THE REQUIREMENT FOR FLAVIN ADENINE DINUCLEOTIDE BY A LIVER MICROSOMAL OXYGENASE CATALYZING THE OXIDATION OF ALKYLARYL AMINES*

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In an earlier communication (Ziegler and Pettit, 1964) it was shown that the liver microsomal mixed function oxygenase (Mason, 1957) catalyzing the oxidative demethylation of N,N-dimethylaniline can be separated into two partial reactions. In the initial reaction the tertiary amine in the presence of NADPH and oxygen is converted to an intermediate N-oxide. The N-oxide is then catalytically dealkylated to yield equimolar amounts of formaldehyde and N-methylaniline.

The data presented in this report demonstrate that flavin adenine dinucleotide is required by the enzyme complex catalyzing the synthesis of the intermediate N-oxide. A fraction isolated from pork liver microsomes treated at an acid pH in the presence of 3M KCl to remove flavin nucleotides will catalyze the NADPH and oxygen dependent synthesis of tertiary alkylaryl N-oxides only when the reaction medium is supplemented with FAD. In contrast to particle bound mixed function oxygenases obtained from microorganisms (Fulco and Bloch, 1962; Conrad et al., 1964), flavin mononucleotide does not restore the activity of the apoenzyme complex.

The microsomes were isolated by differential centrifugation from pork liver homogenates prepared in 0.25M sucrose by a modification of the method of Hogeboom, et al. (1948). The oxygenase activities were measured by following the accumulation of aldehyde and N-oxide in the assay medium. Formaldehyde and the N-oxide of N,N-dimethylaniline were estimated by methods described earlier (Ziegler and Pettit, 1964). The N-oxides of N,N-diethylaniline and N,N-ethylmethylaniline were measured by essentially the same procedure as that used to estimate N,N-dimethylaniline-N-oxide, since the

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parent tertiary amines of all of these compounds readily form the yellow p-nitroso derivatives. The millimolar extinction coefficients of the p-nitroso derivatives, calculated from a standard curve obtained under the assay conditions with the synthetic p-nitroso derivatives at 420mμ were 6.7cm^2 for p-nitrosodiethylaniline, 7.1cm^2 for p-nitrosoethylmethylaniline, and 7.3cm^2 for p-nitrosodimethylaniline. All of the nucleotides used in this study were obtained from Sigma Chemical Company and were used without further purification. Protein bound acid extractable flavin was estimated by the method of de Bernard (1957). The cytochrome c reductase and diaphorase activities were measured spectrophotometrically by methods previously published (Ernster et al., 1962).

Table I

Flavin nucleotide requirements of pork liver microsomal alkylaryl amine oxygenase

Fraction	Flavin mμ moles mg prot.	Supplements[b] to medium	Oxygenase Activity[a] Substrates		
			N,N-dimethyl aniline	N,N-diethyl aniline	N-ethyl,N-methyl aniline
Microsomes	0.27	none	3.8	1.6	3.3
		FAD	4.0	1.7	3.5
		FMN	4.0	1.6	3.5
S_1	0.36	none	2.2	--	--
		FAD	3.8	--	--
		FMN	2.3	--	--
Sephadex treated S_1	0.16	none	0.29	0.18	0.29
		FAD	1.5	0.65	1.2
		FMN	0.31	0.28	0.33

[a]The activity is expressed as mμmoles of N-oxide formed per min. per mg protein which represents the total alkylaryl amine oxygenase activity of the S_1 fractions The activity of the microsomes with N,N-dimethylaniline as substrate was 6.2 mμmoles substrate oxygenated per min. per mg prot., which represents the sum of N-oxide and formaldehyde produced during the reaction. The microsomes had been frozen four days which does not produce complete dissociation of the n-oxide dealkylase from the tertiary amine oxygenase (cf. Ziegler and Pettit, 1964).

[b]The basic assay medium contained in μmoles/ml: potassium phosphate, pH 7.0 - 100; semicarbizide - 1.0, NADPH - 1.0, tertiary amine substrate - 5.0. The concentration of enzyme protein, 2-3 mgs/ml. Incubation time - 10 min.; temp. - 38°. Flavin nucleotides were added where indicated to give a final concentration of 5 mμmoles per ml.

Flavin nucleotides were dissociated from the pork liver microsomes by the following procedure. Two hundred and twenty mgs of KCl were added per ml of a pork liver microsomal suspension (50 mgs protein per ml) containing

0.25M sucrose and 0.1M Tris buffer, pH 7.8. After incubating for at least one hour at 0-3°, the pH*** was adjusted to 5.5 with 1M acetic acid. Two to three minutes later sufficient 1M KOH was added to give a pH reading of 5.8. The suspension was then centrifuged at 50,000 rpm for 10 minutes. The turbid supernatant (S_1) fraction which contained about one-third of the original protein was decanted. Approximately 4-5 mls of the S_1 fraction were passed through a column of G-25 Sephadex (2 cm. in dia., 18 cm. long) to separate the flavin nucleotides from the protein. The protein was washed off the column with a solution of 3M KCl adjusted to pH 5.8 with acetic acid. The flavin nucleotides remained near the top of the column as a clearly discernable yellow fluorescent band. The Sephadex used in the column was routinely pretreated at room temperature overnight with the acid 3M KCl solution.

