Purification and Characterization of NADPH–Cytochrome *c* Reductase from the Midgut of the Southern Armyworm (*Spodoptera eridania*)

By Daune L. CRANKSHAW, Krystyna HETNARSKI and Christopher F. WILKINSON
Department of Entomology, Cornell University, Ithaca, NY 14853, U.S.A.

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1. NADPH–cytochrome *c* reductase was solubilized with bromelain and purified about 400-fold from sucrose/pyrophosphate-washed microsomal fractions from southern armyworm (*Spodoptera eridania*) larval midguts. 2. The enzyme has a mol.wt. of 70035 ± 1300 and contained 2 mol of flavin/mol of enzyme consisting of almost equimolar amounts of FMN and FAD. 3. Aerobic titration of the enzyme with NADPH caused the formation of a stable half-reduced state at 0.5 mol of NADPH/mol of flavin. 4. Kinetic analysis showed that the reduction of cytochrome *c* proceeded by a Bi Bi Ping Pong mechanism. 5. Apparent K_m values for NADPH and cytochrome *c* and K_i values for NADP^+ and 2'-AMP were considerably higher for the insect reductase than for the mammalian liver enzyme. 6. These are discussed in relation to possible differences in the active sites of the enzymes.

NADPH–cytochrome *c* reductase (EC 1.6.2.4) is an integral part of the microsomal electron-transport pathway and as such plays an important role in the cytochrome *P*-450-mediated oxidation of drugs, insecticides and other lipophilic foreign compounds in mammals (Brodie *et al.*, 1958; Conney, 1967; Nakatsugawa & Morelli, 1976; Lu *et al.*, 1976), insects (Agosin & Perry, 1974; Wilkinson & Brattsten, 1972) and other species (Terrier, 1968; Bend *et al.*, 1977).

The enzyme was first isolated in a membrane-bound form from pig liver (Horecker, 1950), but has subsequently been solubilized from hepatic microsomal fractions of several species and characterized as a flavoprotein capable of reducing cytochrome *c*, several dyes, ferricyanide and quinones, as well as cytochrome *P*-450 (Williams & Kamin, 1962; Masters *et al.*, 1965*b*; Glazer *et al.*, 1971; Iyanagi *et al.*, 1978). The mechanism of electron transfer by NADPH–cytochrome *c* reductase has been described by several workers (Masters *et al.*, 1965*a,b*, 1975; Iyanagi & Mason, 1973; Dignam & Strobel, 1977; Iyanagi *et al.*, 1978).

Solubilization of the enzyme from the hepatic microsomal membranes has been effected with a variety of proteinases (Phillips & Langdon, 1962; Masters *et al.*, 1967; Pederson *et al.*, 1973) and detergents (Iyanagi & Mason, 1973; Vermilion & Coon, 1974; Yasukochi & Masters, 1976; Dignam & Strobel, 1975, 1977). Subsequent purification procedures employing Sephadex gel, ion-exchange or affinity chromatography have yielded electrophoretically

homogeneous enzyme preparations purified 150–200-fold, with specific activities of 30–69 units/mg of protein. The purified enzyme contains equimolar amounts of FMN and FAD, and molecular weights reported range from 57 700 to 71 000 with the proteinase-solubilized material and from 78 000 to 80 000 with the detergent-solubilized enzyme. Only the latter form can be successfully reconstituted with soluble cytochrome *P*-450 and phospholipid to support oxidase activity (Levin, 1977; Estabrook, 1978; Coon, 1978).

In contrast with the numerous reports on the NADPH–cytochrome *c* reductase from mammalian liver, information on the enzyme from insect tissues is limited. The reductase has been solubilized from housefly (*Musca domestica*) microsomes by butan-2-ol (Wilson & Hodgson, 1971 *a,b*) and detergent (Capdevila *et al.*, 1975) treatment and was purified about 40-fold in each case. The butan-2-ol-solubilized enzyme was shown to be a flavoprotein with a mol.wt. of approx. 57 000. A lipase-extracted housefly reductase has recently been purified 400-fold; it reportedly contains equal molar concentrations of the flavins FMN and FAD, and has a mol.wt. of 65 000–70 000 (Mayer & Prough, 1977).

In the course of studies on the microsomal enzymes in the midgut tissues of larvae of the southern armyworm (*Spodoptera eridania*) and other lepidopterous species, we have attempted to solubilize and purify the major microsomal electron-transport components. This paper describes the purification and partial characterization of the proteinase-solubilized NADPH–cytochrome *c* reductase from the southern armyworm larval midgut and for comparative pur-

Abbreviation used: SDS, sodium dodecyl sulphate.

poses also includes data on the enzyme obtained from rat liver.

Experimental

Chemicals

Bovine serum albumin was obtained from Pentax Biochemicals, and egg albumin from Miles Laboratories, both of Kankakee, IL, U.S.A. Phosphorylase *a*, carbonic anhydrase, cytochrome *c* (type VI), NADPH (type III), NADP⁺, NADH, Coomassie Blue R, Coomassie Blue G, 2,6-dichlorophenol-indophenol, FAD, FMN (grade III), D-amino acid oxidase, phenylmethanesulphonyl fluoride, poly(vinylpyrrolidone) (PVP-10), *NN*-methylenebisacrylamide, glycine, SDS, mersalyl, menadione, *p*-hydroxymercuribenzoate and D-phenylglycine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose was obtained from Whatman, Clifton, NJ, U.S.A., Sephadex G-100 from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A., and 2-mercaptoethanol from Bio-Rad Laboratories, Rockville Centre, NY, U.S.A. Neotetrazolium chloride was purchased from Calbiochem, La Jolla, CA, U.S.A., and 2'-AMP from P-L Biochemicals, Milwaukee, WI, U.S.A. All other solvents and chemicals employed were of analytical reagent grade.

