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RAT BRAIN NADPH-DEPENDENT DIAPHORASE

A POSSIBLE RELATIONSHIP TO CYTOCHROME P_{450} REDUCTASE

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Abstract—An enzyme (NADPH-dependent diaphorase) present in rat brain microsomes has been solubilised and shown to utilise both nitrobluetetrazolium and cytochrome *c* as electron acceptors, when reduced by NADPH. The kinetics of the enzyme have been determined using cytochrome *c* (K_m = 1.3 μM), NADPH (K_m = 1.4 μM) and the V_{max} (4.7 nmol/min/mg solubilised microsome protein). The subunit M_r is approximately 73,000 D and that of the native enzyme is 170,000–180,000 D, indicating that the enzyme is probably a dimer. Evidence is also provided to show that the enzyme is a flavoprotein, and that it has equimolar amounts of FAD and FMN with respect to the subunit concentration. It seems a possibility that the rat brain diaphorase enzyme may be cytochrome P_{450} reductase, EC 1.6.2.4.

For many years it has been possible to visualise discrete, albeit sparse, populations of neurons histochemically, on the basis of their "NADPH-dependent diaphorase" activity [1, 2]. Although striatal and some neocortical diaphorase positive neurons were found to contain somatostatin and neuropeptide Y [3], this was not a general observation throughout the CNS. Thus NADPH diaphorase does not seem to be associated specifically with any particular neurotransmitter system.

NADPH-diaphorase is an enzyme which appears to act by transferring electrons between NADPH and various electron acceptors, including tetrazolium dyes. Its possible physiological function is unknown. It is, however, of interest that in Huntington's disease (HD) NADPH-diaphorase positive neurons may be selectively preserved, while being surrounded by degenerating cells [4].

In this study we have extended our previous characterisation [5] of rat brain microsomal NADPH-diaphorase and demonstrated that the enzyme can utilise other substrates such as cytochrome *c* in addition to the artificial electron acceptor dye, nitroblue tetrazolium (NBT). A comparison of the determined characteristics of NADPH diaphorase with those of the enzyme cytochrome P_{450} reductase, has revealed striking similarities.

MATERIALS AND METHODS

Materials. Sephacryl S-300, cytochrome *c*, polyacrylamide gel reagents and molecular mass marker kits were obtained from Sigma (Poole, Dorset). NADPH was from Park Biochemicals (Northampton). SKF 525A was a gift from Smith, Kline and French Ltd. (Welwyn Garden City). All other chemicals were obtained from B.D.H. (Poole, Dorset) or Sigma.

Preparation of solubilised microsomes. Wistar rats (300 g) were stunned, decapitated and the brains rapidly removed and transferred into ice-cold buffered (0.32 M) sucrose. Following homogenisation in 10 vol. of sucrose solution, the homogenate was centrifuged at 1000 *g* for 10 min to remove debris. The supernatant was centrifuged at 17,500 *g* for 30 min and the P2 discarded. The supernatant was then recentrifuged at 100,000 *g* for 1 hr to yield a crude microsomal pellet. This was resuspended and solubilised in 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% Triton-X 100 for 20 min at room temperature, followed by a further 100,000 *g* centrifugation to remove unsolubilised material. The protein concentration of the solubilised microsomes was estimated and they were then stored as 1 ml aliquots in liquid nitrogen.

Estimation of native relative molecular mass by gel exclusion chromatography. This was carried out using a 1.2 × 70 cm Sephacryl S-300 column as described previously [6]. Basically, 2 ml samples of crude enzyme containing 20% glycerol, 2 mg blue dextran and 20 mg potassium ferricyanide were applied to the column and 0.5 ml fractions collected, the flow rate being 20 ml/hr of 50 mM Tris–HCl buffer (pH 7.5) containing 0.1% Triton X-100.

The presence of cytochrome *c* reductase activity was detected by the addition, to each aliquot, of sufficient cytochrome *c* (oxidised) and NADPH to give final concentrations of 30 and 100 μM respectively. These were then incubated at 37° for 30 min and the absorbance read in a spectrophotometer at 550 nm. These data were then related to the elution pattern of relative molecular mass standards, of M_r 15.2 to 480 kD.

