ON THE BINDING OF AFLATOXIN B_1 AND ITS METABOLITES TO HEPATIC MICROSOMES

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Received October 23,1972; revised December 11,1972

SUMMARY: The metabolism of aflatoxin B_1 was studied using the cytochrome P450-dependent mixed function oxidase system of rat liver microsomes. An aflatoxin metabolite produced in the presence of microsomes and NADPH and not produced in the presence of SKF-525A seems to become covalently bound to microsomes. The bound metabolite is observed as a spectral peak at 412 nm by means of difference spectroscopy. This metabolite appears to be related to either aflatoxin B_{2a} or its precursor.

Aflatoxin B_1 (AFB_1), a mycotoxin and a mycohepatocarcinogen, is metabolized by several animal species although both qualitative and quantitative differences have been noted (1-6). The major metabolites reported either in vivo (7-9) and/or in vitro (4, 5, 10-12) are the 4-hydroxy derivative (M_1), the O-demethylation product, and the 2-hydroxy-2,3-dihydro derivative. The latter is also known as aflatoxin B_{2a} or the aflatoxin hemiacetal. Aflatoxin M_1 has been shown to be nearly as toxic as aflatoxin B_1 (8, 13), whereas aflatoxin B_{2a} is relatively non-toxic (14, 15).

Current studies on the mechanism of action of aflatoxin(s) are concerned with the identification of specific molecular species involved in producing the toxic and/or the carcinogenic effect.

This communication presents evidence that AFB_1 is bound non-covalently to liver microsomes, whereas the metabolite of this carcinogen binds tightly and possibly covalently to liver microsomes.

MATERIALS AND METHODS: Chromatographically pure unlabelled AFB_1 was obtained from Calbiochem and labelled (^{14}C and ^{3}H) AFB_1 was provided as a gift

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This investigation was supported in part by USPHS Core Program Grant CA-13038 and Institute GRSG allocation RR 05648-06.

from Dr. T. C. Campbell of Virginia Polytechnic Institute and State University, Blacksburg, Virginia. The purity of this compound was established spectrographically in this laboratory. SKF-525A (2-diethylaminoethyl 2,2-diphenylvalerate) was kindly provided as a gift from Smith, Kline and French Co. NADP, DL-isocitrate and isocitrate dehydrogenase were obtained from Sigma Chemical Co.

Livers excised from Charles River male rats (220-280 grams) and perfused with isotonic saline until free of blood, were frozen at -20°C. Within 24 hrs., the livers were thawed and microsomes were isolated from a 0.25 M sucrose (containing 1 mM EDTA) homogenate (1 g liver in 4 ml) by differential centrifugation as described elsewhere (16). Protein in the microsomal suspension was assayed by the method of Lowry et al. (17). Before use aminopyrine demethylase and cytochrome c reductase activities and contents of cytochromes P450 and b_5 of microsomes were verified (16, 18, 19).

Four sets of incubation mixtures comprised of 24 ml each and containing the following constituents were prepared: a) microsomes (2.75 mg/ml) in buffer (0.07 M) containing AFB_1 (0.133 mM), NADP (0.33 mM), DL-isocitrate (8 mM) and Sigma type IV, isocitrate dehydrogenase (16); b) same as A but containing SKF-525A (0.60 mM); c) same as A but without AFB_1; d) microsomes (2.75 mg/ml) in buffer containing AFB_1 (0.133 mM) alone.

In each case NADPH generating system of NADP, DL-isocitrate and isocitrate dehydrogenase was preincubated at 37° in buffer containing $MgCl_2$ (5 mM) and $MnCl_2$ (1 mM) for 10 min.; next, microsomes and SKF-525A (where indicated) were added followed by an additional preincubation for 2 min.; and finally the reaction was started with the addition of AFB_1. Reaction mixtures were incubated for an additional 40 min., immediately cooled in ice, and recentrifuged at 105,000 x g at 4° for 90 min. The supernatants were discarded and the microsomal pellets were stored frozen (-20°) overnight under a layer of 0.05 M phosphate buffer containing 0.25 M sucrose. On the following day, after washing, the microsomal pellet were suspended in 0.1 M phosphate buffer (pH 7.4), protein determined (17) and difference spectra recorded on diluted portions.