Animals

Southern armyworm (*Spodoptera eridania*) larvae were fed on bean plants through the first five instars and were maintained under greenhouse conditions as described previously (Krieger & Wilkinson, 1969). The sixth-instar larvae used as the source of the microsomal preparations were kept in an environmental chamber maintained at a temperature of 26°C, with 16 h light and 8 h dark, and 50–60% relative humidity.

Male rats of Sprague–Dawley descent (about 200 g) were purchased from Blue Spruce Farms, Altamont, NY, U.S.A. Induction of armyworm larvae and rats with pentamethylbenzene and phenobarbital respectively was carried out as described previously (Crankshaw *et al.*, 1979).

Enzyme preparations

Armyworm midgut microsomal fractions and those from rat liver were isolated, stored and washed as described previously (Crankshaw *et al.*, 1979). Protein concentrations were determined by the method of Lowry *et al.* (1951) or by that of Bensadoun & Weinstein (1976), with bovine serum albumin as standard.

Anaerobic proteolytic digestion was effected, under N₂ to prevent lipid peroxidation, by gentle agitation of the washed microsomal suspensions (10 mg of protein/ml in 0.05 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA) at 0–4°C with bromelain at either 15 µg/mg of microsomal protein for 3 h (rat liver) or

5 µg/mg of microsomal protein for 2 h (armyworm gut). The resulting digests were centrifuged at 105 000g for 60 min in an International Equipment Co. (IEC) B-60 ultracentrifuge with an IEC A-211 fixed-angle rotor, and the supernatants were concentrated to a volume of less than 10 ml in an Amicon ultrafiltration cell with a PM-30 Diaflo membrane (Amicon Corp., Lexington, MA, U.S.A.).

Concentrated 105 000g supernatants of the bromelain digests (4–9 ml) of rat liver or armyworm gut microsomal preparations were loaded on to a column (2.6 cm × 55 cm for the rat liver preparation and 2.6 cm × 95 cm for the midgut preparation) of Sephadex G-100 equilibrated with 0.05 M-Tris/HCl (pH 8.0; 0–4°C) containing EDTA (1 mM). The column was eluted with the same buffer at a flow rate of 30–40 ml/h, and 1–2 ml fractions were collected. Fractions containing NADPH-cytochrome *c* reductase activity were pooled for ion-exchange chromatography.

The combined reductase-containing fractions from the Sephadex G-100 column were adsorbed on a DEAE-cellulose (DE-52; Whatman) column (1.3 cm × 16 cm) packed and equilibrated with 0.05 M-Tris/HCl (pH 8.0; 0–4°C) containing EDTA (1 mM). Fractions of the rat liver enzyme were eluted with a linear 0–0.5 M-KCl gradient (total volume 150 ml) in the same buffer. Elution of the insect enzyme was effected with a linear 0–0.2 M-KCl gradient (total volume 300 ml) in 0.05 M-Tris/HCl (pH 8.0; 0–4°C) containing EDTA (1 mM) and glycerol (20%) after the loaded column had been washed with 30 ml of the same buffer.

Elate fractions containing reductase activity were combined, diluted 3-fold with 0.05 M-Tris/HCl (pH 8.0; 0–4°C) containing 20% glycerol, and loaded on a second DEAE-cellulose column (1.0 cm × 13 cm) equilibrated with the same buffer containing 0.1 mM-EDTA. The rat liver enzyme was eluted with a 0.15–0.35 M-KCl linear gradient in 0.05 M-Tris/HCl (pH 8.0; 0–4°C)/glycerol (20%)/EDTA (0.1 mM) (total volume 60 ml); the armyworm enzyme was similarly eluted with a linear 0.05–0.15 M-KCl gradient (150 ml). Fractions containing reductase activity were pooled and stored at –15°C.

Enzyme assays

The NADH- or NADPH-mediated reductions of different electron acceptors by the various enzyme preparations were performed as described previously (Crankshaw *et al.*, 1979). One unit of enzyme activity is that which reduces 1 µmol of electron acceptor/min.

Kinetic analyses to obtain *K_m* and *K_i* values employed the standard method of Wilkinson (1961) and utilized the IBM-370 computer at Cornell University.

In microsomal preparations, cytochrome *b₅* was measured from the difference spectra of samples reduced with sodium dithionite by the method of

Omura & Takesue (1970) by using the absorption coefficient $185 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$. After removal of interfering coloured materials by Sephadex-gel chromatography, the cytochrome content of column fractions was determined from the A_{423} of sodium dithionite-reduced samples by using the absorption coefficient $171 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ (Omura & Takesue, 1970).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis employing 0.1% SDS was conducted as described by Laemmli (1970), and the 7.5% gels ($0.4 \text{ cm} \times 10 \text{ cm}$) were fixed in 10% (w/v) trichloroacetic acid overnight and stained for protein with Coomassie Blue R (Johnson *et al.*, 1971). Molecular weights were determined by comparison with known protein standards (Weber & Osborn, 1969) run simultaneously with the enzyme samples. The protein standards were phosphorylase α , bovine serum albumin, egg albumin and carbonic anhydrase. Gels were scanned with an Isco UA-5 Absorbance Monitor and model 659 gel-scanner attachment (Instrument Specialties Co., Lincoln, NE, U.S.A.).