While the preceding method does not remove all of the protein bound acid extractable flavin nucleotides (Table I), virtually all of the amine

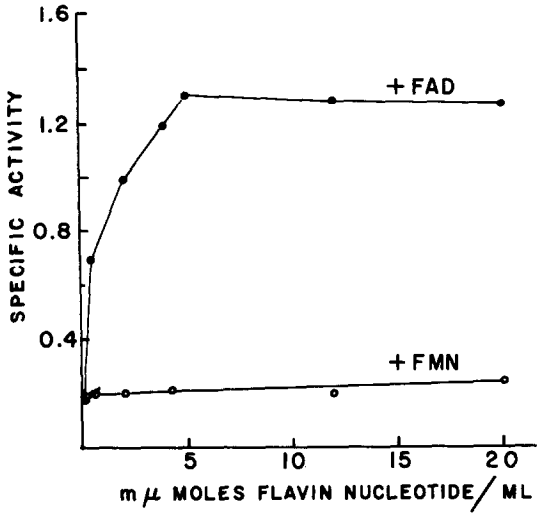

Fig. I - Response of the N,N-dimethylaniline oxygenase activity of the Sephadex treated S_1 fraction to flavin nucleotides. The basic assay conditions were as indicated in Table I. The concentration of enzyme protein was 2.6 mgs per ml. Specific activity expressed as millimicromoles of N-oxide formed per min. per mg prot.

*** These pH values represent readings obtained at 0-3° with a Metrohm E-300 pH meter fitted with a number EA 120T electrode. The readings are not corrected for variations from true pH produced by high salt concentrations.

oxygenase activity of the S_1 fraction is lost after passage through the Sephadex column. The activity of this fraction can be partially restored by adding FAD to the assay medium, and the stimulation of the oxygenase activity obtained by adding FAD is essentially the same for the three substrates listed in Table I. Approximately two millimicromoles of FAD per mg protein is required to saturate the enzyme (Fig. I). Other nucleotides (AMP up to 1 μmole/ml) or FMN (Table I and Fig. I) have essentially no effect on the activity of the apoenzyme complex.

The lower specific activity of the Sephadex treated S_1 fraction (even when supplemented with FAD), as compared to that of the original microsomes, can be attributed to irreversible inactivation of the enzyme complex by the acid 3M KCl solution. After adjusting the pH to 5.5-5.8 there is a slow but progressive loss in the amine oxygenase activity of the microsomal preparations. The fractions were routinely assayed immediately after they were obtained and the difference between the specific activities of the S_1 and Sephadex treated S_1 is due primarily to the longer time at the acid pH required to obtain the latter fraction.

There is no immediately apparent correlation (cf. Table II) between the flavin requirements of the tertiary amine oxygenase and the flavoprotein catalyzed diaphorase of cytochrome c reductase (Williams and Kamin, 1962; Danielson, Ernster and Ljunggren, 1960) also present in liver microsomes. The NADPH cytochrome c and 2,6-dichloroindophenol reductase activities are decreased after removing the flavin nucleotides from the S_1 fraction. However, unlike the tertiary amine oxygenase activity of this fraction, both the diaphorase and cytochrome c reductase activities are stimulated

Table II

Effect of flavin nucleotides on liver microsomal NADPH dehydrogenase activities

Fraction	Supplements[a] to medium	mμmoles NADPH oxidized/min/mg prot. by Cyt. c	DCIP
Microsomes	none	81	110
	FMN	86	112
	FAD	82	110
Sephadex treated S_1	none	28	21
	FMN	80	66
	FAD	58	53
	FMN + FAD	78	64

[a]Final concentration of flavin nucleotides in the assay medium was 20 mμmoles/ml.

by either FMN or FAD. At all the levels tested FMN consistently produced a greater stimulation of these activities than that observed with equivalent amounts of FAD, and the addition of both flavin nucleotides did not produce a greater stimulation than that obtained with FMN alone. While these data suggest that the microsomal NADPH dehydrogenase activities measured by the reduction of 2,6-dichloroindophenol or cytochrome c are not related to the tertiary amine oxygenase activity, additional studies will be required to establish this point.

REFERENCES

Conrad, H. E., Lieb, K., and Gunsalus, I. C., Fed. Proc. 23, 429 (1964).
Danielson, L., Ernster, L., and Ljunggren, M., Acta Chem. Scand. 14, 1837 (1960).
de Bernard, B., Biochim. et Biophys. Acta 23, 510 (1957).
Ernster, L., Danielson, L. and Ljunggren, M., Biochim. et Biophys. Acta 58, 171 (1962).
Fulco, A. J., and Bloch, K., Biochim. et Biophys. Acta 63, 545 (1962).
Mason, H. S., Science, 125, 1185 (1957).
Williams, C. H. and Kamin, H., J. Biol. Chem. 237, 587 (1962).
Ziegler, D. M. and Pettit, F. H., Biochim. Biophys. Res. Comm. 15, 188 (1964).