The other portions of these microsomal suspensions were adsorbed onto Sephadex G-25 columns (size: 20.5 x 1.2 cm), and eluted with 0.02 M phosphate buffer (pH 7.4). Fractions 2 through 4 (5 ml each) which contained cytochrome P450 were pooled, the volume adjusted to 24 ml with buffer and then recentrifuged at 105,000 x g for 2 hr. The pellet was resuspended in buffer and adjusted to an equivalent cytochrome P450 concentration (0.85 mμmole/ml) for recording difference spectra. The studies were repeated three times with consistent results.

Difference spectra (details given in the legend to Fig. 1), cytochromes P450 and b_5 (19) and cytochrome c reductase assays (18) were done employing recording spectophotometers.

RESULTS AND DISCUSSION: Difference spectra were obtained on microsomes that were pretreated by incubation for 40 min. with various combinations of AFB_1, NADPH and SKF-525A; the latter compound is a well known inhibitor of microsomal mixed function oxidase system (20). After pretreatment these microsomes were reisolated by centrifugation, washed and then used for obtaining the difference spectra. Spectrum A, Fig. 1, is the spectral difference between two sets of microsomes pretreated with NADPH in the presence and the absence of AFB_1. This spectrum shows two peaks, one at ~363 nm due to bound AFB_1, and the other at ~412 nm due to a bound AFB_1 metabolite. Spectrum B, Fig. 1, represents the difference between microsomes pretreated with AFB_1, NADPH and SKF-525A and microsomes pretreated with NADPH alone. Under these conditions the formation of AFB_1 metabolite is prevented and only the peak due to the bound unmetabolized AFB_1 (363 nm) is observed. These data are interpreted to mean that the formation of AFB_1 metabolite with a spectral peak at 412 nm in the form bound to microsomes requires NADPH-dependent microsomal mixed function oxidase system and that the inhibition of this enzyme by SKF-525A prevents the appearance of this metabolite. Additional support for this interpretation is provided by the data shown in Table 1, spectra A, C, D and E. Spectrum E in particular shows the dependence of the appearance of 412 nm peak on NADPH.

When the pretreated microsomal preparations described in Table 1 were passed through Sephadex G-25 columns as described in the section on methods, the difference

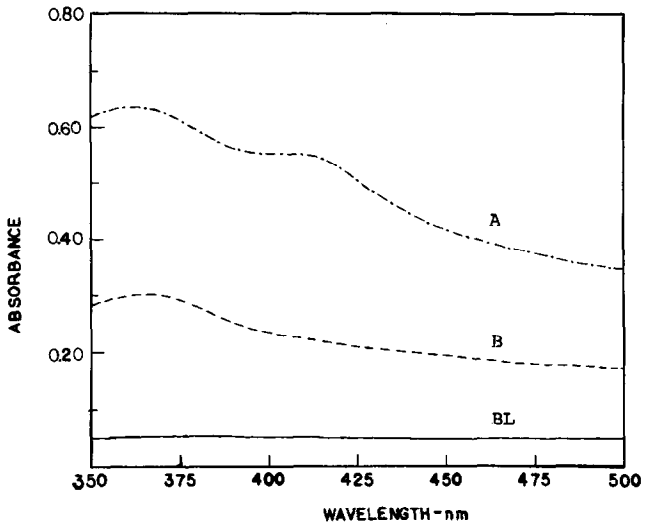

Figure 1: Difference spectra before sephadex gel filteration of the reisolated liver microsomes (mcs) pretreated with various combinations of aflatoxin B_1 (AFB_1), NADPH and the inhibitor, SKF-525A. Microsomal suspensions of equal protein concentration containing an equivalent of about 0.82 mμmole cytochrome P450/ml of 0.1 M potassium phosphate buffer, pH 7.4 were used. The concentrations of the incubation mixtures and the procedures for the reisolation of microsomes are given in the text. The reference cuvette in each case contained microsomes reisolated from an incubation mixture of [mcs + NADPH]. The contents of the sample cuvette were as follows: Spectrum A, -·-·-: microsomes reisolated from a mixture of [mcs + NADPH + AFB_1]; Spectrum B, - - - -: microsomes reisolated from a mixture of [mcs + AFB_1 + NADPH + SKF-525A]; Baseline (BL), ——: same as in the reference.

spectra of the resulting microsomes indicated that (1) the peak at 363 nm due to bound AFB_1 was eliminated (Spectra A and E) and (2) the peak at 412 nm due to the bound AFB_1 metabolite was retained (Spectra A and D). These results suggest that AFB_1 is attached weakly by non-covalent linkage to microsomes, whereas the metabolite of AFB_1 is bound tightly and possibly covalently to this particulate fraction. It has been shown by Gurtoo and Johns (21) that under similar conditions of gel filteration, non-covalently bound dichlorindophenol (DCl) is separated from the xanthine oxidase-DCl complex.