Comparison of cytochrome c *reductase and NADPH-diaphorase activities in rat brain microsomes*. In order to investigate whether cytochrome *c* reductase and NADPH-dependent diaphorase activity might be attributable to the same enzyme, a non-denaturing gradient polyacrylamide (1.5 mm thick, 150 mm wide, 3–30%) gel was loaded

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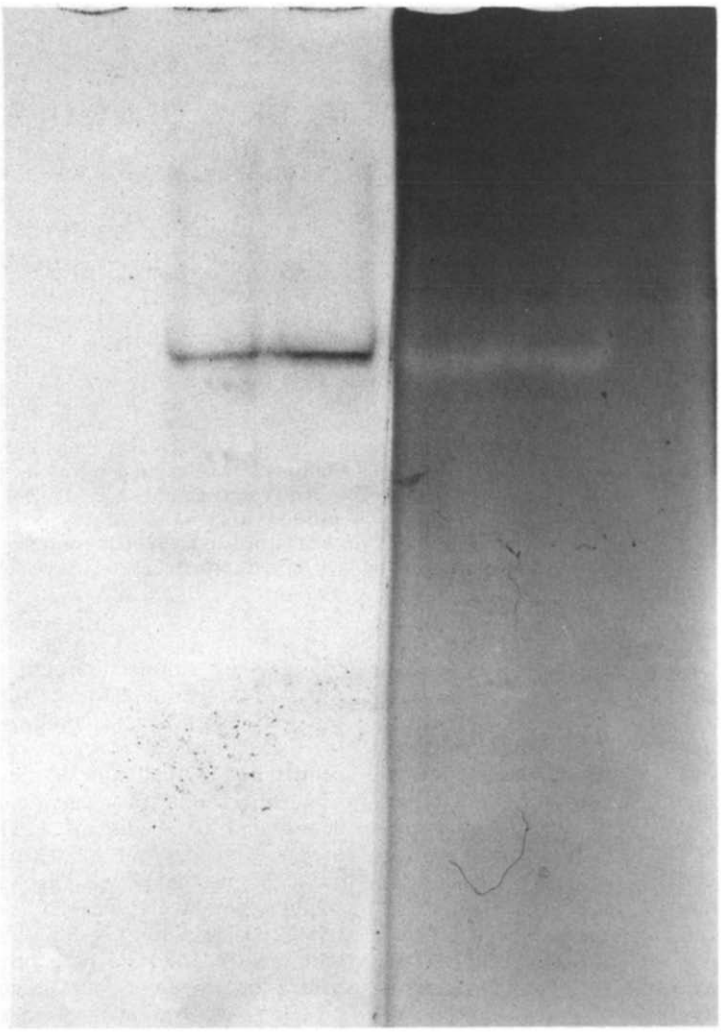

Fig. 1. Solubilised microsomes electrophoresed under non-denaturing gradient gel conditions. This shows the colocalisation of NADPH-dependent diaphorase activity directed against NBT and cytochrome *c* reductase activity. It also incidentally shows that there is a single enzyme present in the solubilised microsomal fraction which is capable of utilising both substrates under the conditions of staining. To emphasise the low contrast between the background oxidised cytochrome *c* (brick red) and the reduced form (peach band) a high definition polychromatic film was used, and then printed on high contrast paper.

with 100 μg of solubilised microsomes and then electrophoresed as previously described [6]. When the tracking dye had reached the bottom of the gel, the run was terminated and the gel split into halves. One half was stained for NADPH-diaphorase, in 50 mM Tris–HCl (pH 7.5) containing 0.25 mM NBT and 0.25 mM NADPH, and the other for cytochrome *c* reductase, using the same buffer and concentrations as for the diaphorase stain (cytochrome *c* being exchanged for NBT). They were then placed back together again and photographed (see Fig. 1).