For detection of reductase activity, electrophoresed gels (Davis, 1964) were rinsed (10 min) in 0.05 M-Tris/HCl (pH 8.0)/1 mM-EDTA and transferred to the same buffer containing 0.1 mM-neotetrazolium chloride and 4 mM-NADPH. At room temperature, the purple-coloured band appeared within 15–30 min. A duplicate gel was stained for protein with Coomassie Blue G (Diezel *et al.*, 1972).

Flavin analysis

The total flavin content was determined spectrophotometrically by measuring the A_{450} and employing a flavin absorption coefficient of $11.7 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ (Masters *et al.*, 1975). FAD and FMN were determined by the microfluorimetric method of Faeder & Siegel (1973) and by enzymic analysis using the apo-(D-amino acid oxidase) assay for FAD (Fonda & Anderson, 1967) and apo-(pyridoxamine 5'-oxidase) assay for FMN (Kazarinoff & McCormick, 1975). Standard samples of FMN and FAD were purified by DEAE-cellulose chromatography with 0.1 M-potassium phosphate (pH 6.8) as eluting buffer (Massey & Swoboda, 1963). Fluorimetric analyses were conducted with an Aminco-Bowman spectrophotofluorimeter, and spectrophotometric measurements made with an Aminco-Chance DW-2 spectrophotometer.

Results

Solubilization

Fig. 1 shows the total reductase activity recovered and the degree of solubilization achieved after anaerobic incubation of washed armyworm midgut

and rat liver microsomal fractions (10 mg/ml) with various concentrations of bromelain for 3 h at 0–4°C. In both cases total reductase activity (105 000g pellet plus supernatant) was greater than that measured in the original washed microsomal preparation after incubation with up to about 30 μg of bromelain/mg of protein; optimal total recovery occurred at 5–15 μg . Of the total activity recovered, about 80% of the armyworm midgut reductase was solubilized (present in the 105 000g supernatant) at a bromelain concentration of 5 μg /mg of protein. The rat liver reductase was somewhat more refractory to solubilization with bromelain and, although perhaps not optimal, bromelain was employed in this investigation at a concentration of 15 μg /mg of protein; this resulted in the solubilization of 60–80% of the total reductase activity recovered. The relative ease with which the armyworm midgut reductase was solubilized is indicated by the fact that 27% of the reductase activity (12% of the protein) was recovered in the 105 000g supernatant after a 3 h incubation in the absence of bromelain. Similar treatment of rat liver microsomal preparations resulted in the release of only 2% of the reductase activity (10% of the protein).

Fig. 1. Effect of bromelain concentration on the solubilization of NADPH-cytochrome *c* reductase activity. Washed microsomal preparations (10 mg of protein/ml) were incubated at 0–4°C with the indicated concentration of bromelain. After 3 h the samples were centrifuged at 105 000g for 1 h and NADPH-cytochrome *c* reductase activity was measured in the pellet and the supernatant. Activities as percentages of original value are shown for armyworm midgut supernatant (○) and pellet+supernatant (●) and for rat liver supernatant (△) and pellet+supernatant (▲).

Solubilization of the armyworm midgut reductase occurred rapidly in the presence of 5 μ g of bromelain/mg of protein, about 70% of the reductase activity (27% of the protein) being solubilized during the 90min centrifugation period without preincubation. Preincubation of the microsomal fraction with bromelain for 2h yielded an additional 10% of the reductase activity and 5–10% of the protein in the soluble fraction. The rat liver enzyme was solubilized at a lower rate, with a 3h preincubation with bromelain (15 μ g/mg of protein) being required to release 80% of the reductase activity.

Purification

Purification of the reductase from concentrated supernatants of bromelain-treated armyworm midgut and rat liver microsomal preparations was effected by passage through Sephadex G-100 and DEAE-cellulose (DE-52) columns as described in the Experimental section. The results summarized in Table 1 clearly show that with the purification procedure employed both reductases behaved quite similarly with respect to final yield and specific activity. Relative to its activity in washed microsomal preparations, the armyworm midgut reductase was purified 405-fold to give a 33.3% yield of enzyme, exhibiting a specific activity of 40.5 units/mg of protein (Table 1).

The elution profiles from Sephadex G-100 (Figs. 2a and 2b) are quite similar for both the armyworm midgut and rat liver preparations. In each case NADPH-cytochrome *c* reductase was clearly separated from cytochrome *b*₅, which was retained for a longer time on the column. At this stage of the purification the reductase-containing fractions appeared quite clear with only a faint trace of yellow coloration.

