corrected with the basal absorbance as described earlier (17) and protein-bound dye was estimated by assuming the molecular extinction of 4×10^{4}. The

amino acid composition of the dye-binding protein hydrolyzate (6N HCl, 110°, 32 h) was obtained by an automatic amino acid analyzer (Hitachi KLA-3B). SDS-polyacrylamide gel electrophoresis of dye-binding protein was carried out along with some reference proteins according to the procedure of Weber et al.(18) under a constant current (3 mA/tube) for 9 h. Protein was stained with Coomassie Briliant Blue R and protein-bound dye was stained with 10% CCl_3COOH.

RESULTS AND DISCUSSIONS

The 50% liver cytosol containing more than 70% of the total protein-bound dye in the liver was dialyzed against 0.01M NaCl in 0.01M Tris-HCl buffer, pH 7.0. 150 ml of the dialyzate pooled from 130 g livers were subjected to chromatography on CM-cellulose (Whatman CM-52). Following the preliminary eluate (nonbasic proteins), the basic proteins were eluted with a NaCl gradient (0.01 - 0.15M) in the same Tris buffer. The second peak protein band in Fig. 1 (the third one if the preliminary eluate was counted) containing 22% of protein-bound dye and 1.9% of proteins in the cytosol was collected, and stored, if necessary, in 50% glycerol at -20°. Glycerol was removed by gel-filtration on Sephadex G-25 and the macromolecular fraction thus obtained was subjected to differential precipitation with $(NH_4)_2SO_4$ at pH 7. The precipitates between 30% and 70% saturation were dissolved in a small volume of 0.1M NaCl in 0.02M Tris-HCl buffer, pH 7.5. 5 ml of the solution were subjected to gel-filtration on Sephadex G-100. The second major protein band which contained most of the protein-bound dye was collected as indicated in Fig. 2. Dye-binding protein precipitated with $(NH_4)_2SO_4$ at

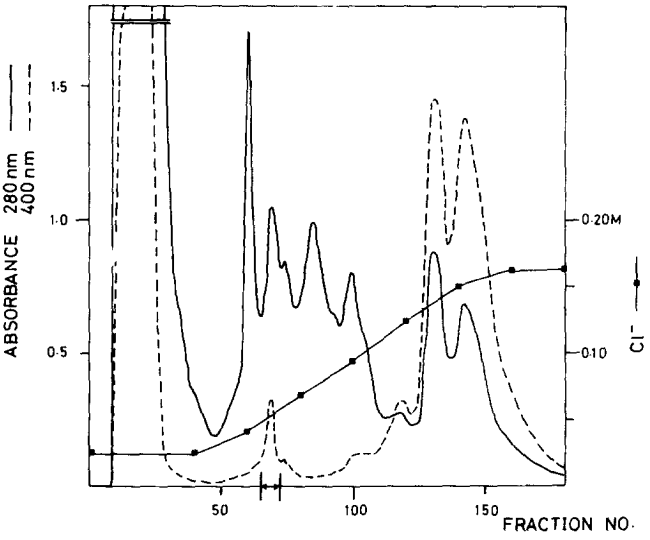

Fig. 1. Elution diagram in chromatography of the liver cytosol of rats given 3'MeDAB on CM-cellulose (3.0 x 26 cm) under NaCl linear gradient elution (0.01-0.15M NaCl in 0.01M Tris-HCl buffer, pH 7.0) at a flow rate of 33 ml/h. Fractions were collected every 10 ml effluent. The dye-binding protein was detected by 400 nm absorbance as well as the spot test using 10% CCl_3COOH (positive ; pink color).

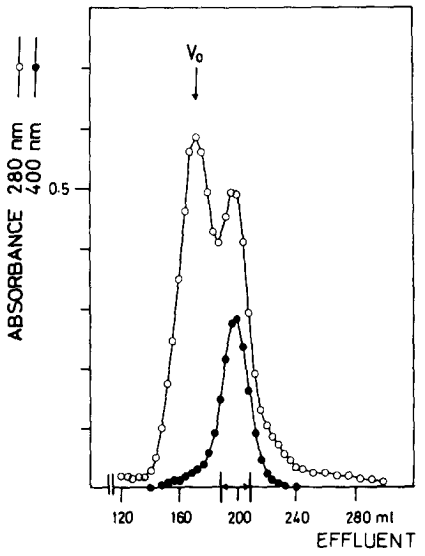

Fig. 2. Gel-filtration of 30-70% saturated $(NH_4)_2SO_4$ precipitate fraction obtained from the basic, dye-binding protein fraction in Fig. 1 on Sephadex G-100 (2.8 x 62 cm). Elution was carried out by running 0.1M NaCl in 0.02M Tris-HCl buffer, pH 7.5 at a flow rate of 20 ml/h and fractions were collected every 5.0 ml.

