Purification and Properties of a Soluble Reduced Nicotinamide-Adenine Dinucleotide (Phosphate) Dehydrogenase from the Hepatopancreas of *Octopus vulgaris*

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1. The oxidation of NADH and NADPH catalysed by the soluble supernatant from the hepatopancreas of *Octopus vulgaris* is due to a single enzyme, which has been purified approximately 100-fold. The enzyme reacts rapidly with potassium ferricyanide, and more slowly with 2,6-dichlorophenol-indophenol. No activity is obtained with oxygen, cytochrome *c*, lipoic acid, vitamin K₁, vitamin K₃, ubiquinone-30, *p*-benzoquinone, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride or methylene blue. 2. GSH, cysteine and mercaptoethanol stimulate the enzymic activity up to fivefold. GSSG is without any apparent effect. When stimulated by GSH the enzyme becomes sensitive to dicoumarol, which produces an inhibition competitive with respect to the activator. 3. The purified enzyme contains an acid-removable flavine component, which has been identified as FMN by spectrofluorimetry and chromatography in three solvent systems. After acid ammonium sulphate treatment the enzymic activity is lost, but it can be almost fully restored by incubation with FMN. FAD produces only a partial reactivation.

Considerable attention has been paid to enzymic systems that oxidize reduced nicotinamide nucleotides in mammalian organs. Several of these systems have been purified and their properties described in detail. Effort has been particularly focused on the soluble NAD(P)H dehydrogenases present in brain (Giuditta & Strecker, 1961; Harper & Strecker, 1962; Banay-Schwartz & Strecker, 1962) and liver tissues (Ernster, Danielson & Ljunggren, 1962; Märki & Martius, 1960; Wosilait, 1960) and on the NADH dehydrogenases of mitochondrial (Singer, 1963) and microsomal (Strittmatter & Velick, 1956) origin. In brain, at least five different soluble enzymes have been identified that catalyse the oxidation of NADH or NADPH (Giuditta, 1964); a similar pattern is thought to exist in liver. Although some properties of these soluble enzymes have been described in detail, little is known of their biochemical role or of possible naturally occurring electron acceptors, and almost no information is available on the NADH and NADPH dehydrogenases of non-mammalian classes of animals.

We have been concerned with the systems of oxidation of reduced nicotinamide nucleotides in the hepatopancreas of *Octopus vulgaris* and have obtained evidence that most, if not all, of the NADH and NADPH dehydrogenase activity present in soluble form in this organ is due to a single flavo-

protein (di Prisco, Casola & Giuditta, 1966). This enzyme is similar to NAD(P)H dehydrogenases of mammalian origin in donor specificity, but differs from them in several other properties.

MATERIALS AND METHODS

Materials. NADH, NADPH, FMN and FAD were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and dicoumarol from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Other reagents were of the highest purity commercially available. All preparations were made in glass-distilled water.

Source of enzyme. The hepatopancreas of *Octopus vulgaris*, made available by the Zoological Station of Naples, was quickly removed and either treated immediately or stored at -20° . All preparations were carried out at $0-4^{\circ}$. Homogenates were obtained by mincing the tissue with fine scissors, followed by grinding with 0.05 M-potassium phosphate buffer, pH 7.5, in a glass Dounce homogenizer (Kontes Glass Co., Vineland, N.J., U.S.A.); the final suspension contained 10% (w/v) of tissue. Acetone-dried hepatopancreas was prepared by mechanical homogenization in 10 vol. of acetone at -20° , by means of a Servall Omnimixer, filtration and suspension twice in 5 vol. of cold acetone. After being dried *in vacuo* the powder was stored at 0° .

Assays of enzymic activity. Assays were carried out in a total volume of 3 ml. at $20-25^{\circ}$ in cells of 1 cm. light-path. Extinction changes were recorded automatically with a Gilford Optical Density Converter and a Beckman model DU monochromator. The reaction was started by the

addition of the enzyme; activities, in enzyme units, were calculated from the linear portion of the progress curves. A unit is defined as the amount of enzyme that oxidizes 1 μ mole of NADH/min. in the presence of the indicated electron acceptor. The composition of the assay mixture is given in the Table and Figure legends. Changes in extinction were recorded at 420 m μ with ferricyanide as acceptor, or at 600 m μ with 2,6-dichlorophenol-indophenol. All rates were corrected for non-enzymic reduction due to reduced nicotinamide nucleotide and (when used) GSH or other thiol compounds.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Fluorimetry. Fluorescence measurements were carried out with a Farrand spectrofluorimeter.