Estimation of the subunit relative molecular mass. Approximately 2 mg of solubilised microsomes were loaded onto preparative non-denaturing gradient gels. After running, the gels were stained with NBT-NADPH, as described above (although as indicated later, staining for cytochrome *c* reductase would also have been appropriate). Bands of enzyme activity were removed from the excised gel fragments by electroelution. The total protein in the electroeluate was estimated and SDS PAGE carried out according to the method of Laemmli [7]. Samples containing 10 μg of protein were treated with mercaptoethanol (2 μl) in 200 μl of buffer (0.025 M Tris–glycine, pH 8.3, 0.1% SDS) and boiled for 2 min. They were then run in duplicate tracks on a gradient SDS gel (1.5 mm, 5–15%), with appropriate marker proteins. After running, the gel was stained with silver, using the following method. The gel was fixed for 30 min in 12% TCA/methanol, washed in distilled water extensively, immersed in 0.00005% dithiothreitol for 30 min, washed briefly with distilled water and immersed in 0.1% silver nitrate for 10 min, rinsed briefly with distilled water to remove excess. The

bound silver was then developed in 3% sodium carbonate containing 0.00005% formalin until contrast was well defined. The reaction was terminated by immersion in 1% acetic acid. The R_f values were read and the subunit relative mass determined.

Kinetic analysis of cytochrome c reductase activity. All assays were performed at 37° in a temperature-controlled double beam spectrophotometer (Beckman model 25) with plotter at a wavelength of 550 nm; which was the absorbance maximum for reduced cytochrome *c*.

The reaction was observed for about 5 min to allow a steady rate to be reached. The rate of reaction, expressed as nmol reduced cytochrome *c* formed/mg/min, could then be determined, using an extinction coefficient of 21 cm^{-1} mM^{-1} at 550 nm. All assays were run in triplicate. The constants K_m and V_{max} for both NADPH and cytochrome *c* were then determined by standard two substrate kinetic analysis [8]. The Lineweaver–Burk plots were constructed with lines of best fit computed by linear regression analysis.

Flavin content. The flavin content of the electroeluted enzyme was determined using the method of Faeder and Siegel [9]. This method relies on the difference in the fluorescence of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) at pH 7.4 and pH 2.6. Flavin standards of 25, 50, 100 nM were measured at both pHs in a fluorimeter (excitation, $\lambda = 450$ nm; emission, $\lambda = 535$ nM). From these values fluorescent extinction constants were calculated. The enzyme was boiled for 2 min to liberate the flavins from the protein, rapidly cooled and then centrifuged at 3000 *g* for 10 min. Fluorescence was measured at each pH as before.

Using the following formula the concentration of the flavins was calculated;

$$F_{7.4} = N_{7.4}[\text{FMN}] + D_{7.4}[\text{FAD}]$$

$$F_{2.6} = N_{2.6}[\text{FMN}] + D_{2.6}[\text{FAD}]$$

where F = total fluorescence, N = FMN constant, D = FAD constant.

Ionic strength. Previous workers [10, 11] have reported that the activity of a liver enzyme capable of NADPH-dependent reduction of cytochrome *c* was greatly influenced by the ionic strength of the buffer solution. To investigate whether rat brain NADPH-diaphorase showed similar characteristics, a series of standard assays were carried out, varying the ionic strength by addition of potassium chloride to the buffer. The standard assay used to determine activity was as follows: 100 μg solubilised microsomal protein, 20 μM NADPH, 50 μM cytochrome *c* in a volume of 1 ml 0.1 mM Tris–HCl buffer, pH 7.5, containing 0.1% Triton X-100; reduction of cytochrome *c* was monitored at 550 nm at 37°.

Inhibitor studies. To obtain further evidence as to the possible identity of the enzyme a number of known and possible inhibitors of NADPH-cytochrome *c* reductase, cytochrome P_{450} and DT-diaphorase, were investigated as follows.

(i) 2′ Monoester phosphates. 2′ AMP and NADP have both been reported to be competitive inhibitors of NADPH-cytochrome *c* reductase [10–12].

(ii) *p*-Chloromercuribenzoate (pCBM) has been reported to inhibit cytochrome P_{450} reductase by binding to thiol groups, preventing the flow of electrons to cytochrome P_{450} and thus inhibiting the oxidation of xenobiotics [10, 11, 13].

(iii) Dithio-bis-nitrobenzoic acid (DTNB) is thought to act in the same way as pCBM.