The reductase-rich fractions eluted from Sephadex G-100 adsorbed on DEAE-cellulose as a narrow yellow band. Elution with a linear 0–0.5M-KCl gradient (150ml) (Figs. 2c and 2d) followed by elution of the enzyme-containing fractions through a second DEAE-cellulose column with a linear 0.1–0.35M-KCl gradient yielded an apparently homogeneous rat liver reductase, as indicated by both native and SDS/polyacrylamide-disc-gel electrophoresis. In contrast with the rat liver NADPH-cytochrome *c* reductase, which was eluted from the DEAE-cellulose columns at 0.27M-KCl, the armyworm midgut enzyme was eluted at 0.09M-KCl (peak fractions). After following a procedure identical with that described for the rat liver enzyme, the peak armyworm reductase fractions from the second DEAE-cellulose column contained one major and several minor protein bands according to native polyacrylamide-disc-gel electrophoresis. The major protein band coincided with a single band observed in gels incubated with NADPH and neotetrazolium chloride for detection of reductase activity. The presence of impurities in this preparation was further suggested by the fact that the specific activities of the peak fractions eluted from DEAE-cellulose were slightly lower than those in fractions to one side of the peak.

Conditions yielding an almost homogeneous midgut reductase were achieved by increasing the length of the Sephadex G-100 column from 55 to 95cm and eluting the reductase from the DEAE-cellulose columns with longer linear KCl gradients, 0–0.2M-KCl (300ml) for the first column and 0.5–0.15M-KCl (150ml) for the second. With these lower salt gradients only a small amount of the coloured materials adsorbed on the first DEAE-cellulose column was eluted with the peak reductase fractions; the remainder was

Table 1. Summary of purification procedures for rat liver and armyworm NADPH-cytochrome *c* reductase
All procedures were conducted as described in the Experimental section. Values in parentheses indicate percentage recovery. Purification ratio is the specific activity after a purification step divided by that of the microsomal fraction.

Step	Microsomal preparations							
	Rat liver				Armyworm midgut			
	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification ratio	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification ratio
Microsomal fraction	164 (100)	86.8 (100)	0.53	1	600.8 (100)	59.0 (100)	0.10	1.0
Solubilized supernatant	60 (37)	75.2 (86.6)	1.21	2.3	201.2 (33.5)	62.0 (105)	0.31	3.1
Sephadex	7.0 (4.3)	51.2 (58.9)	11.97	22.6	20.4 (3.4)	42.4 (71.9)	2.1	21.0
DEAE-cellulose	1.3 (0.79)	40.9 (47.2)	32.0	60.4	3.8 (0.63)	28.6 (48.4)	7.5	75.0
DEAE-cellulose	0.37 (0.23)	20.7 (23.9)	56.1	144	1.05 (0.17)	19.7 (33.3)	40.5	405

Fig. 2. Elution profiles for Sephadex G-100 gel filtration (a and b) and DEAE-cellulose ion-exchange chromatography (c and d) of bromelain-solubilized rat liver (a and c) and armyworm midgut (b and d) NADPH-cytochrome *c* reductase. All procedures were carried out as described in the Experimental section. $\circ$, A_{280} ; Δ , reductase activity; $\square$, cytochrome b_5 concentration; ----, KCl.

eluted at higher salt concentrations but contained no reductase activity. There was some indication that elution through a second Sephadex G-100 column before DEAE-cellulose chromatography resulted in a slightly purer final product, though in most cases this step was not included in the procedure employed.

When stored at -15°C the armyworm midgut reductase eluted from the second DEAE-cellulose column retained 80% or more of its activity for over a year and was stable for months at $0-4^{\circ}\text{C}$.

Scans of SDS/polyacrylamide disc gels of the purified armyworm midgut reductase (Fig. 3) indicate a shoulder on the main protein band; this leads to a somewhat asymmetric protein band on gels run at relatively high enzyme concentrations ($40\mu\text{g}$).

The microsomal fraction of armyworm midgut is able to utilize both NADPH and NADH for the

reduction of various electron acceptors, such as cytochrome *c*, ferricyanide and 2,6-dichlorophenol-indophenol. A comparison of the NADPH- and NADH-mediated reductions of these acceptors measured in microsomal fractions with those catalysed by the purified NADPH-cytochrome *c* reductase (Table 2) clearly shows that only the material with NADPH-mediated activity co-purifies with the reductase.

Physical properties

The molecular weight of the purified armyworm midgut reductase was determined to be 70035 ± 1300 on the basis of its mobility relative to the standards phosphorylase *a*, bovine serum albumin, egg albumin and carbonic anhydrase on 0.1% SDS/polyacrylamide-gel electrophoresis. In agreement

Fig. 3. SDS/polyacrylamide-gel electrophoresis of purified armyworm midgut NADPH-cytochrome *c* reductase. Samples containing 40 μ g (a) and 8 μ g (b) of protein were electrophoresed in the presence of 0.1% SDS as described in the Experimental section.

with a previous report (Pederson *et al.*, 1973), the molecular weight of the bromelain-solubilized rat liver reductase was found to be $71\,000 \pm 500$ under similar electrophoretic conditions.

Activity of the purified armyworm midgut reductase increased linearly as a function of protein concentra-

tion over the range 0.05–1.8 μ g, and 0.4–0.6 μ g was employed in routine assays. The pH profiles of the purified enzymes from rat liver and armyworm midgut are quite similar to one another (Fig. 4), both enzymes exhibiting a sharp increase in activity as the pH of 0.3M-potassium phosphate buffer is increased from 6.0 to 7.5 and thereafter maintaining a rather broad optimum up to pH 9.0.

