PURIFICATION AND PROPERTIES OF THE PRINCIPAL LIVER PROTEIN CONJUGATE OF A HEPATIC CARCINOGEN

Sam Sorof, Valerie M. Kish, and Brahma Sani

The Institute for Cancer Research
Fox Chase, Philadelphia, Pennsylvania 19111

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SUMMARY: The principal carcinogen-protein conjugate of rat liver cytosol resulting from a hepatic carcinogen has been purified and partly characterized. The azoprotein conjugate (h_2-5S) has been reproducibly isolated 88-91% pure in 50 mg amounts from rats fed the azo dye, 3'-methyl-4-dimethylaminoazobenzene. The subunit molecular weight is 43,000, indicating that the molecule (m.w. 60,000-80,000) exists as a dimer. The subunits are apparently not disulfide-linked. There are 1.4 moles (probably less) of azocarcinogen bound per mole of average protein (2 subunits). The azoprotein appears not to be derived from liver arginase as was previously considered, and differs from the previously isolated minor conjugate which derives from the liver protein, ligandin.

Certain cellular proteins are primary targets of chemical carcinogens in vivo and in culture. During transformation to malignancy in rat liver by aminoazo dyes and N-2-fluorenylacetamide (2-acetylaminofluorene) (1-3), and in mouse skin as well as in cell culture by polycyclic aromatic hydrocarbons (4,5), metabolites of the carcinogens are covalently bound to mainly very few protein species. The carcinogen-protein conjugates isolated from cytosol are relatively basic proteins, and belong to the electrophoretic classes of proteins which have been termed "h".

The feeding of hepatocarcinogenic aminoazo dyes to rats leads to the formation of a predominant species of liver conjugate, termed "slow h_2-5S" azoprotein (1,2). The conjugate derives from a target protein whose subcellular localization, nature, and function are unknown. The present communication reports the purification and some properties of this azoprotein conjugate.

MATERIALS AND METHODS

Adult rats (♀ ♂) were fed a diet containing 0.058% by weight of the liver carcinogen, 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), for 15-18 days (2,6). Livers were perfused with 0.25 M sucrose, and homogenized (Potter-Elvehjem)

in the same medium (1:1 w/v). The homogenates were centrifuged at 105,000 x g for 1 hr. yielding clear cytosol fraction (37-42 mg protein per ml), to which solid NaCl and trishydroxymethylaminomethane (tris) were added to 0.10 M and pH 7.2-7.4, respectively, before storage at -60°C.

Purification of principal liver azoprotein (1-4°C; 35 days). Pools of ca 2 liters of extract, containing 76 g to 82 g protein from 2.3 kg to 3.9 kg liver and 291 to 335 rats, were dialyzed (6 x 24 liters, 45 hr. total) against 0.002 M sodium veronal, 0.0192 M veronal, pH 7.0 + 0.003 M NaCl. The proteins were applied to a column of CM-cellulose (31 cm high x 15.5 cm diameter) which had been equilibrated with that buffer. The proteins were then eluted with 10 liters of the above buffer, followed by 32 liters of a linear gradient of NaCl, starting with that buffer to a limiting solution of 0.10 M NaCl in the veronal buffer. The main azoprotein fractions located by assay in 88% formic acid (6,7) were pooled (between ca 0.026 M - 0.041 M NaCl), and concentrated by ultrafiltration (Amicon Corp., UM-10) to 50-70 ml and 22-39 mg protein per ml.

The combined fractions were dialyzed against 0.01 M tris-Cl buffer, pH 7.0, clarified by centriguation, and applied to another column of CM-cellulose (30 cm x 2.2 cm) which had been equilibrated with the tris-Cl buffer. The column was washed with 100 ml of that buffer, and then eluted with 800 ml of a linear gradient of KCl, starting with the tris-Cl buffer to a limiting solution of the buffer containing 0.145 M KCl. The pool of the principal azoprotein (spanning ca 0.005 M - 0.063 M KCl) was ultrafiltered to 5-10 ml and 26-75 mg protein per ml.

The proteins were dialyzed against 0.02 M sodium veronal buffer, pH 8.6 + 0.03 M NaCl (6), and electrophoresed in a column of ethanolyzed cellulose (95 cm x 3.1 cm) at 80 ma for 165 hr. (6). The eluted pool of principal azoprotein (h_2) was adjusted to pH 7.4 with solid tris-HCl, and ultrafiltered to 4-8 ml and 14-35 mg protein per ml.

The pool was dialyzed (for ultraviolet spectrophotometry) against 0.01 M

tris-Cl buffer, pH 7.4 + 0.20 M NaCl. The proteins were filtered through a column of Sephadex G-200 gel (222 cm x 3.4 cm) in this buffer (2,6). The profile contained 1 peak of azoprotein. Its retardation volume corresponded to a molecular size (60,000-80,000) which was indistinguishable from that of the principal azoprotein in liver extract (2). The pool of azoprotein was ultrafiltered to 4.5-8.8 ml containing 52-61 mg of purified h_2-5S azoprotein.