70% saturation was dissolved in a small volume of 0.5% ampholyte solution and dialyzed against the same solution. The dialyzate was then subjected to isoelectric focusing as described by Vesterberg and Svensson (19). As shown in Fig. 3, a major protein band with two closely located peaks having

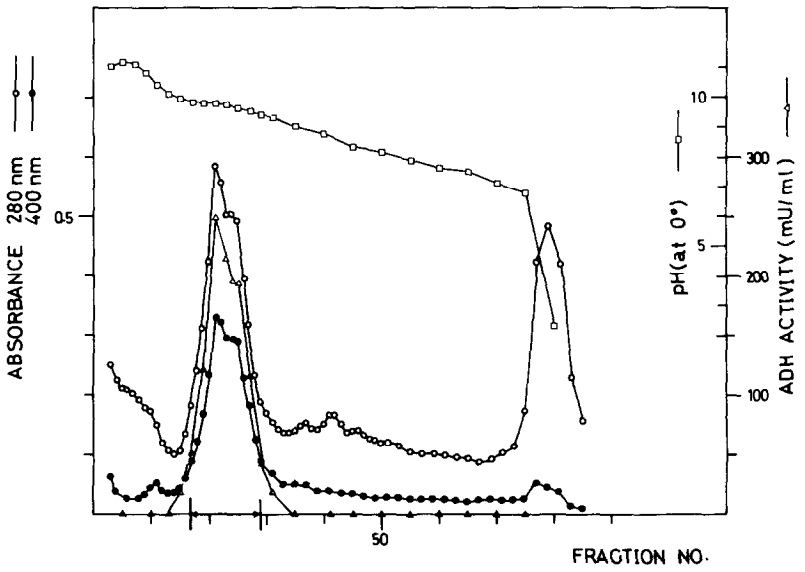

Fig. 3. Isoelectric focusing profile of the dye-binding protein peak band in Fig. 2. The total 24 layers (each 4.6 ml) with sucrose (0-50%) and ampholyte (0.5-1.5%) concentration gradients were housed in a LKB 8101 column (110 ml) and the dye-binding protein sample was mixed into the 11th and 12th layers from the bottom. Electrophoresis was carried out for 64 h at 8° (440 volts for the first 24 h and 500 volts for the last 40 h and a final current of about 1 mA). Fractions of each 1.3 ml volume were collected. pH of each fraction was measured at 0°.

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the same specific ADH activity as well as specific dye-binding was separated. The isoelectric focusing profile seems to suggest a microheterogeneity (7,20) of the target protein. The dye-binding protein was precipitated at 70% saturation of $(NH_4)_2SO_4$ after been diluted with 4 volumes of 0.1M NaCl-0.02M Tris-HCl buffer, pH 7.5, and dissolved in a small volume of the same salt solution. 4-5 ml of the solution were subjected to gel-filtration on

Sephadex G-200. As shown in Fig. 4, a single protein band with parallel ADH activity and aminoazo dye absorbance was eluted in a region correspond-