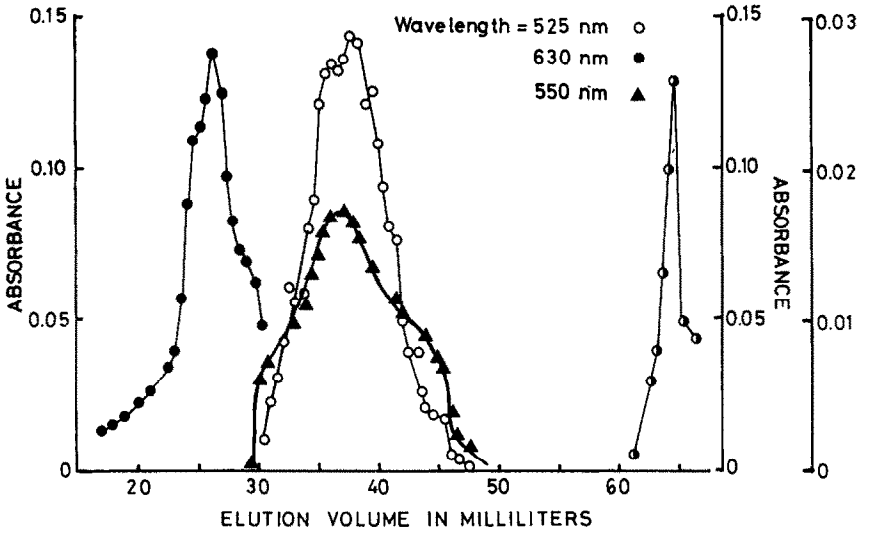

Fig. 2. The elution profile from a Sephacryl S-300 column of solubilised microsomes assayed for diaphorase reductase activity against NBT and cytochrome *c*. The column was previously equilibrated with 50 mM Tris–HCl (pH 7.5) buffer containing 0.1% Triton X-100; and calibrated with standard molecular weight proteins of 15.2 to 480 kD. It can be seen that the peaks for NBT diaphorase and cytochrome *c* reductase activity are coincidental. The eluate was monitored at 630 nm for blue dextran (Ve), and at 400 nm for potassium ferricyanide (Vo). NBT diaphorase 525 nm, cytochrome *c* reductase 550 nm.

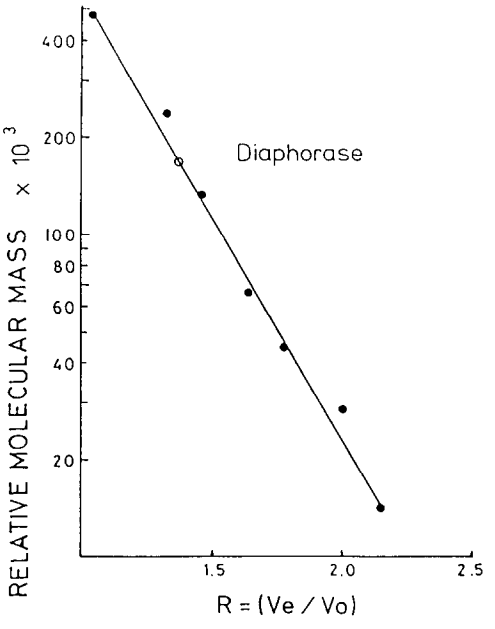

Fig. 3. Determination of M_r for NADPH-diaphorase (○) by comparison with standard molecular weight markers (●) run on the Sephacryl S-300 column. The R (Ve/Vo) value for the diaphorase enzyme corresponds to a M_r value of 171,000 D (± 3.100 D).

(iv) SKF525A (β-diethylamino ethyl-3,3'-diphenyl propyl acetate) is a powerful inhibitor of cytochrome P_{450} and binds to its xenobiotic oxidation binding site [13].

(v) Dicoumarol, has been reported to be a potent inhibitor of DT-Diaphorase (EC 1.6.99.2) [14].

2'AMP, NADP and pCMB were tested over a range of both inhibitor and cytochrome c concentrations (N = 6 for each) to determine overall K_i and to determine the class of inhibition. This was carried out by replotting the slopes derived from Lineweaver–Burk plots, against inhibitor concentration [8]. Other inhibitors were tested in standard assays at concentrations of 100–500 μM.

Protein determinations. Protein concentrations were measured by the method of Lowry [15], using bovine serum albumin as standard.

RESULTS

NADPH-dependent diaphorase was solubilised from rat brain microsomes.

Relative molecular mass estimations of the enzyme from non-denaturing PAGE (staining with NBT) and from gel exclusion chromatography were in good agreement. Electrophoresis of the enzyme gave an M_r = 170,000–180,000 (data not presented). The average molecular mass as determined by exclusion chromatography of the non-denatured enzyme was M_r = 171,000 ± 3100 (Figs 2 and 3).

