SHORT COMMUNICATIONS

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Purification and properties of dichlorophenol-stimulated NADH oxidase from rat uterus

The enzymatic oxidation of NADH in various tissues is stimulated by the presence of a phenolic compound[1]. This catalytic requirement is relatively nonspecific; apparently the primary necessity is an aromatic hydroxyl group[2]. Several mechanisms involving free radicals have been proposed to explain the participation of catalytic quantities of a phenolic compound, Mn^{2+}, and molecular O_2 in the oxidation of NADH[1-7]. Among the supportive evidence for this free radical mechanism is inhibition by 2-mercaptoethanol[8], cysteine[5,8,9], and GSH. Presumably, this inhibition is a result of the reaction of these compounds with free radical intermediates produced in the chain reaction from either the phenol or NADH or both. The present study was initiated to fractionate further this dichlorophenol-stimulated NADH oxidase activity and to test other sulfhydryl and disulfide group inhibitors.

The enzyme assay mixture (1.0 ml total volume) contained (in μmoles) $MnCl_2$, 0.05; 2,4-dichlorophenol, 0.05; NADH, 0.15; 0.5 ml of 0.05 M phosphate buffer (pH 7.0); and 0.005–0.01 ml enzyme preparation and inhibitor where indicated. The reaction was initiated by addition of NADH and the disappearance of NADH was followed in 2 min. The unit of activity is defined as change in absorbance per min at 37° at 340 mμ; specific activity is defined as units/mg protein. Protein was estimated either colorimetrically[10] or spectrophotometrically[11], using bovine serum albumin as a standard.

Uterine tissue from 140–160 g Holtzmann rats was stripped of excess fatty and vascular tissues. A 10% (w/v) homogenate was made in 0.05 M sodium, potassium phosphate buffer (pH 6.0), in an all-glass homogenizer. All fractionation steps were carried out at 5°. After centrifugation at 500 × *g* for 10 min, the sediment was successively re-homogenized with each of the following in a volume equal to that of the supernatant discarded from the previous step: 0.05 M phosphate buffer (pH 6.0), 0.25 M sucrose, 0.2 M NaCl and finally 1 M KCl. The 1 M KCl supernatant was slowly brought to 20% saturation with respect to solid $(NH_4)_2SO_4$, centrifuged, and the supernatant then brought to 45% saturation. The precipitate from the 45% saturation treatment was dissolved in 1 M KCl (0.1 the volume of the first 1 M KCl supernatant).

A sample of the second 1 M KCl fraction (not exceeding 150 mg protein) was placed on a Bio-Gel P-200 column (4.5 cm × 48 cm packed volume) equilibrated with the phosphate buffer at 5°. Enzyme activity was eluted as a single broad peak after passage of 400 ml of phosphate buffer. Those fractions which had high activity and low protein content were pooled and concentrated to 1–2 ml by ultrafiltration in a collodion bag. This concentrated protein solution (19 mg) was fractionated on a CM-cellulose column (1 cm × 25 cm packed volume) equilibrated with the phosphate buffer. Elution with the phosphate buffer containing 0.2 M NaCl resulted in a relatively sharp peak of enzyme activity contained in 4 or 5 fractions of 1.5 ml each.

Results of a typical purification are shown in Table I. These fractionation proce-

TABLE I

PURIFICATION OF UTERINE DICHLOROPHENOL-STIMULATED NADH OXIDASE

The units of enzyme activity refer to change in absorbance of NADH per min and specific activity as units/mg protein.

Fraction	*Total protein (mg)*	*Total enzyme units*	*Specific activity*	*Purification*	*Percentage recovery of enzyme*
10% homogenate	1670	117	0.07	(1.0)	100
Second 1 M KCl extract	140	73	0.52	7.4	63
Bio-Gel P-200	19	66	3.50	50.0	56
CM-cellulose	2	26	13.45	192.1	22

dures result in almost a 200-fold purification with a recovery of 22% of the total activity.

Table II shows the effect of a group of compounds on the activity of dichlorophenol-stimulated NADH oxidase. Compounds such as cysteine, GSH and 2-mercaptoethanol are capable of destroying S–S bond integrity as well as trapping free radicals. A reciprocal plot of activity *vs.* [NADH] (μM) in the presence of 1 μM 2-mercaptoethanol indicates a reversible mixed competitive and noncompetitive inhibition. According to WEBB[12] this mixed type of inhibition can be ascribed to two separate sites. Those compounds which destroy S–H bond integrity (*i.e.*, GSSG, *p*-chloromercuribenzoate, *N*-ethylmaleimide, and iodoacetate) are effective inhibitors of the enzyme activity only when incubated with the enzyme before the addition of dichlorophenol or $MgCl_2$. If the order of addition is reversed no inhibition is noted and occasional slight stimulation occurs. Order of addition did not influence the effectiveness of compounds such as 2-mercaptoethanol. LINEWEAVER–BURK[13] plots of the inhibition data obtained with *N*-ethylmaleimide and chloroacetophenone illustrate noncompetitive inhibition with respect to NADH concentration. Inhibition by GSH could be reversed by the stoichiometric addition of GSSG but only to extent of the original activity; the reverse situation could also be demonstrated. When 10 μg of α-tocopherol

TABLE II

INHIBITORS OF DICHLOROPHENOL-STIMULATED NADH OXIDASE ACTIVITY

Data are expressed as per cent of control activity. The indicated compound, either 0.1 mM or 1 mM, was pre-incubated with the enzyme and 0.05 M phosphate buffer (pH 7.0) for 10 min prior to addition of 0.05 μmole $MnCl_2$, 0.05 μmole 2,4-dichlorophenol, and 0.15 μmole NADH in a total volume of 1.0 ml.

Inhibitor	*0.1 mM*	*1.0 mM*	*Inhibitor*	*0.1 mM*	*1.0 mM*
2-Mercaptoethanol	17	0	NaHS	43	62
Thioglycerol	43	0	$HgCl_2$	70	51
Thioglycolic acid	22	0	$Pb(NO_3)_2$	30	9
Cysteine	17	5	Chloroacetophenone	75	61
GSH	85	13	*p*-Chloromercuribenzoate	56	43
GSSG	108	37	*N*-Ethylmaleimide	68	52
Cystine	56	51	Iodosobenzoate	69	56
Homocystine	82	63	Arsenite	56	56
Cystamine	63	56			

was pre-incubated with the enzyme before the addition of an amount of *p*-chloromercuribenzoate sufficient to produce half-maximal inhibition of activity, no significant inhibition was detected. If this order of addition was reversed, no appreciable protection by α-tocopherol was manifested.

If compounds such as 2-mercaptoethanol were acting only by destroying S–S bond integrity, a LINEWEAVER–BURK plot would demonstrate noncompetitive inhibition, provided the S–S bond involved was not at the active site. Free radical trapping would demonstrate competitive inhibition with respect to either NADH concentration or H_2O_2. The latter possibility is minimal since small amounts of added H_2O_2 did not reverse or reduce the inhibition. The mixed competitive and noncompetitive inhibition shown by 2-mercaptoethanol is indicative that two separate inhibition sites are involved. Thus, an assignment of a sensitive S–S bond necessary for the dichlorophenol-stimulated NADH oxidase is indicated. This labile group would not be present at the active site for NADH.

In addition it would appear that a labile –SH group is present which can undergo reversible oxidation–reduction and that this group can be protected by α-tocopherol. CORWIN AND SCHWARTZ[14] report similar results with α-ketoglutarate and they suggest that this action is due to protecting the sensitive –SH groups from oxidation.

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