THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 236, No. 9, September 1961
Printed in U.S.A

Preliminary Communications

Flavin Component of Reduced Diphosphopyridine Nucleotide Dehydrogenase*

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(Received for publication, May 1, 1961)

An electron transport particle containing the complete sequence of enzymes and carriers necessary to link both reduced diphosphopyridine nucleotide and succinate to oxygen has been prepared from beef heart mitochondria by D. E. Green and his colleagues (3, 4). By altering slightly the isolation procedure, another particle (DPNH oxidase) containing only the intact DPNH chain has been prepared from the same source (5–7). From the particulate DPNH oxidase, Mackler has isolated recently a soluble DPNH dehydrogenase (8). The key step in this procedure, *i.e.* exposing the particles at 44° to 9% ethanol and pH 4.8, had been devised previously by Mahler *et al.* (9) for the detachment of a DPNH cytochrome *c* reductase from pig heart and was used also by de Bernard for obtaining the same enzyme from electron transport particles (10).

The soluble DPNH dehydrogenase from beef heart DPNH oxidase utilizes 2,6-dichlorophenol indophenol, ferricyanide, and cytochrome *c* as electron acceptors, and contains bound flavin and nonheme iron in the ratio of 1:2 (8). The present study was undertaken in order to obtain further information about the flavin component of the enzyme.

The flavin was released completely and quantitatively by the following treatment: heating the enzyme for 6 minutes at 100°; rapidly cooling to 5°; acidification with perchloric acid to a final concentration of 10%; and centrifugation to remove the denatured protein. No additional flavin was removed from the residue by digestion with commercial trypsin (Nutritional Biochemicals Corporation) (11, 12). From each milligram of enzyme approximately 10 mμmoles of flavin were released as measured either by a microbiological assay[1] for total riboflavin or by the decrease in light absorption at 450 mμ upon treatment with hydrosulfite (see Table I, Column 1).

Larger quantities of the enzyme (30 to 50 mg) were deproteinized as described above by means of heat and acid, and the liberated flavin was separated from salts by adsorption and elution from a 2 × 10 cm column of Florisil (13), and then chromatographed on a 1.5 × 10 cm column of diethylaminoethyl cellulose, with a modification[2] of previous methods (14, 15) for the separation of flavins on this adsorbent. In the elution profile the dehydrogenase flavin emerged as a single, symmetrical peak at a position identical to that of authentic FMN[3].

Descending (16) or circular (17) paper chromatography of the dehydrogenase flavin, liberated by treatment with heat and acid, was carried out in two solvent systems: (*a*) *n*-butanol-acetic acid-water (4:1:5, organic phase); or (*b*) 5% Na_2HPO_4. In these systems the dehydrogenase flavin migrated as a single spot, visualized under ultraviolet light by its greenish yellow fluorescence, and had R_F values identical with those of FMN. No separation was observed when the dehydrogenase flavin and FMN were admixed before chromatography. When added to the intact enzyme before denaturation with heat and acid, FAD was recovered quantitatively in the supernatant fraction as judged by paper chromatography. The dehydrogenase flavin was unaffected by treatment with nucleotide pyrophosphatase from snake venom but was degraded by a specific 5′-nucleotidase from the green gram plant (18) to yield a product which migrated on paper chromatograms with the same R_F value as riboflavin.

It should be noted that when the dehydrogenase was subjected to heat deproteinization at 100° for periods of 1 minute or less and the subsequent treatment with acid was also omitted, the liberated flavin migrated in the above solvent systems with R_F values lower than FMN; however, when eluted from the paper, the flavin reacted quantitatively as FMN in the specific TPNH cytochrome *c* reductase assay, described below. Although the reason for this anomalous migration is not clear, it is possible that the shorter periods of heat denaturation and the omission of the acid leaves materials in the supernatant fraction which hinder the migration of the flavin.

With solutions which have been standardized to the same molal concentration by their absorbancy at 450 mμ, the fluorescence of the dehydrogenase flavin is equal to that of FMN and both values are approximately 7 times larger than that of FAD (19). Treatment of FMN or the dehydrogenase flavin with nucleotide pyrophosphatase did not alter the fluorescence of either material whereas, under the same conditions, the fluorescence of FAD increased markedly as it was degraded to FMN.

Additional proof for the identity of the dehydrogenase flavin with FMN was obtained by assaying the material with enzyme systems specific for FMN (TPNH cytochrome *c* aporeductase from yeast (20) and for FAD (D-amino acid oxidase coupled with lactic dehydrogenase (21)). The dehydrogenase flavin produced the same response as an equimolal amount (standardized spectrophotometrically at 450 mμ) of FMN in the former system and no response in the FAD-dependent system. Flavin analyses of the DPNH dehydrogenase by various methods are summarized in Table I. These enzymatic assays have been used also to quantitate the amount of acid-extractable FAD (0.08 mμmole per mg of protein) and FMN (0.12 mμmole per mg of protein) in the DPNH oxidase preparation from which the dehydrogenase was derived. FMN is recovered to the extent of 50 to 60% in

* Paper VII in the series "Flavin Nucleotides and Flavoproteins." For paper VI see (1). This work was supported, in part, by a grant (H-5457) from the National Heart Institute, National Institutes of Health. A preliminary account of this work was presented at the 45th Annual Meeting of the American Society of Biological Chemists, Atlantic City, April, 1961 (2).