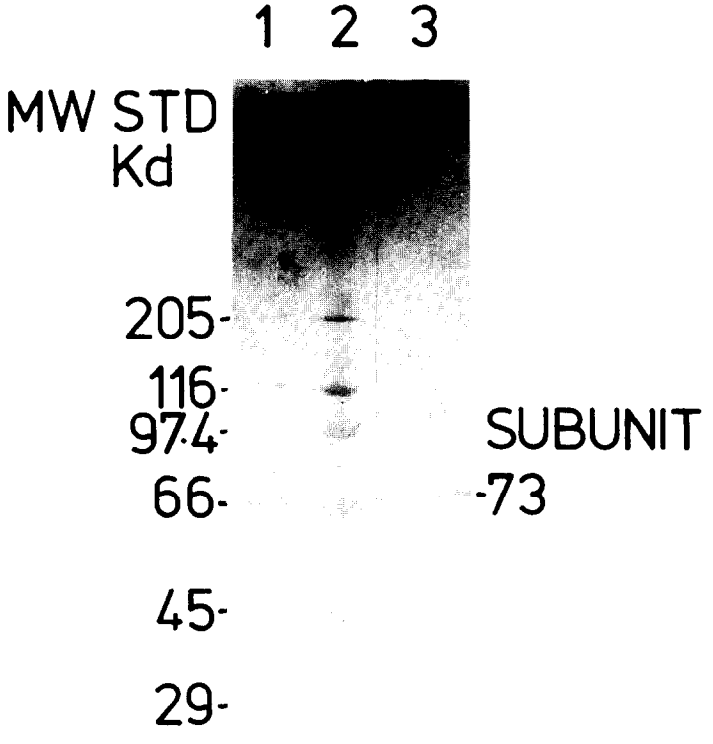

Fig. 4. Silver stained SDS gradient gel. Track 1: standard molecular weight proteins at the masses indicated. Track 2 is a sample of electroeluted enzyme processed for SDS electrophoresis. It can be seen that there is only a single band present in the sample track visible (the standards have about 0.5 μg protein in each band) this is an indication of the purity of the electroeluted enzyme.

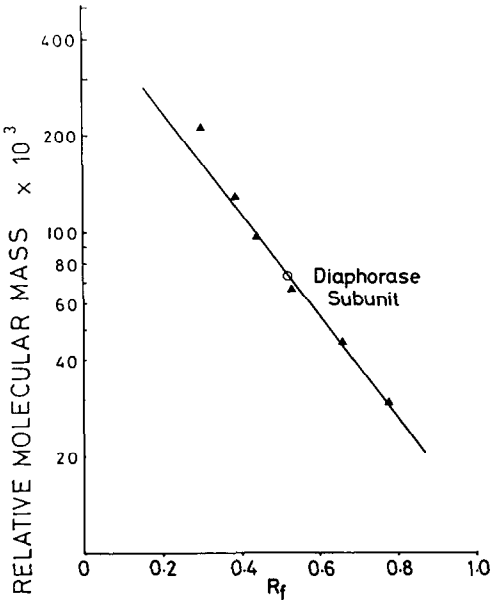

Fig. 5. Determination of M_r for diaphorase subunit. R_f values of the SDS standard molecular weight markers were plotted on a log scale to give a straight line. The R_f of the diaphorase enzyme subunit indicated a M_r = 73,000 D.

The relative molecular mass of the enzyme's subunit, as determined by SDS-PAGE, was 73,000 (N = 3) (Figs. 4 and 5). From this it may be suggested that the enzyme is likely to be a dimer. Comparison of the ability of the solubilised microsome preparation to reduce NBT and cytochrome *c* revealed a coincidentally mobile protein (under non-denaturing PAGE) that utilised both substrates.

Kinetic analysis of the enzyme indicated that the K_m for NADPH was 1.4 μM and for cytochrome *c*, 1.3 μM, with a V_{max} of 4.7 nmol substrate reduced/min/mg protein. These data compare with previously determined values [8] of K_ms for NADPH (1.1 μM) and for NBT (7.3 μM) with a V_{max} of 30 nmol/min/mg protein. The Lineweaver–Burk plots and their replots were straight and consecutive, indicating that no inhibition by product or substrate was taking place (Figs 6 and 7).