In response to increasing ionic strength (NaCl in 0.05M-Tris/HCl), the activity of the purified rat liver reductase increased by approx. 70% as the concentration of NaCl was increased from 0 to 0.4M. Over a similar range of salt concentration the armyworm midgut reductase exhibited only a 15–20% increase in activity.

Within the range of 7–36°C both the rat liver and armyworm midgut reductases exhibited a linear increase in the rate of reduction of cytochrome *c*, and Arrhenius plots of activity data obtained over this temperature range are shown in Fig. 5. Activation energies were calculated to be 31.65 kJ/mol for the armyworm midgut reductase and 60.16 kJ/mol for the rat liver enzyme. At temperatures above 36°C thermal inactivation of both enzymes occurred, activity at 47°C being only about 50% of that measured at 36°C.

Kinetic analysis

The interaction of the midgut reductase with NADPH and cytochrome *c* was analysed kinetically by varying the concentration of NADPH (12.9–258 μ M) at various fixed concentrations of cytochrome *c* ranging from 23 to 143 μ M. Double-reciprocal plots of the rate of cytochrome *c* reduction versus NADPH

Fig. 4. Effect of pH on NADPH-cytochrome *c* reductase activity

Reductase assays with the purified armyworm midgut (○) and rat liver (△) enzymes were conducted in 0.3M-potassium phosphate buffer at the pH values indicated as described in the Experimental section.

Table 2. Electron donor and acceptor specificity of NADPH-cytochrome c reductase

All procedures were carried out as described in the Experimental section. Abbreviation: DCPIP, dichlorophenol-indophenol. Purification ratio is the specific activity of the purified preparation relative to that of the microsomal fraction.

Donor	Acceptor	Specific activity (units/mg of protein)		
		Microsomal fraction	Purified preparation	Purification ratio
NADPH	Cytochrome c	0.148	45.50	307
NADH	Cytochrome c	0.189	0.44	2
NADPH	Ferricyanide	0.410	43.10	105
NADH	Ferricyanide	0.794	8.80	11
NADPH	DCPIP	0.115	12.70	110

Fig. 5. Effect of temperature on NADPH-cytochrome c reductase activity
Assays of the purified armyworm midgut (0.55 μg/ml) (○) and rat liver (0.38 μg/ml) (Δ) reductases were conducted at temperatures ranging from 7 to 47°C, as described in the Experimental section.

Fig. 6. Double-reciprocal plots of armyworm midgut NADPH-cytochrome c reductase activity with various NADPH and cytochrome c concentrations
Assays were conducted as described in the Experimental section, employing cytochrome c concentrations of 23.75 (●), 47.5 (□), 95 (Δ) and 142.5 μM (○).

concentration at each concentration of cytochrome c yielded a series of parallel lines (Fig. 6), suggesting that the interaction of NADPH and cytochrome c with the reductase occurs through a Bi Bi Ping Pong reaction mechanism (Masters *et al.*, 1965a; Wilson & Hodgson, 1971b; Mayer & Prough, 1977). A double-reciprocal plot of the V_{max} , observed at each cytochrome c concentration against cytochrome c concentration yielded a straight line from which an apparent K_m of $29.8 \pm 9.5 \mu M$ (mean $\pm$ S.E.M., $n = 3$) was measured for cytochrome c. Lineweaver-Burk plots of data obtained with various concentrations of cytochrome c at a saturating concentration of NADPH gave an apparent K_m value for cytochrome c of $34.1 \pm 1.9 \mu M$, and a K_m of $46.6 \pm 4.2 \mu M$ was determined for NADPH when assayed with a fixed concentration of cytochrome c. The apparent K_m values

for the purified rat liver reductase, obtained by a similar Lineweaver-Burk analysis, were 6.3 ± 1.0 and $13.1 \pm 1.1 \mu M$ for NADPH and cytochrome c respectively.
Analysis of data for inhibition of the midgut reductase by NADP⁺ and 2'-AMP gave K_i values of 15 and 201 μM respectively the inhibition in each case being competitive, as illustrated in Fig. 7 for 2'-AMP. These data were obtained by using 0.05M-phosphate buffer

Fig. 7. Double-reciprocal plot for inhibition of armyworm midgut NADPH-cytochrome *c* reductase activity by 2'-AMP

Assays were conducted as described in the Experimental section (except with 0.05M-potassium phosphate buffer) with cytochrome *c* in the presence (Δ) or absence (○) of 0.1 mM-2'-AMP.

rather than the usual 0.3M to facilitate their comparison with previously reported values (Phillips & Langdon, 1962; Mayer & Prough 1977).

The kinetic parameters for the armyworm midgut reductase are summarized in Table 3, where they are compared with those obtained for reductases purified from other sources. For the armyworm midgut reductase, K_m values for NADPH and cytochrome *c* are approx. 10-fold greater than those measured for the enzyme from mammalian liver and 1.5–3-fold greater than those reported for a partially purified reductase from housefly abdomens. A similar trend can also be seen with K_i values for 2'-AMP and NADP⁺, which are substantially greater with the two insect-derived reductases than with those from mammalian liver.