Paper chromatography. The ascending technique was used, with Whatman no. 1 paper, or Whatman no. 3MM paper for preparative chromatography. The solvent systems used were: water saturated with 3-methylbutan-1-ol (Whitby, 1950); 2-methylpropan-2-ol-water (3:2, v/v) (Huennekens & Kilgour, 1955); butan-1-ol-acetic acid-water (4:1:5, by vol.) (Kilgour, Felton & Huennekens, 1957). After the chromatogram had been dried, the spots were observed with a long-wave SL-3660 UV Mineralight lamp (Ultra-Violet Products Inc., San Gabriel, Calif., U.S.A.).

RESULTS

Distribution of the dehydrogenase activity. Homogenates of *Octopus hepatopancreas* catalysed the oxidation of both reduced nicotinamide nucleotides when potassium ferricyanide was used as electron acceptor. After centrifugation at 105 000g for 2 hr., only a part of the dehydrogenase activity was found in the soluble fraction; a large part remained associated with the resuspended pellet. Further washing of the particulate fraction did not result in any significant increase of soluble activity. The two enzyme fractions differed in their response to dicoumarol and to GSH. Table 1 shows that the particle-bound activity was strongly inhibited by dicoumarol and stimulated about 50% by 0.5 mM-GSH, whereas the soluble fraction was practically insensitive to the inhibitor and stimulated much more (four- to five-fold) by GSH.

The soluble activity was purified to determine whether the oxidation of NADH and NADPH was carried out by the same enzyme or by two different systems.

Enzyme purification. The most suitable source of enzyme was found to be the acetone-dried powder of hepatopancreas, since this material was free from most of the abundant lipid originally present. Control experiments indicated that the catalytic properties of the enzyme extracted from acetone-dried powders were indistinguishable from those of the soluble enzyme obtained by high-speed centrifugation of the homogenate. Acetone-dried powder (3g.) was suspended in 30 ml. of 0.05 M-potassium phosphate buffer, pH 7.5, containing 0.2 mM-EDTA,

Table 1. *Distribution of NADH- and NADPH-ferricyanide reductase activities in the hepatopancreas of Octopus vulgaris*

The assay mixture contained reduced nicotinamide coenzyme (0.1 mM), K₃Fe(CN)₆ (0.2 mM) and potassium phosphate buffer, pH 7.5 (0.05 M). EDTA (3 mM) was added when GSH was present; at this concentration, it did not affect the enzymic rate. The activities are expressed as μ moles of substrate oxidized/min./g. of fresh tissue.

	Activities		
	No addition	With 0.2 mM-dicoumarol	With 0.5 mM-GSH
NADH			
Homogenate	15.8	7.2	27.0
Resuspended precipitate	9.0	3.8	13.5
Supernatant	2.9	2.7	12.4
NADPH			
Homogenate	12.3	5.5	20.2
Resuspended precipitate	8.4	3.4	12.8
Supernatant	2.2	2.1	11.1

and stirred at 0° for 20 min. The suspension was centrifuged at 105 000g for 3 hr., and alumina C γ gel added to the supernatant (100 mg. dry wt./100 mg. of protein). After recentrifugation, the supernatant was discarded, and the gel was washed twice with the same buffer and eluted with two 20 ml. portions of 0.5 M-potassium phosphate buffer, pH 7.5, containing 0.2 mM-EDTA. The two eluates were combined, and solid ammonium sulphate was added with stirring to give 95% saturation. One of the most remarkable properties of the enzyme is its solubility in concentrated ammonium sulphate solutions. This step therefore resulted in a high degree of purification, since most of the contaminating proteins could be removed by centrifuging. The ammonium sulphate was removed from the supernatant by dialysis against 0.05 M-potassium phosphate buffer, pH 7.5, containing 0.2 mM-EDTA. The enzyme obtained by this procedure was used either directly or after freeze-drying, although the latter procedure resulted in losses of up to 40% of the activity. The enzyme in concentrated ammonium sulphate solutions retained most of its activity for several weeks. A summary of the purification procedure is given in Table 2. The slight increase in total activity after the ammonium sulphate step was found on every occasion.