Flavin analysis revealed that electroluted enzyme (giving a single band on silver-stained SDS gel) of assumed subunit molecular weight of 73,000 daltons, gave the following flavin contents in two separate trials: enzyme concentration, 0.84 and 0.87 μmol, FAD concentration 0.79 and 0.88 μmol, FMN concentration 0.79 and 0.88 μmol.

This gives a specific flavin content of 64.7 nmol/mg and 69.3 nmol/mg solubilised microsomal protein respectively for the two trials. These data clearly demonstrate that there is one mole of FAD and FMN to each mole of subunit.

Ionic strength was found to greatly influence the rate of reduction of cytochrome *c*, with increasing ionic strength increasing the rate to a maximum at 0.8 M (see Fig. 5). The form of the graph was similar to that of Phillips and Langdon [10], who also reported peak activity at the unusually high level of 0.8 M.

2′AMP and NADP might be expected to give similar inhibition. Both were strictly competitive and the replots of the slopes against inhibitor concentration gave straight lines. When the appropriate data were compared to those of Phillips and Langdon [10] for cytochrome *c* reductase, following suitable recalculation to take account of differing ionic

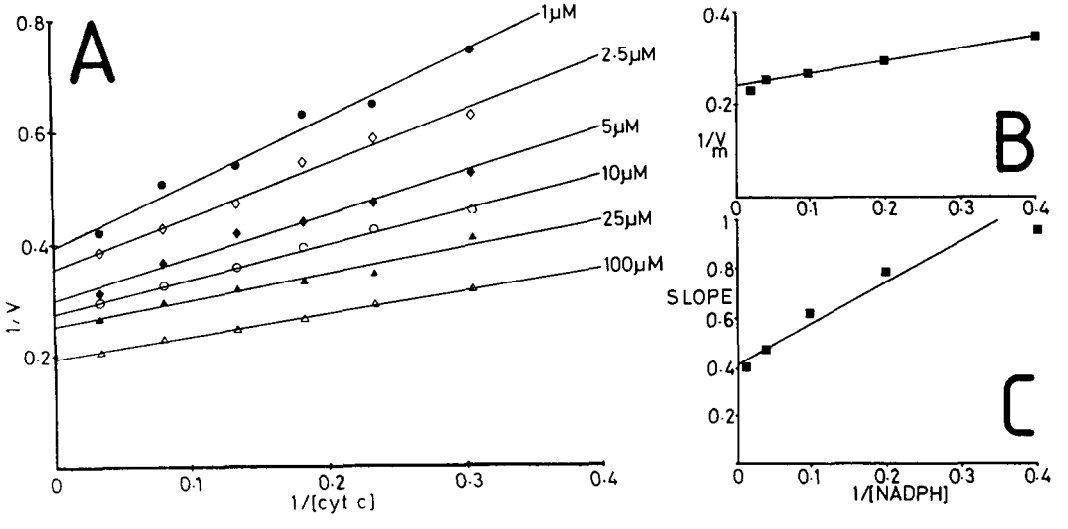

Fig. 6. A kinetic analysis of the reductase enzyme; determination of the K_m for cytochrome *c*. Lineweaver–Burk plots were drawn from hyperbolic regression fits to the data points, which were the average of three trials performed in triplicate. The inverse of the apparent V_{max} for each of the lines, from graph A was replotted against the reciprocal of the NADPH concentration (graph B). The slope of the lines was also plotted against the inverse of the NADPH concentration (graph C). From these replots the overall K_m and V_{max} values for each substrate were derived. K_m cyt.*c* = 1.7 μM, K_m NADPH = 1.15 μM. V_{max} = 4.1 nmol/min/mg solubilised microsomal protein.

