CHEMICAL STRUCTURE OF POLAR DYES ISOLATED FROM THE LIVERS OF RATS ADMINISTERED CARCINOGENIC AMINOAZO DYES

Tohru Higashinakagawa, Mitsuo Matsumoto and Hiroshi Terayama

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo, Japan

Received August 11, 1966

Studies on the chemical structure of "polar dyes" isolated from the livers of rats administered carcinogenic aminoazo dyes have been carried out by Miller et al.(7,9), Terayama et al.(3,5,11,12) and others (4,8) to elucidate what metabolic change in the administered dye is involved in the dye-protein binding.

The present paper reports our latest data suggesting the binding of the carcinogenic aminoazo dye with the methionine residue of rat-liver proteins through the 4-amino group, probably at a stage of oxidation of the N-methyl group of MAB.

MATERIALS AND METHODS

Preparation of Polar Dyes: By means of a stomach tube, albino rats were administered 25 mg of aminoazo dye (3'-Me-DAB, DAB or MAB) dissolved in 1 ml olive oil and sacrificed about 40 hours later. Defatted liver powder was subjected to Pronase hydrolysis and the enzymatically prepared polar dye was then subjected to ethanolic alkali hydrolysis as described in the previous paper (11). The crude polar dye

---

Abbreviations: 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; DAB, 4-dimethylaminoazobenzene; MAB, 4-monomethylaminoazobenzene; 3-MeS-MAB, 3-methylmercapto-4-monomethylaminoazobenzene; AB, 4-aminoazobenzene.

thus prepared was dissolved in acetone and placed on a silica-gel column. A small amount of non-polar dye contamination was eliminated as a preliminary fraction and the adsorbed polar dye was eluted with 50% methanol. The polar dye was then subjected to paper chromatography according to the method of Hanaki and Terayama (3) and was fractionated into four major components which were referred to as P1 (Rf 0.52), P2a (Rf 0.58), P2b (Rf 0.36) and P3 (Rf 0.8), respectively. Each component was subjected to column chromatography on an Amberlite CG-50 (H^+ form) to remove free amino acid contamination according to the reported procedure (5). The polar dye components thus prepared were used for the following studies.

Preparation of N-^{14}C-Methyl- MAB and DAB: N-^{14}C-methyl-MAB was prepared from ^{14}C-methyliodide and "cold" AB, and N-^{14}C-methyl-DAB from ^{14}C-methyliodide and "cold" MAB. The products were purified by alumina column chromatography using a benzene-petroleum ether mixture as an eluating solvent.

Administration of L-^{35}S-Cystine or L-^{35}S-Methionine: ^{35}S-Cystine (106.2 mc/mmole), and ^{35}S-methionine (56.1 mc/mmole) were purchased from the Radiochemical Centre, Amersham, England. Fifteen μc/young rat (40g) of ^{35}S-cystine, 30 μc/adult rat (150g) of ^{35}S-cystine, or 20 μc/adult rat (100g) of ^{35}S-methionine were injected intraperitoneally at the same time of 3'-Me-DAB administration. Polar dyes were prepared as described above.

Methylation of Polar Dye: Into an aqueous solution of polar dye (P1), drops of dimethylsulfate and sodium hydroxide were alternately added for 1 hour at 80°C. After the reaction the mixture was neutralized and the polar dye was extracted with n-butanol, and then fractionated by paper chromatography as described above.

Detection of Sulfur in Polar Dyes: The presence of sulfur was detected by two different methods. The first method was carried out with

paper chromatograms of the HCl-$SnCl_2$ reduction products of the polar dye by spraying a H_2PtCl_6-KI solution according to Winegard and Toennies (13). As the second detection method, a sodium azide reaction was adopted according to the procedure of Feigl (1).

Nitrous Acid Test: In order to clarify the status of the 4-amino group (primary, secondary, or tertiary) in the polar dye, the nitrous acid test was carried out using diluted sodium nitrite in HCl according to the procedure described in the previous paper (10).

RESULTS AND DISCUSSION

The presence of sulfur in the polar dye components, P1, P2a, P2b and P3, was shown by the sodium azide reaction, using about 20 μg of each component of the polar dye. In order to make this point surer, another test for sulfur-containing amino acids was carried out with paper chromatograms of HCl-$SnCl_2$ reduction products (6) of the polar dye by spraying with the H_2PtCl_6-KI solution. With all components of the polar dye examined, only a spot corresponding to a p-phenylenediamine derivative was bleached, suggesting the presence of a sulfur-containing amino acid in the p-phenylenediamine moiety. This point was also supported by spraying a ninhydrin solution on the paper chromatogram because the blue color was developed only at a spot corresponding to the p-phenylenediamine moiety.

If the amino acid bound to the aminoazo dye is sulfur-containing, then it must be either cysteine or methionine. As an approach to solve this question, we compared radioactivities of polar dyes prepared from rats which had been injected with either ^{35}S-cystine or ^{35}S-methionine.

As shown in Table I, the incorporation of the radioactivity of methionine into the polar dye was much greater than that of cystine.

Recently Scribner et al. (9) found 3-MeS-MAB in the hexane extract of alkali-treated dye-bound protein and speculated that the methionine

Table I. Comparison of radioactivity of polar dyes prepared from ^{35}S-cystine treated rat liver with that from a ^{35}S-methionine treated one.