Evidence for a single enzyme. An indication that a single enzyme was oxidizing both reduced nicotinamide nucleotides was provided by the data presented in Table 3. The rate with NADH was somewhat higher than that with NADPH, but the ratio

Table 2. *Purification procedure*

The assay mixture contained potassium phosphate buffer, pH 7.5 (0.05 M), NADH (0.1 mM) and $K_3Fe(CN)_6$ (0.2 mM). Enzyme units are defined in Table 1.

Fraction	Volume (ml.)	Total enzymic activity (units)	Protein (mg./ml.)	Specific activity (units/mg.)	Yield (%)	Times purified
(1) Acetone-dried powder extract	30	11.70	15	0.026	100	—
(2) Alumina C_γ eluate	40	8.70	3.2	0.068	74	2.6
(3) $(NH_4)_2SO_4$ supernatant (dialysed)	52	9.75	0.067	2.800	83	107.8

Table 3. *Constancy of kinetic behaviour of the enzyme at different stages of purification*

V_{NADH} , V_{NADPH} and V_{GSH} refer to the rates obtained in the presence of the indicated compound; reduced co-enzymes were 0.1 mM and, when added, GSH and dicoumarol were 0.5 mM and 0.2 mM respectively. Other conditions were as in Table 1.

	V_{NADH}/V_{NADPH}	$V_{GSH}/V_{Coenzyme}$		Dicoumarol inhibition (%)	
		NADH	NADPH	NADH	NADPH
Extract	1.38	4.5	5	48	46
Alumina C_γ eluate	1.41	3.3	3.6	51	47
$(NH_4)_2SO_4$ supernatant	1.45	3.4	4.3	52	48

Table 4. *Non-additive rate of reaction in the presence of both substrates*

The assay mixture contained potassium phosphate buffer, pH 7.5 (0.05 M), $K_3Fe(CN)_6$ (0.2 mM) and the indicated concentration of substrate. The theoretical mixed-substrate velocity was calculated by means of the equation $v_t = (V_a\alpha + V_b\beta)/(1 + \alpha + \beta)$ (Dixon & Webb, 1964). V_a , V_b , K_a and K_b were obtained graphically from double-reciprocal plots similar to those illustrated in Fig. 4.

Substrate	...	NADH	NADPH	NADH + NADPH
Concn. (mM)	...	0.1 (a)	0.1 (b)	0.1 + 0.1
K_m (μM)	...	34 (K_a)	22 (K_b)	—
Relative concn.	...	2.95 ($\alpha = a/K_a$)	4.55 ($\beta = b/K_b$)	—
V_{max} (unit/ml.)	...	0.252 (V_a)	0.171 (V_b)	—
v_t (unit/ml.)	...	—	—	$\left\{ \begin{array}{l} 0.172 \text{ (experimental value)} \\ 0.178 \text{ (theoretical value)} \end{array} \right.$

of the velocities remained practically constant throughout the purification procedure.

The experiment reported in Table 4 provided evidence that the purified preparation contained a single enzyme. At equimolar concentrations (0.1 mM) of both coenzymes added together, so that the sum was 0.2 mM, a near-saturating concentration, the observed rate approached the average of the two rates observed at saturating concentrations of each coenzyme separately, as was to be expected from a single enzyme. Moreover, the theoretical value of v_t , calculated on the assumption that both nucleotides were oxidized by the same enzyme (Dixon & Webb, 1964), was in good quantitative agreement with the experimental velocity.

Stimulation by GSH. Fig. 1 shows the effect of the GSH concentration on the enzymic activity;

maximal stimulation was observed at about 0.5 mM-GSH, with either reduced nicotinamide nucleotide. Higher concentrations of GSH were less stimulatory. An inhibitory effect of dicoumarol on the stimulation brought about by GSH is illustrated in Fig. 2, which shows that maximal inhibition occurred at a concentration of 0.2 mM. Dicoumarol inhibited only in the presence of GSH. Fractions of different purity were found to be equally stimulated by GSH and equally inhibited by dicoumarol when this inhibitor was added in the presence of GSH. A similar degree of stimulation by GSH and of inhibition by dicoumarol was found with either NADH or NADPH, favouring the hypothesis that a single enzyme, rather than a mixture of two, was catalysing the oxidation of both nicotinamide nucleotides.

Fig. 1. Effect of GSH concentration on the reaction rate. The assay mixture contained potassium phosphate buffer, pH 7.5 (0.05 M), $K_3Fe(CN)_6$ (0.2 mM) and EDTA (3 mM).