Gel electrophoresis. Polyacrylamide gels [10% in 50 mM $NaPO_4$, pH 7.0, and 0.1% sodium dodecyl sulfate (SDS); 9 cm x 0.5 cm] were pre-electrophoresed for 30 min. to remove persulfate ion. Proteins were processed according to 2 methods: (A) 4 M urea (ultrapure), 1% SDS, 1% β-mercaptoethanol (v/v) at 37°C for 16 hr. (8); (B) 8 M urea (ultrapure), 50 mM β-mercaptoethanol; then 0.1 M iodoacetamide, followed by 0.2 M β-mercaptoethanol and 1% SDS. Gels were stained for protein with Coomassie Blue and scanned spectrophotometrically, and the areas were measured by planimetry. The determination of the molecular weight of the azoprotein subunit (treated by method B) was based on a linear plot of the relative mobilities of 7 standard purified proteins (treated by method A) in gel electrophoresis.

RESULTS

In 3 preparations the principal azocarcinogen-protein conjugate was purified 112- to 157-fold relative to its specific concentration in liver extract, and 204- to 286-fold compared to that in whole liver. The overall yield of azoprotein dyes relative to all protein dyes in liver extract was 1.6-2.0%.

The isolated azoprotein was 88-91% pure according to gel electrophoresis at levels of 2-60 µg protein (method A). Gels containing 60 µg protein, when stained in 5% trichloracetic acid, revealed only a single azoprotein band, which was coincident with the main component. Two azoprotein-negative components were detected ("minor" 4-8%; "trace" 3-4%). A scan of the proteins stained by Coomassie Blue is shown in Fig. 1. Reduction by β-mercaptoethanol followed by alkylation with iodoacetamide (method B) yielded similar results.

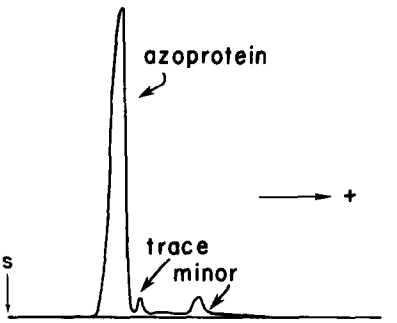

Fig. 1: Gel electrophoretic profile of the purified h_2-5S azoprotein. A 10 µg sample of protein was treated according to method A and was resolved from the starting position S by gel electrophoresis in SDS, as described in the text. Proteins stained with Coomassie Blue were scanned spectrophotometrically at 600 mµ.

The subunit molecular weight of the principal azoprotein is 43,000 (minor component 28,000; trace component 38,000). Accordingly there are 2 subunits in the h_2-5S azoprotein, which was reported to have a molecular weight of 60,000-80,000 (2). The subunits are apparently not disulfide-linked, inasmuch as omission of β-mercaptoethanol prior to gel electrophoresis in method A still yielded protein having the same subunit size. According to analysis[1], the average subunit of the h_2-5S azoprotein contains 0.7 mole of azo dye, equivalent to 1.4 moles of dye per mole of average protein (2 subunits). However, these values are likely elevated artifically by the use of the Folin assay for protein (based on bovine serum albumin), inasmuch as the amino acid analysis of the azoprotein in progress indicates the presence of a low content of tyrosine[2].

DISCUSSION

This communication is the initial report of the isolation of a principal species of carcinogen-protein conjugate from liver undergoing

1. Corrected for the purity of h_2-5S preparations, and based on the spectrophotometric absorptions at 520 mµ of 3'-Me-DAB (ε = 62,000) and h_2-5S azoprotein in 88% formic acid.
2. The gray-brown biuret color of the azoprotein precluded its use for protein assay.

transformation to malignancy. Purified conjugate in 50 mg amounts is reproducibly provided. The way is now open to the further characterization of this conjugate, and ultimately to the identification of the normal protein from which the conjugate derives. Such information is essential to the determination of whether or not that protein is a critical target of chemical carcinogens in oncogenesis *in vivo* and in transformation *in vitro*.

The presence of 1.4 moles (probably less) of azo dye per mole of azoprotein implies that the carcinogen may react predominantly with one amino acid in the target protein.

An azoprotein has previously been purified from livers of rats given a single dose of azocarcinogen (9,10). The conjugate is apparently the h_3 azoprotein, which is present in minor amount in liver cytosol of rats undergoing hepatocarcinogenesis (1,2,6). It is the azo dye conjugate of "ligandin", the basic liver protein which reversibly combines with anionic metabolites of corticosteroids (10), anionic metabolites of estradiol-17β (11), polycyclic aromatic hydrocarbon (12), bilirubin and cholecystographic agents (13), and azo dye-glutathione adduct (14). The principal hydrocarbon-protein conjugate which has been isolated from skin undergoing carcinogenesis and from cultured cells undergoing transformation may also be derived from ligandin (4,5). In contrast, the present findings further substantiate that in liver carcinogenesis by azo dyes the principal azoprotein (h_2-5S) is not a ligandin-azo dye complex. In addition to previously cited differences (1,2), the principal azoprotein has a larger subunit weight (43,000) and more dye content (1.4 dye residues per 2 subunits) than has the azo dye-ligandin complex [23,000 and 0.2, respectively (14,9)].

Liver arginase was previously speculated to be the principal target protein of azocarcinogen in rat liver tumorigenesis (1). In disagreement a difference was previously encountered in the molecular sizes of arginase and the principal liver azoprotein (16). That difference and now the non-identity of the subunit molecular weights of rat liver arginase

[30,800 (15)] and of the principal azoprotein (43,000) weigh against the validity of the hypothesis. Amino acid data further supporting this conclusion will be reported.

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