Patterson and Roberts (12) have demonstrated spectographically the formation of B_{2a} from AFB_1; initially a good correlation between the formation of B_{2a} and increase in absorbance at 400-416 nm was seen but with time absorbance at higher wavelengths increased and the incubation mixtures turned yellow. These changes appeared to be related to the formation of quinone type degradation products of B_{2a}. These and additional observations made by Patterson and Roberts (12) were confirm-

Table 1: Spectrophotometric absorption maxima[a] of difference spectra obtained with microsomes pretreated with various combinations of aflatoxin B_1, NADPH and an inhibitor, SKF-525A.

Difference Spectrum[c]	Incubation condition for microsomes prior to reisolation[b]: Sample Cuvette	Reference Cuvette	Absorption maxima (nm) of difference spectra before and after Sephadex treatment: Before	After[d]
A	AFB_1 + Mcs. + NADPH	Mcs. + NADPH	363,412	--,412[e]
B	AFB1 + Mcs. + NADPH + SKF	Mcs. + NADPH	363, --[g]	f
C	AFB_1 + Mcs. + NADPH	AFB_1 + Mcs. + NADPH + SKF	--[g],412	f
D	AFB_1 + Mcs. + NADPH	AFB_1 + Mcs.	--[g],412	--[g],412
E[h]	AFB_1 + Mcs.	Mcs. + NADPH	363,-- [g]	--[g],--- [g]

[a]Absorption maxima wavelengths are approximate estimates because of the broadness of peaks (see Fig. 1).

[b]Sample and Reference cuvettes contained microsomes (Mcs) reisolated from reaction mixtures containing various combinations of aflatoxin B_1, NADPH, and SKF-525A as shown in the table.

[c]Experimental conditions employed for the reaction mixtures are described in the text and legend to Fig. 1.

[d]After incubation, microsomes (Mcs) were pelleted, passed over Sephadex G-25, and cytochrome P-450 fractions were pooled and adjusted to equivalent P-450 contents (0.85 mμmole/ml) for recording difference spectra.

[e]Increased absorption was seen above 412 nm in some samples and may be due to degradation products of aflatoxin B_{2a} (12).

[f]Difference spectra not recorded.

[g]No peaks were seen.

[h]Since washed microsomes are employed no interference due to the absorption of NADPH occurs in the wavelength range studied. Similar results were obtained when NADPH was omitted from the reference cuvette preparation.

ed in the present studies. Since the absorption peak of the bound metabolite is at 412 nm it is suggestive that the metabolite is either B_{2a} or its precursor.

Since the conversion of aflatoxin B_1 to B_{2a} involves the olefinic double bond, it is possible that an epoxide intermediate (22-25) could covalently bind a nucleophylic group of microsomal protein and quickly rearrange to a monohydroxy derivative B_{2a} that subsequently degrades to resonating quinone compounds having yellow color. A number of epoxides are highly unstable and rearrange quickly in presence of proteins to yield monohydroxy compounds (23, 25). The alkylation of epoxides to the S-group of glutathione has been demonstrated (22).

Present results and those of Wogan et al. (9) who found that 50% of the total radioactivity in the liver following i.p. injection of ^{14}C-AFB_1 was contained in microsomes are consistent with the suggestion that a metabolite(s) binds covalently to liver microsomal fractions; however, the covalent binding of aflatoxin B_{2a} degradation products, though remote and not consistent with published data, cannot be excluded completely from the results presented here.

Initial studies employing labelled (^{14}C and ^{3}H) AFB_1 are in agreement with the spectral data presented in this communication.

ACKNOWLEDGEMENTS: The author gratefully acknowledges the valuable criticism of Drs. C. Wenner, T. Gessner, and C. Dave of this Institute. Skilled technical assistance of Mr. L. Motycka is also acknowledged.

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