The purified reductase was inhibited by micromolar concentrations of the mercurials, *p*-hydroxymercuribenzoate and mersalyl, and the presence of mercaptoethanol or NADPH provided only slight protection against inhibition by the former (Table 4). Two known inhibitors of microsomal drug metabolism, 4,5-dichloro-1,2-methylenedioxybenzene and 1-phenylimidazole, did not inhibit the reduction of cytochrome *c* by the purified midgut reductase. At

Table 3. Summary of data for NADPH-cytochrome *c* reductase

Enzyme source	K_m (μM)		K_i (μM)	
	NADPH	Cytochrome <i>c</i>	2'-AMP	NADP ⁺
Pig liver*	3.8	5.4	—	2.0
Pig liver†	3.4	2.6	54	4.8
Housefly‡	23.5	16.6	640	14.0
Armyworm midgut	32.1	46.6	201	15.0

* Williams & Kamin (1962).

† Phillips & Langdon (1962).

‡ Mayer & Prough (1977).

Table 4. Inhibition of armyworm midgut NADPH-cytochrome *c* reductase

Assays were conducted as described in the Experimental section.

Inhibitor	Concentration (M)	Percentage of original activity
<i>p</i> -Hydroxymercuribenzoate	2×10^{-6}	100
	4×10^{-6}	44.7
	6×10^{-6}	16.2
	4×10^{-6} + mercaptoethanol (2×10^{-5})	67.9
	4×10^{-6} + NADPH (9×10^{-5})	59.1
Mersalyl	1×10^{-5}	61.1
	1×10^{-4}	0
4,5-Dichloro-1,2-methylenedioxybenzene	1×10^{-3}	110.0
1-Phenylimidazole	1.25×10^{-3}	102.4
Menadione	1×10^{-3}	113.7
Alizarin	1×10^{-3}	53.5

Fig. 8. Absolute absorption spectrum of armyworm midgut NADPH-cytochrome *c* reductase

The oxidized (A) and reduced (excess NADPH) (B) absorption spectra of the enzyme (6.8 nmol in 0.05M-Tris/HCl, pH 8.0, containing 1 mM-EDTA and 20% glycerol) are shown in (a). The inset (b) shows absorption spectra obtained during the aerobic titration of the reductase with NADPH. Spectrum A is that of the oxidized enzyme, B was obtained after addition of 3.16 nmol of NADPH, C after addition of excess (>4.7 nmol) NADPH and D is the spectrum of the semiquinone form of the enzyme obtained by allowing air oxidation after addition of excess NADPH. See the text for full explanation.

1 mM the quinone alizarin, which has also been shown to inhibit microsomal oxidase activity (Desmarchelier *et al.*, 1973), inhibited reductase activity by 53%. In contrast, another quinone, menadione, had a slight stimulatory effect on reductase activity at 1 mM.

Flavin analysis

The absolute spectrum of the purified midgut reductase (Fig. 8) exhibited absorbance maxima of 287, 385 and 455 nm. Aerobic reduction of the preparation with NADPH led to a decrease in the absorbance of the 385 and 455 nm peaks and an increase in absorbance at wavelengths above the isosbestic point of 500 nm (Figs. 8 and 9), suggesting that the semiquinone of the flavin is formed during the titra-

tion (Beinert, 1956). No further change in absorbance at 455 or 585 nm was observed above a concentration of 4.75 nmol of NADPH/10.7 nmol of flavin, a ratio of approx. 0.5 reducing equiv./mol of flavin (Fig. 9). After full aerobic reduction of the enzyme had been achieved by addition of excess NADPH (line C, Fig. 8) the gentle bubbling of O_2 through the sample caused some reoxidation of the reduced flavin (line D, Fig. 8), but failed to restore it to fully oxidized state.

From the absorbance at 450 nm and the absorption coefficient of $11.7 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ (Masters *et al.*, 1975), the total flavin content of preparations of the purified enzyme was determined to be 25.6 nmol/mg of protein or 1.8 mol of flavin/ml of enzyme. The species of flavin present in the midgut reductase was

examined by spectrofluorimetric and enzymic analysis of flavin extracts. By using the fluorimetric method of Faeder & Siegel (1973), which utilizes heat to extract the flavin from the enzyme, both FMN and FAD were measured in almost equal amounts (56 and 44% respectively) (Table 5). The sum of the FMN and

Fig. 9. Aerobic titration of armyworm midgut NADPH-cytochrome *c* reductase with NADPH

Absorbance changes at 455 (○), 500 (□) and 585 (△) nm are plotted against the concentration of NADPH added. The titration was conducted with 0.48 mg of purified enzyme (6.8 nmol, 11.9 nmol of flavin) in 0.05 M-Tris/HCl (pH 8.0)/1 mM-EDTA/20% glycerol, with 3 μ l portions (1.58 nmol) of NADPH.

FAD extracted and measured by this method accounted for 93% of the total flavin determined spectrophotometrically (450 nm absorbance) in the enzyme sample employed. Recoveries of FMN and FAD standards subject to exactly the same procedure were 110 and 70% respectively (Table 5), and fluorimetric assay of a heat-derived flavin extract of the enzyme D-amino acid oxidase indicated the presence of only FAD in a yield of 73% of the flavin initially measured spectrophotometrically (450 nm). If the FAD values determined in the reductase extract are corrected for the 30% loss observed with the standard FAD and with D-amino acid oxidase, the final FMN/FAD ratio is almost exactly 1:1.