Fig. 2. Effect of dicoumarol concentration on the reaction rate in the presence of 0.5 mM-GSH. Other conditions were as in Fig. 1.

The fact that enzymic activity became sensitive to dicoumarol only when GSH was also present prompted us to look at this property more closely. Double-reciprocal plots obtained at a constant concentration of either NADH or NADPH and different GSH concentrations showed clear competition between the inhibitor and the activator (Fig. 3), suggesting that dicoumarol and GSH interact at the same site on the protein. On the other hand, the data plotted in Fig. 4, which shows the effects of varying the concentration of either of the two coenzymes on the reaction rate in the presence and in the absence of GSH, indicated that the interaction of the activator with each of the coenzymes was of a mixed type. It is likely therefore that the site of interaction for NADH and NADPH is different from that of GSH. Other thiols such as cysteine and mercaptoethanol also activated the enzyme to an extent comparable with that obtained with GSH, and the stimulation brought about by these compounds was also dicoumarol-sensitive. In each case, non-enzymic reduction of ferricyanide

Fig. 3. Relationship between the effects of GSH concentration and dicoumarol inhibition. The lines are double-reciprocal plots. Conditions were as in Fig. 1. $\circ$, No dicoumarol; $\bullet$, 0.2 mM-dicoumarol.

Fig. 4. Initial velocity as a function of reduced nicotinamide coenzyme concentration, in the presence and absence of GSH. Conditions were as in Fig. 1. $\circ$, No GSH; $\bullet$, 0.5 mM-GSH.

by the thiol was greatly diminished by EDTA. EDTA, however, did not prevent the reduction of ferricyanide by another reducing agent, ascorbate, the effect of which could not therefore be ascertained. The addition of GSSG neither inhibited the reaction in the absence of thiols, nor prevented the activation in their presence. This could indicate that the activation might not proceed through direct reduction of thiol groups.

Other catalytic properties. Some kinetic constants were calculated from the data plotted in Figs. 3 and 4 (Table 5). The K_m values for NADH and NADPH, in the presence and in the absence of GSH, were obtained graphically (Fig. 4); the K_m for GSH with

Table 5. *Kinetic constants of the enzyme*

Except for variations necessary for the determination of constants, conditions were as in Table 1.

	K_m or K_i (μM)		V_{max} (units/ml.)	
	NADH	NADPH	NADH	NADPH
Substrate	34	22	(100)	64
Substrate+ GSH	21	13	171	124
GSH	37	43	} (100)	78
Dicoumarol	58	62		

both coenzymes was also obtained (Fig. 3), and the K_i for dicoumarol was determined by the method of Dixon & Webb (1964). The affinity constants for the activator and the inhibitor are independent of the nature as well as the concentration of the substrate. As shown in Table 5, all these constants fell in the range 10–60 μM ; the apparent affinity of the enzyme for NADPH is higher than that for NADH, although V_{max} is higher in the latter case.

The purified enzyme did not catalyse the oxidation of NADH or NADPH by the following electron acceptors: oxygen, methylene blue, lipoic acid, *p*-benzoquinone, ubiquinone-30, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride, cytochrome *c*, vitamin K_1 and vitamin K_3 . All the assays were carried out in the presence and absence of GSH. The only acceptor, other than ferricyanide, the reduction of which was catalysed by the enzyme was 2,6-dichlorophenol-indophenol, which oxidized both nicotinamide nucleotides at approximately half the rate (in terms of $\mu moles/min.$) observed with ferricyanide. Rates in the presence of GSH could not be recorded because of the instantaneous reduction of the 2,6-dichlorophenol-indophenol by the thiol even in the presence of EDTA.

Prosthetic group. Solutions of the dehydrogenase after the final ammonium sulphate step were dialysed exhaustively, freeze-dried and dissolved in the minimum volume of water. Perchloric acid was added in the cold to a final concentration of 7% (w/v), the precipitate discarded, the supernatant carefully neutralized with 6*N*-potassium hydroxide and the precipitate of potassium perchlorate discarded. The supernatant showed a fluorescence spectrum similar to that of flavine, with an excitation maximum at 445 $m\mu$ and a fluorescence maximum at 530 $m\mu$ (Fig. 5). Since the neutralized supernatant still contained some unidentified impurities that lowered the R_F of the flavine in several chromatographic systems, a purification was carried out by chromatography of the solution on Whatman no. 3MM paper with water saturated with 3-

Fig. 5. Fluorescence spectrum of a perchloric acid supernatant of purified enzyme (see the text). The exciting light was at 445 $m\mu$.