[1] We are indebted to Dr. M. Burger, Wisconsin Alumni Resaerch Foundation, for carrying out these assays.

[2] Flavins are adsorbed onto a column of diethylaminoethyl cellulose and eluted with phosphate buffer, pH 5.0, which varied in concentration from 0.005 to 0.05 M.

[3] The abbreviation used is: FMN, riboflavin 5′-phosphate.

the preparation of the soluble DPNH dehydrogenase from the particles.

Following the observation that deproteinized preparations of the DPNH dehydrogenase contained only FMN, attempts were made to resolve the enzyme reversibly. It was found that dialysis of the enzyme against 0.02 M phosphate buffer, pH 7.5, for 24 hours caused a progressive loss in activity of the enzyme which also paralleled the loss of bound flavin (8); partial reactivation of the enzyme with added FMN could be demonstrated with preparations dialyzed for periods not exceeding 12 hours. A rapid, and more satisfactory, resolution was achieved by passing the enzyme (6 mg) through a small (1 × 7 cm) column of Florisil in the cold. Approximately 75% of the protein was recovered in the effluent whereas all of the flavin remained adsorbed onto the column; the latter material could be recovered by elution of the column with 5% aqueous pyridine and shown to be FMN by paper chromatography. None of the enzyme-bound iron is lost by this procedure. The addition of FMN to the apoenzyme, prepared in this manner, restored the activity in the cytochrome *c* assay system, as shown in Fig. 1. The Michaelis constant (K_m) for FMN is 2.3×10^{-4} M. The apoenzyme was not reactivated by the addition of FAD. The activity of the enzyme with indophenol as the terminal acceptor drops sharply (from 40 to 3 μmoles of DPNH oxidized per minute per mg of protein) upon resolution of the flavin and the activity with this acceptor is not increased by the addition of FMN.

Despite the present findings that the DPNH dehydrogenase flavin is quantitatively accounted for as FMN in denatured preparations and that activity is restored to the reversibly resolved enzyme by FMN, but not FAD, some doubt remains as to the identity of the flavin while it is bound to the enzyme. For example, with DPNH cytochrome *c* reductase from pig heart (9) the prosthetic group was believed to be a flavin dinucleotide (not FAD) which was readily degraded to a mononucleotide during deproteinization. More recently, it has been reported that both a soluble DPNH dehydrogenase, obtained by treatment of beef heart electron transport particles with snake venom (22, 23), and a lipoflavoprotein DPNH dehydrogenase from beef heart (24), contain only FAD. It is possible that the present DPNH dehydrogenase contains bound FAD which is readily converted to FMN during deproteinization, but our data do not support this hypothesis since every method of detaching the flavin (heat, acid, dialysis, adsorption on Florisil, or even dilution of the enzyme) leads only to the release of FMN and not of FAD.

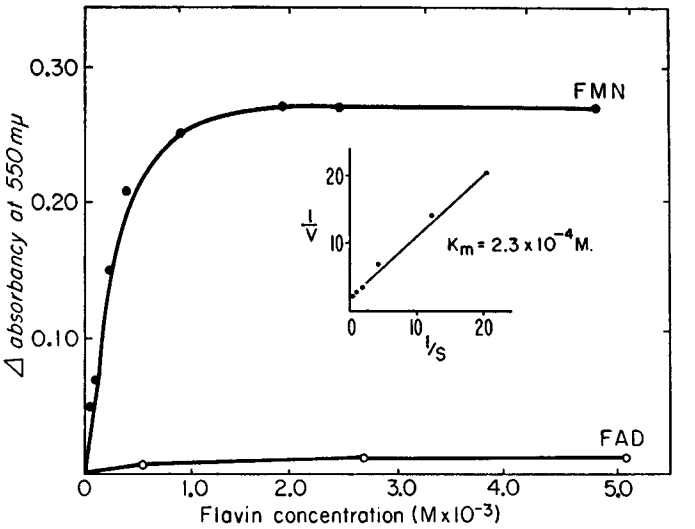

FIG. 1. Assay system for DPNH dehydrogenase consisted of the following components placed in a 1.5-ml cuvette: 0.15 μmole of DPNH, 1.0 mg of cytochrome *c*, 1000 μmoles of phosphate buffer, pH 8.5, and flavin as indicated in a total volume of 1.0 ml. The blank cuvette contained the same components. The change in absorbancy at 550 mμ was measured in a Beckman model DU spectrophotometer, after addition of 0.01 ml of enzyme containing 6.0 μg of protein to the experimental cuvette, and was corrected for a blank omitting DPNH. Data are expressed as change in absorbancy at 550 mμ per minute.