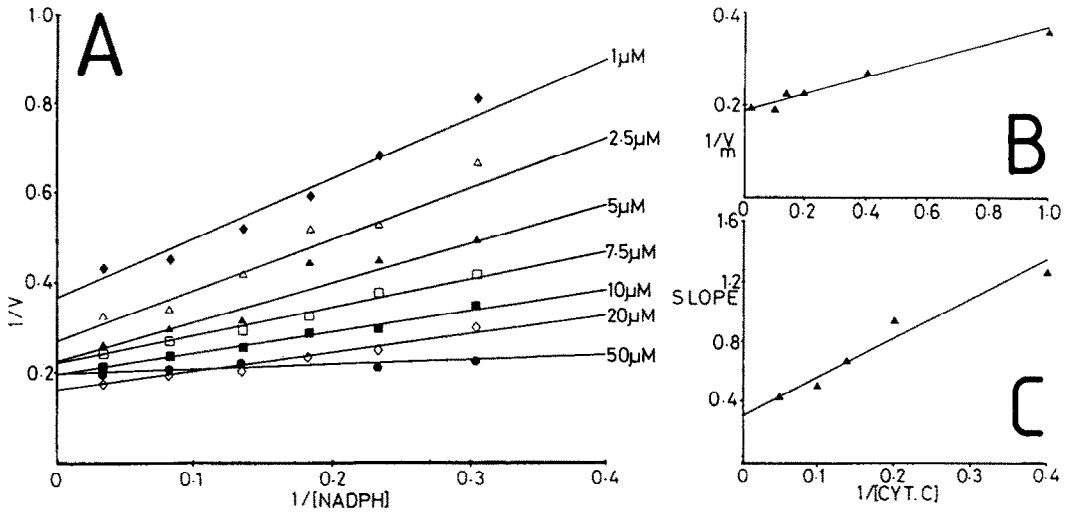

Fig. 7. Kinetic analysis of the reductase enzyme; determination of the K_m for NADPH. The Lineweaver–Burk plots were produced in the same way as indicated for Fig. 6. The replots were against the inverse of the cytochrome *c* concentration. The derived data from these replots give the following values: K_m NADPH = 1.65 μM, K_m cyt.*c* = 0.97 μM, V_{max} = 7.7 nmol/min/mg protein.

strength and specific activity of the enzyme, the K_i values obtained under these conditions (54 mM ionic strength, saturating substrates) are very similar: for 2′AMP, cytochrome *c* reductase, 7.9 μM, rat brain diaphorase, 8.2 μM; for NADP, cytochrome *c* reductase, 7.5 μM, rat brain diaphorase 7.1 μM.

The overall K_i data derived from the replots of the double reciprocal plots, gave identical values of 62.5 μM for both inhibitors.

pCMB on replotting gave a hyperbolic curve. This is associated with non-competitive inhibition, and as such no K_i can be derived. DTNB was also found to act in the same way, but with lower potency.

SKF 525A, a potent inhibitor of cytochrome P_{450}, failed to influence the reduction of cytochrome *c*.

Similarly the DT-diaphorase inhibitor dicoumarol was also without effect on the reduction of cytochrome *c*.

Other substrates found to be reduced by this enzyme were; potassium ferricyanide, dichlorophenolindolphenol (DCPIP) and menadione. The order of activity being as follows; potassium ferricyanide > menadione > DCPIP > NBT > cytochrome *c*. However, the last three were found to be the most suitable as their high extinctions rendered the assays very sensitive.

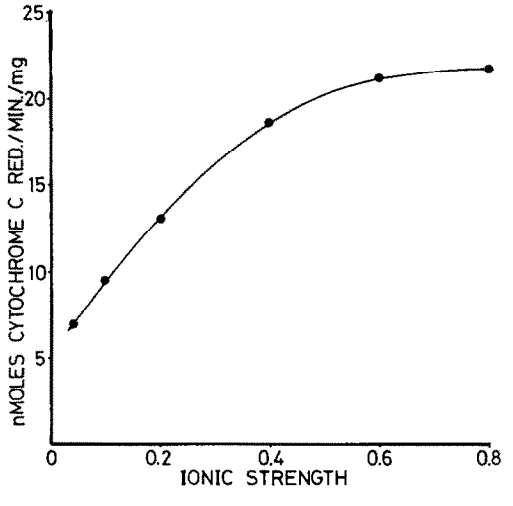

Fig. 8. Effect on ionic strength on the rate of reduction of cytochrome c. Using a standard assay of 50 mM Tris–HCl pH 7.5 0.1% Triton X-100, saturating substrates and 100 μg protein, the ionic strength was increased by addition of KCl and reduction monitored at 550 nm at 37°.