methylbutan-1-ol. After elution of the yellow spot and concentration by freeze-drying, the solution exhibited the same fluorescence properties. Chromatography of a sample in two different solvent systems, 2-methylpropan-2-ol-water (3:2, v/v) and butan-1-ol-acetic acid-water (4:1:5, by vol.), gave in each case a fluorescent spot with the same R_F as authentic FMN. Heating with 5% (w/v) trichloroacetic acid for 10 min. at 100° followed by neutralization with 0.12*M*-dipotassium hydrogen phosphate did not increase the fluorescence of the purified extract. These results indicated that purified fractions of the enzyme contained FMN.

To confirm that FMN was indeed the prosthetic group of the dehydrogenase, a sample of purified enzyme in 95% saturated ammonium sulphate was adjusted to pH 2.0 by the addition of 2*N*-hydrochloric acid. After 5 min. the sample was dialysed against 1*M*-potassium phosphate buffer, pH 7.5, and samples were incubated at 0° with 0.5 *mm*-FMN for 3 hr. Table 6 shows that this procedure resulted in restoration of over 80% of the original activity. Incubation with 0.5 *mm*-FAD also restored about 55% of the initial activity. GSH stimulated in both cases, and this stimulation was dicoumarol-sensitive. Similar results were obtained with NADPH as substrate. A control sample incubated without flavines showed no spontaneous recovery of activity. A sample of enzyme not pretreated with acid ammonium sulphate gave no increase in activity after incubation with FMN or FAD. This evidence leaves little doubt of the flavoprotein nature of the enzyme.

Table 6. *Effect of incubation with flavines, after removal of the prosthetic group, on the NADH-ferricyanide reductase activity*

The assay conditions were as in the previous Tables.

Enzyme sample	Activity (unit/ml.)		
	No addition	With 0.5 mm-GSH	With 0.5 mm-GSH and 0.2 mm-dicoumarol
Control, dialysed	0.120	0.382	0.178
pH 2.0, dialysed (1)	0.013	0.057	0.025
(1), Incubated with 0.5 mm-FMN	0.097	0.345	0.165
(1), Incubated with 0.5 mm-FAD	0.067	0.225	0.108

DISCUSSION

The present results indicate conclusively that a soluble dehydrogenase that catalyses the oxidation of both NADH and NADPH is present in phosphate homogenates of the hepatopancreas of *Octopus vulgaris*. This conclusion is based on the constancy of the ratio of NADH and NADPH oxidation during the purification procedure, and on the similarity of other kinetic parameters such as the stimulation by GSH and the inhibition by dicoumarol. Further evidence that a single enzyme catalyses the oxidation of both nucleotides is derived from the non-additivity of reaction rates when the assay is carried out with mixed substrates.

The enzyme is stimulated severalfold by GSH and other thiols, and it is inhibited by dicoumarol only in the presence of these activators. Kinetic studies reveal that there is competition of GSH with inhibitor but not with the reduced nicotinamide coenzymes. These data suggest a common site of interaction for activator and inhibitor that is different from the substrate-binding site.

The flavoprotein nature of the purified enzyme is shown by the fluorescence spectra of acid extracts and by the identification of the flavine as FMN in three different chromatographic systems. Further indications that the flavine is FMN derive from the lack of fluorescence enhancement after acid hydrolysis and from reactivation experiments with enzyme exposed to acid in the presence of ammonium sulphate. Incubation with FMN restored 80% of the original activity, whereas only 55% was restored by FAD.

In its ability to oxidize both nicotinamide nucleotides the enzyme resembles the soluble NAD(P)H dehydrogenases described in mammalian liver (Ernster *et al.* 1962; Märki & Martius, 1960; Wosilait, 1960) and brain (Giuditta & Strecker, 1961; Harper & Strecker, 1962). It differs from

them, however, in its lack of reactivity with vitamin K₁ and vitamin K₃, in its relative resistance to dicoumarol inhibition and in the nature of its prosthetic group.

The presence of a single soluble NAD(P)H dehydrogenase in the hepatopancreas of *Octopus vulgaris* contrasts markedly with the complex pattern of NADH and NADPH oxidation in the central nervous system of another closely related cephalopod, *Sepia officinalis*, where recent investigations carried out in this Laboratory have shown the presence of at least five different soluble enzymes (E. Aloj & A. Giuditta, unpublished work).

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