Expt. No.	^{35}S-Amino acid	Polar dye component	Sp. activity (c.p.m./μmole dye*)
I - 1 (young)	Cystine	P1	100
I - 2 (adult)	Cystine	P1	75
II (adult)	Methionine	P1	4,000
		P2b	6,000

*Calculated from the absorbancy at 400 mμ in neutral media, assuming the molecular extinction coefficient as 2×10^4.

residue of rat-liver protein bound MAB at position 3. Methionine as an amino acid residue responsible for the dye-protein binding seems to be supported by our data, but the site of the binding does not seem likely to be position 3 as shall be discussed below.

In our previous paper (12) it was reported that P2a and P2b had a secondary amino group, and P1 had a primary one at position 4 based on the nitrous acid test. This was re-examined and it was found that P1, P2a and P2b had a secondary amino group. In order to see if any substitution may be present at position 3, we have studied the N-methylation of polar dye (P1) to obtain a polar dye having a tertiary amino group at position 4. Using dimethylsulfate, we obtained a component (Rf 0.69) which has a tertiary amino group at position 4 by the nitrous acid test. The U.V. and visible absorption spectra of this component were quite different from those of 3-Me-DAB, which were characterized by the lack of absorption in the visible region in acidic media, and resembled N-methyl-N-isopropyl-4-aminoazobenzene. This fact suggests that position 3 is not substituted in the polar dye, at least in P1 component. Since we got p-phenylenediamine by peroxytrifluoroacetic acid oxidation of P1, followed by $SnCl_2$ reduction (14), the nucleus

of phenylenediamine moiety may not be involved in the amino acid binding, and the binding may take place at the 4-amino group.

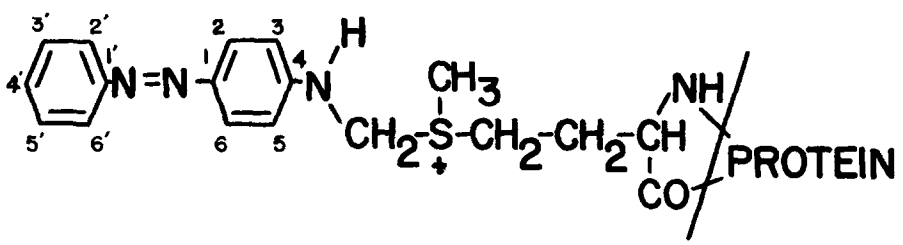
The next experiment was carried out to see if the N-methyl group in the aminoazo dye is involved in the binding. The results shown in Table II indicate that the N-methyl carbon of MAB is completely retained in the polar dye, while half of the N-methyl carbons of DAB is lost during the dye-protein binding. Since the polar dye has a secondary amino group at position 4, the amino acid must be linked with the dye through the N-methyl group, probably in a form of a methylene bridge.

Table II. Radioactivity of polar dyes prepared from the livers of rats administered either N-^{14}C-methyl-MAB or DAB.

Expt. No.	Administered ^{14}C-labelled dye	Sp. activity (c.p.m./10 mμmoles dye)* Polar dye components				
		Unfr.**	P1	P2a	P2b	P3
I	MAB (205)	208	208	223	205	205
II - 1	DAB (309)	148	-	-	-	-
II - 2	DAB (333)	-	170***		185	168

*Calculated as described in Table I
**Unfractionated polar dye (i.e. mixture of four components)
***P1 + P2a

From the experimental evidence reported here together with the findings reported previously, we may draw a conclusion that DAB is first N-demethylated to become MAB, which is then subjected to a second oxidation at the N-methyl group, and it may bind with the methionine residue of a specific rat-liver protein (2) at this second oxidation stage:



Among the three components, P1, P2b and P3, it was also observed that P2b was converted into P1 and P3 during the course of experiment, while P1 was converted into P3. A similar conversion was demonstrated by hydrogen peroxide oxidation of P2b. Studies are in progress to clarify these points.

ACKNOWLEDGEMENT

This work was supported by grants from the National Cancer Institute, U. S. A. (CA-05246-06) and the Ministry of Education, Japan.

REFERENCES

1) F. Feigl, Spot Test in Org. Analysis , Elsevier Publishing Co., New York, 5th English Ed., pp 87 (1957).
2) J.J.Freed and S.Sorof, Biochem. Biophys. Res. Communs., 23,1 (1966).
3) A.Hanaki and H.Terayama, Gann, 53, 285 (1962).
4) P.E.Hughes, Cancer Res., 19, 472 (1959).
5) K.Kusama and H.Terayama, Gann, 48, 181 (1957).
6) M.Matsumoto, J. Japan. Biochem. Soc. (Seikagaku), 36, 181 (1965).
7) E.C.Miller, J.A.Miller, R.W.Sapp and G.H.Weber, Cancer Res., 9, 336 (1949).
8) G.Prodi, Italian J. Biochem., 12, 199 (1963).
9) J.D.Scribner, J.A.Miller and E.C.Miller, Biochem. Biophys. Res. Communs., 20, 560 (1965).
10) H.Terayama, K.Kusama, K.Teruya, S.Kuroda and T.Nakayama, Gann, 49, 85 (1958).
11) H.Terayama and S.Kanda, Gann, 51, 389 (1961).
12) H.Terayama and M.Takeuchi, Gann, 53, 293 (1962).
13) H.M.Winegard and G.Toennies, Science, 108, 506 (1948).
14) Unpublished data.