TABLE I

Flavin analyses of DPNH dehydrogenase

Method of estimation	Flavin (mμmoles per mg of protein)*		
	Intact enzyme	Heat- and acid-denatured extract	
		Experiment 1	Experiment 2
1. Total flavin by reduction with hydrosulfite†	9.5	8.1	
2. Riboflavin by microbiological assay	11.0	9.8	
3. FMN by TPNH cytochrome *c* reductase		7.4	7.2
4. FAD by D-amino acid oxidase-lactic dehydrogenase		<0.4	0.7
5. As in Method 4 except that 0.66 mμmole of FAD was added		1.0	

* For denatured preparations, this value was calculated on the basis that all of the flavin was recovered after heat and acid treatment.

† With a value of Δ_{450} (difference of extinction coefficients between oxidized and reduced flavin at 450 mμ) of 11.3×10^3 cm² per mole.

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The Isolation of an α'-Alkoxy-β-acyl-α-glycerophosphorylethanolamine from Bovine Erythrocytes*

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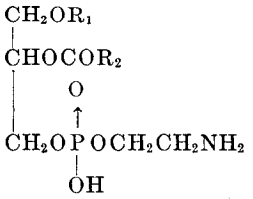
(Received for publication, June 15, 1961)

Several investigators (1–4) have reported on the occurrence of a phospholipid stable to acid and alkali, but which is not a sphingolipid. Carter *et al.* (5) were able to isolate from egg yolk phospholipids, which had been subjected to a mild alkaline hydrolysis, an α-fatty ether of glycerophosphorylethanolamine. Svennerholm and Thorin (6) obtained by an alkaline treatment of phospholipids from human and calf brain a compound similar in structure to that found by Carter *et al.* (5) and presented suggestive evidence that this glycerol ether phospholipid might have occurred as a fatty acid ester derivative. In this communication, evidence is presented for the occurrence of an α'-alkoxy-β-fatty acyl-α-glycerophosphorylethanolamine in bovine erythrocytes. The preparation of this compound, free of any diacyl analogues, was effected through a short term alkaline treatment of the ethanolamine phospholipid fraction.

In an investigation of the chemistry of the phospholipids of bovine erythrocytes, we noted that the "phosphatidylethanolamine" fraction had a fatty acid ester to P value in a range from 1.20 to 1.30 but contained no lysophosphatidylethanolamine and less than 2% vinyl ether plasmalogen. It has now been established that these results can be attributed to the presence of α'-glycerol ether containing phospholipids of the following structure:



in which R_1 and R_2 are long chain alkyl groups. Furthermore, it has been shown that the above compound can be obtained free of the usually encountered diacyl derivative (phosphatidylethanolamine) through rapid attack by a chloroform-methanol (1:10, volume for volume) mixture which was 0.45 N with respect to NaOH, at low temperature. The "phosphatidylethanolamine" fraction used in these experiments was isolated from bovine erythrocyte by a previously described technique (7). Certain of its chemical characteristics are described in Table I.

The presence of a glycerol ether in the phosphatidylethanolamine fraction, as well as in the more purified component Fraction IV (Table I), was established as follows. "Phosphatidylethanolamine,"[1] 1098 mg, was subjected to an acetolysis essentially as described by Bevan *et al.* (9), and the mixture was diluted with 2 volumes of water and extracted twice with 2 volumes of diethyl ether. The diethyl ether extract, which showed a deep infrared absorption band at 9.0 μ (expected for glyceryl ethers (5)) and contained no phosphorus, was refluxed in 1 N ethanolic KOH for 2 hours, then extracted with diethyl ether. This latter extract, which weighed 317 mg, had an infrared pattern expected for a glycerol ether, except for the presence of a weak absorption band at 5.85 μ. This latter band was probably due to contaminant, nonesterified fatty acids which could be

Table I

Chemical composition of "phosphatidylethanolamine" fraction of bovine erythrocytes and phospholipids present after short term deacylation

Phospholipid was subjected to a 3-minute reaction at 10° with a chloroform-methanol-0.5 N NaOH mixture. Details are provided in the text.

	P	N	N:P	Fatty acid ester / P*	Plasmalogen / P†	Iodine uptake
	%					
"Phosphatidylethanolamine"	4.06	1.85	1.00	1.22	0.03	67.1
Products isolated by silicic acid chromatography						
Fraction IV	4.06	1.91	1.03	0.99	Nil	65.0
Fraction V‡	5.56	2.60	1.03	0.25	Nil	30.4

* See Footnote 1. Assayed by its infrared absorption at 5.75 μ with triolein as a standard.

† Assayed by the potentiometric method of Norton (8).

‡ Several other experiments have resulted in a Fraction V with a lesser fatty acid ester to P value (0.10) and with a concomitantly higher P value (*e.g.* 6.5 to 6.7%).

* Aided by grants from the Life Insurance Medical Research Fund and the National Science Foundation.

[1] The fatty acid ester to P molar ratio of 1.22 of this fraction could be considered as a mixture of 23% of a diacyl phospholipid and 77% of an α'-alkoxy-β-fatty acyl phospholipid.