Attempts to confirm the presence of both FMN and FAD in the purified reductase preparation were made by measuring the ability of neutralized acid flavin extracts of the flavoprotein to restore enzyme activity to the apoenzymes of D-amino acid oxidase (FAD-specific) and pyridoxamine 5'-oxidase (FMN-specific). Although this method confirmed the presence of both flavins in the reductase extract, the ratio of FAD/FMN was estimated to be 1:2. The apparent discrepancy between these results and those obtained fluorimetrically may in part reflect the known lability of FAD even under mildly acidic conditions.

Discussion

The flavoprotein NADPH-cytochrome *c* (P-450) reductase was purified 405-fold after solubilization with bromelain of pyrophosphate-washed microsomal fractions from southern armyworm (*Spodoptera eridania*) larval midguts. Since the pyrophosphate

Table 5. Flavin analysis of the midgut NADPH-cytochrome *c* reductase

Samples of purified standard flavins (12.5 nmol/ml consisting of equal amounts of FMN and FAD), D-amino acid oxidase (2.23 mg/ml, 15.75 nmol of flavin/ml) and purified armyworm midgut reductase (1.3 mg/ml, 36.67 nmol of flavin/ml) were heat-extracted and assayed fluorimetrically for FMN and FAD as described in the Experimental section. Enzymic analysis was conducted on a neutralized acid extract of the purified reductase (0.33 mg/ml) as described in the Experimental section. Abbreviation: N.D., not detected.

	Flavin employed* (nmol/ml)	Flavin recovered (nmol/ml)		FAD/FMN ratio
	FAD+FMN	FAD	FMN	
Fluorimetric assay				
Standards FMN		—	6.90	—
	12.50	4.58	—	—
FAD		11.2	N.D.	—
D-Amino acid oxidase	15.75	—	—	—
Reductase	36.67	33.99	—	—
	—	14.85	19.14	1:1.3
Enzymic assay				
Reductase	8.4	1.9	3.8	1:2

* Determined spectrophotometrically by method of Faeder & Siegel (1973). Expressed in terms of nmol/ml of original sample volume.

wash usually leads to a 20–30% increase in specific activity of the microsomal reductase (Crankshaw *et al.*, 1979), the overall purification factor relative to activity in unwashed microsomal fraction is about 500. The specific activity of the purified insect midgut preparation (40.5 μ mol of cytochrome *c* reduced/min per mg of protein) is only slightly lower than that of the enzyme purified from rat liver by a similar procedure (Table 1), and the latter in turn concurs with values reported elsewhere for the purified mammalian liver enzyme (Pederson *et al.*, 1973; Iyanagi & Mason, 1973; Masters & Ziegler, 1971; Omura & Takesue, 1970).

The only previous attempts to purify NADPH-cytochrome *c* reductase from insect tissues have been with whole houseflies (*Musca domestica*), where various degrees of enzyme purification have been reported after treatment of microsomal fractions with butan-2-ol (Wilson & Hodgson, 1971a), Triton X-100 (Capdevila *et al.*, 1975) and pancreatic lipase (Mayer & Prough, 1977). Of these, only the latter succeeded in achieving a high degree of purification (460-fold) and in producing a final preparation with a specific activity of 25–30 μ mol of cytochrome *c* reductase/min per mg of protein (calculated from results provided). Purification factors of the preparations obtained by butan-2-ol and Triton X-100 treatments were 44 and 35 respectively.

The solubilization and purification procedure employed in this study is very similar to that previously found satisfactory for the rat liver enzyme; it provided an almost homogeneous enzyme protein as judged by high-resolution polyacrylamide-gel electrophoresis (7.5% gels containing 0.1% SDS). The purified armyworm midgut reductase had a mol.wt. of 70035 ± 1300 . This is in close agreement with the 71000 ± 500 obtained in this study for the purified rat liver enzyme and agrees with the values of 68000–71000 reported for other proteinase-solubilized reductases from mammalian liver (Williams & Kamin, 1962; Masters & Ziegler, 1971; Pederson *et al.*, 1973; Iyanagi & Mason, 1973). It is somewhat higher than the reported molecular weights of the enzyme purified from butan-2-ol (57000) (Wilson & Hodgson, 1971a) and lipase (68000) (Mayer & Prough, 1977) extracts of houseflies, although this does not necessarily indicate any significant differences in the enzymes. It is well known that different active forms of the enzyme are released from the microsomal membranes by various classes of solubilizing agents and in addition it is probable that whole-insect preparations of this type contain endogenous proteinases, which catalyse the uncontrolled proteolytic solubilization of the reductase during the early stages of purification (Wilkinson & Brattsten, 1972). This problem was encountered during the initial phases of this study and led to the routine inclusion of phenylmethanesulphonyl fluoride (0.5 mM) in the media

during tissue dissection and preparation of the microsomal fractions (Crankshaw *et al.*, 1979). More meaningful comparisons of molecular weight will be possible only after purification of the native forms of the reductases obtained by detergent solubilization. The results of such studies with the mammalian liver enzyme indicate a native mol.wt. of 75000–80000 (Iyanagi & Mason, 1973; Vermilion & Coon, 1974; Dignam & Strobel, 1975, 1977; Yasukochi & Masters, 1976; Knapp *et al.*, 1977; Iyanagi *et al.*, 1978), but no results are yet available for a detergent-solubilized insect reductase.