DISCUSSION

Previously we demonstrated the presence of a single enzyme in solubilised brain microsomes responsible for NADPH diaphorase activity and have determined kinetic and other biochemical parameters for the enzyme, using the artificial electron acceptor NBT [5]. In this study we report that the same enzyme responsible for the reduction of NBT at the expense of NADPH also reduces cytochrome *c*. The evidence being that not only does the reductase activity appear coincidently on non-denaturing PAGE, but also that the same M_r value is obtained using gel exclusion chromatography with either substrate. It is thus to be anticipated that the K_m for NADPH would be similar for both substrates. Indeed this proved to be the case (see above) and their very similar values imply that the NADPH interacts with the enzyme before the substrate which is to be reduced.

The molecular mass for the native enzyme using NBT was determined by non-denaturing PAGE and gel exclusion chromatography to be approximately

170,000 D. This was confirmed using cytochrome *c*, which yielded very similar values. The finding that the subunit molecular mass gave a value of approximately 73,000 D suggests that the enzyme is probably a dimer. The discrepancy between the values for the native enzyme and that for its two subunits, may be explained by the association of detergent with the enzyme following solubilisation. Under denaturing conditions, such as in the presence of mercaptoethanol and SDS, this layer of Triton X-100 would be stripped away giving an accurate estimate of the true M_r of the subunit.

The inhibitor studies reveal that rat brain diaphorase is inhibited competitively by 2′monoester phosphates and non-competitively by thiol binding compounds such as pCMB and DTNB. Also the failure of dicoumarol to influence the rate of cytochrome *c* reduction by rat brain diaphorase indicates that the enzyme responsible for the reduction cannot be DT-diaphorase (dicoumarol is reported [14] to halve the rate of DT-diaphorase mediated menadione reduction at 10^{-8} M). Dicoumarol also has no effect upon histochemical diaphorase staining of the brain using NBT (S. W. Davies, personal communication). Similarly the failure of SKF 525A to affect the rate of reduction of cytochrome *c* demonstrates that cytochrome P_{450} has no role in the reduction reaction.

Despite our detailed investigations, the function of brain NADPH-diaphorase and the identity of its natural substrate(s) remain unknown. It is also not yet certain whether NADPH-diaphorase activity detected histochemically is synonymous with the activity studied here biochemically. This question will shortly be resolved immunocytochemically, using an antibody we have raised to the purified enzyme.

We have, however, been struck by a number of similarities between the characteristics we have determined for NADPH-diaphorase and those reported for the hepatic enzyme, cytochrome P_{450} reductase (EC 1.6.2.4), a component of the mixed function oxidase system for detoxification of xenobiotics (Table 1). The cytochrome P_{450} hydroxylation system is almost certainly present in the central nervous system and cytochrome P_{450} and the attendant reductase enzyme have been neuronally localised by immunocytochemistry [16]. It has not been extensively studied, probably because its role is assumed to be minor. It is, however, noteworthy that the brain contains approximately 33% of the activity of the liver [17], while being protected from many xenobiotics by the blood–brain barrier.

We are, therefore, suggesting that the brain enzymes, NADPH-diaphorase and cytochrome P_{450} reductase, may be identical. However, the presence of a very similar enzyme cannot be ruled out.

In HD, where there is massive striatal neuronal loss (possibly linked to an endogenous toxic agent), NADPH-diaphorase positive neurons are spared. If this enzyme is indeed cytochrome P_{450} reductase or of similar function, then one might speculate that neurons displaying a relatively enhanced enzyme activity would be better able to withstand toxic cell damage.

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Table 1. Comparison of rat NADPH-diaphorase and cytochrome P_{450} reductase

	Diaphorase	Cytochrome P_{450} reductase	Ref.
(i) Microsomal membrane bound	+	+	[18]
(ii) High cytochrome *c* affinity	+	+	[18]
(iii) K_m cytochrome *c*	1.3 μM	2 μM	[10]*
(iv) FMN:FAD:subunit	1:1:1	1:1:1	[19] [11]
(v) Subunit M_r	73,000	68,000 79,500	[20]† [17]†
(vi) K_i 2′ AMP‡	8.2 μM	7.9 μM	[10–12]
(vii) K_i NADP‡	7.1 μM	7.5 μM	[10–12]
(viii) pCMB inhibition	+	+	[10, 11]
(ix) Dicoumarol inhibition	−	−	[10, 12]

* Value for hepatic enzyme.
† Several other studies report intermediate values.
‡ Value for specific conditions.

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