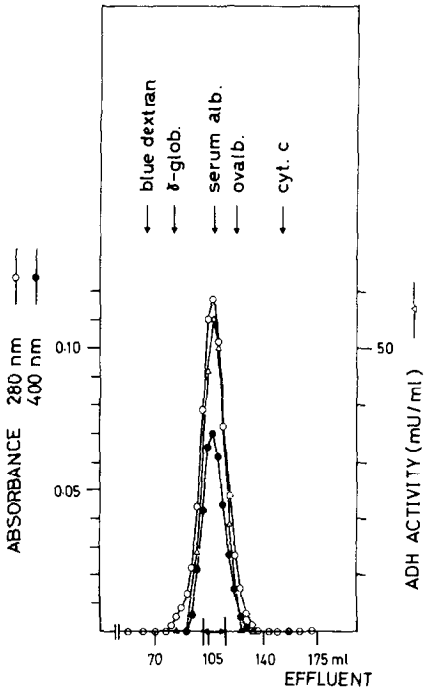

Fig. 4. Gel-filtration of the isoelectric focusing fraction with a peak at pH 9.7 on Sephadex G-200 (2.5 x 38 cm). The same NaCl-Tris buffer solution as in Fig. 2 was run at a flow rate of 10 ml/h and fractions were collected every 3.5 ml effluent. The following crystalline proteins served as molecular weight markers: γ-globulin (150 000), serum albumin (68 000), ovalbumin (43 000) and cytochrome c (12 400).

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ing to the molecular weight of 6.8×10^4. The dye-binding protein was precipitated with $(NH_4)_2SO_4$ with a yield of about 4-5 mg protein from 130 g livers.

Assay of protein-bound dye revealed the presence of 1.1 mole bound dye per mole protein. The purified dye-binding protein migrated as a single band in electrophoresis on cellulose acetate (veronal buffer, pH 8.6, μ=

0.05). Again the alcohol dehydrogenase activity as well as aminoazo dye absorbance were found to be associated exactly with the protein band.

The dye-binding protein subjected to SDS-polyacrylamide gel electrophoresis

Table 1. Properties of the basic, aminoazo dye-binding proteins and liver ADHs

	Dye-binding proteins			Liver alcohol dehydrogenase			
	Tokuma-Terayama	Ketterer et al.	Sorof et al.	Rat	Horse E-ADH[a]	Horse S-ADH[a]	Human
Amino Acid Composition (mole%)							
Lys	8.4	9.4[7]		8.5[24]	8.0[23]	7.9[22]	8.0[25]
His	3.1	1.5		2.4	1.9	1.9	1.6
Arg	2.6	6.3		2.7	3.2	3.0	2.5
Asp	6.9	10.3		7.2	6.7	6.8	7.6
Thr	5.8	3.1		6.1	6.4	5.9	6.3
Ser	6.0	3.8		6.9	7.0	6.7	5.6
Glu	6.7	11.1		6.9	7.8	8.2	7.2
Pro	6.4	4.6		5.8	5.3	5.7	5.5
Gly	10.7	5.6		9.3	10.2	10.4	10.4
Ala	9.8	7.6		9.0	7.5	7.8	9.0
Cys/2	2.9	0.5		4.2	3.7	3.5	3.8
Val	9.8	5.1		9.3	10.4	10.6	10.8
Met	1.1	4.1		1.3	2.4	2.4	2.0
Leu	7.9	13.8		8.0	6.7	6.9	7.7
Ileu	6.2	5.4		6.4	6.4	6.2	5.5
Tyr	0.9	3.0		1.1	1.1	1.0	1.4
Phe	4.9	5.0		4.5	4.8	4.6	4.4
Try	---	---		0.5	0.5	0.5	0.7
Mol. wt. ($\times 10^{-4}$)	6.8	4.5[7] 5.0±0.6[29]	6-8[1]	6.5±0.4[26] 8.0[30]	8.0[21]	8.0[27]	8.0[28] 8.7[32]
Subunit mol. wt. ($\times 10^{-4}$)	3.8-4.0	2.3[31]	4.3[5]	3.7-4.1[26] 4.0[24]	4.0[21]	4.0[27]	4.0[28]
Isoelectric point	9.7	9[31]			6.8[33]	10[22]	

a : E-ADH and S-ADH indicate ethanol- and steroid-active isozymes, respectively.

gave one protein band corresponding to the molecular weight of 3.8 - 4.0 x 10^4. The amino acid composition of the protein is similar to those of liver ADHs reported by other investigators (21-24) as shown in Table 1, being poor in the contents of methionine and tyrosine.

The results presented in this paper seem to suggest that the dye-binding protein isolated by us may be same with the "slow h_2" protein and be identical to the rat liver alcohol dehydrogenase.

A part of expenditures for the present study was covered by the Grant-in-Aid for Scientific Research from the Ministry of Education, Japan.

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