In agreement with the results obtained with the enzymes isolated and purified from mammalian liver (Iyanagi & Mason, 1973; Vermilion & Coon, 1974; Masters *et al.*, 1975, 1976; Dignam & Strobel, 1977) and the housefly (Mayer & Prough, 1977), the purified armyworm midgut reductase contains approx. 2 mol of flavin/mol of enzyme, which from fluorimetric determinations is made up of nearly equimolar concentrations of FAD and FMN. Plots of the aerobic titration of the purified enzyme with NADPH (Fig. 9) are identical with those reported for the mammalian liver reductase (Masters *et al.*, 1975; Iyanagi *et al.*, 1978; Vermilion & Coon, 1978) and indicate the formation of a stable half-reduced state (semiquinone). Formation of the half-reduced state can be observed spectrophotometrically by the increase in absorbance at 585 nm. In addition to its formation after addition of 0.5 mol of NADPH/mol of flavin to the fully oxidized enzyme, it is produced after addition of excess NADPH to the enzyme aerobically and allowing air oxidation to proceed until a stable partially reoxidized spectrum is observed (Fig. 8). In view of these apparent similarities, it is probable that the mechanism of reduction of both one- and two-electron acceptors by the armyworm midgut reductase is similar to that proposed for the mammalian liver enzyme, i.e. through a redox cycle between the fully reduced and 'half-reduced' forms of the enzyme (Masters *et al.*, 1965a).

From an enzymic standpoint, the purified armyworm midgut enzyme exhibits properties very similar to those previously described for NADPH-cytochrome *c* reductase purified from other sources. In the presence of NADPH (but not of NADH) it catalyses the reduction of several one- and two-electron acceptors (ferricyanide, 2,6-dichlorophenol-indophenol etc.). Kinetic analyses and the competitive inhibition of the enzyme by 2'-AMP and NADP⁺ indicate that the reduction of cytochrome *c* occurs through the Bi Bi Ping Pong mechanism described by others (Masters *et al.*, 1965a; Wilson & Hodgson, 1971b; Mayer, & Prough, 1977).

Despite these overall similarities there is considerable evidence suggesting that the nature of the active site of the armyworm midgut enzyme may be quite different from that of the enzyme isolated from mam-

malian liver. The apparent K_m of 32 and $46\mu\text{M}$ measured with the armyworm midgut reductase for NADPH and cytochrome *c* respectively (Table 3) are 3–8-fold higher than those determined for the mammalian liver enzyme, both in this laboratory and elsewhere (Williams & Kamin, 1962; Phillips & Langdon, 1962; Fan & Masters, 1974; Dignam & Strobel, 1977). This suggests that the affinity of the armyworm midgut reductase for NADPH and cytochrome *c* is substantially lower than that of the enzyme from mammalian liver. The corresponding K_m values for the housefly enzyme are also considerably greater than those for the mammalian liver enzyme, though lower than those measured with the armyworm midgut preparation.

A similar trend is apparent on comparing the K_i values for the competitive inhibitors 2'-AMP and NADP⁺ with the various enzymes (Table 3). Thus apparent K_i values of $15\mu\text{M}$ (NADP⁺) observed for the purified armyworm midgut reductase are about 4-fold greater than those observed with the mammalian liver enzyme and the even higher K_i of $640\mu\text{M}$ measured for 2'-AMP with the purified housefly enzyme led Mayer & Prough (1977) to suggest that in the enzyme from this species the 2'-AMP-binding site was almost non-existent. Certainly the ability of both 2'-AMP and NADP⁺ to bind to and inhibit the insect-derived NADPH-cytochrome *c* reductase is much decreased compared with that observed in the mammalian liver enzyme. This difference is further emphasized by the fact that a detergent-solubilized armyworm midgut reductase failed completely to bind to the same NADP⁺ and 2'-AMP affinity columns, which have been successfully employed to purify the mammalian liver enzyme (D. L. Crankshaw, H. K. Hetnarski & C. F. Wilkinson, unpublished work).

Other differences between the armyworm midgut and mammalian liver reductase relate to their response to changes in ionic strength. In the case of the purified rat liver reductase, an increase in salt (NaCl) concentration from 0 to 0.4M resulted in an approx. 70% increase in cytochrome *c* reduction. In contrast, only a slight increase in the activity of the purified armyworm midgut enzyme was observed over this same range of salt concentration. A similar lack of response to changes in ionic strength has been reported previously for the housefly enzyme (Wilson & Hodgson, 1971*b*; Mayer & Prough, 1977), suggesting that it might be characteristic of reductases isolated from insect tissues.

The almost 2-fold difference in the activation energies of the armyworm midgut (31.65 kJ/mol) and rat liver (60.16 kJ/mol) reductases further suggests the existence of real differences at the active sites of the enzymes. This is of considerable interest from the viewpoint of biochemical evolution, since it suggests that in the insect, a poikilothermic organism, the

reductase is adapted in a way that will allow it to function more efficiently under variable ambient temperatures, which are usually lower than those maintained in warm-blooded animals. Precisely how this is accomplished remains unknown, but since a lower activation energy implies that less energy is required for formation of the enzyme-substrate complex, it could well be associated with structural modifications of the protein leading to changes in substrate binding etc. at the active site. Whether such changes relate in any way to the major physiological role of the enzyme in cytochrome *P*-450-mediated foreign compound metabolism in insects remains to be established.

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