

Purification and Characterization of Microsomal Cytochrome b_5 and NADH Cytochrome b_5 Reductase from *Pisum sativum*¹

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ABSTRACT

In this communication we document the reproducible protocols for the purification of milligram quantities of cytochrome b_5 and NADH-cytochrome b_5 reductase from the microsomal fraction of *Pisum sativum*. The cytochrome b_5 component of this NADH linked electron transport chain was found to have a molecular mass of 16,400 daltons and the reductase a molecular mass of 34,500 daltons. These components could be reconstituted into a functional NADH oxidase activity active in the reduction of exogenous cytochrome c or ferricyanide. In the latter assay the purified reductase exhibited a turnover number of 22,000 per minute. The amino-terminal amino acid sequence of the cytochrome b_5 component was determined by sequential Edmond degradation, thus providing crucial information for the efficient cloning of this central protein of plant microsomal electron transfer.

The microsomal electron transport systems of mammalian tissue play important roles in a variety of oxidative reactions including fatty acid desaturation and the support of mixed function oxidative biotransformations catalyzed by the Cyt P-450 monooxygenases. Reductive reactions of the latter class involve NADPH dehydrogenation by a flavoprotein, NADPH-Cyt P-450 reductase, containing both FAD and FMN prosthetic groups, followed by sequential one electron transfers to the cytochrome P-450 heme center. In addition, some P-450 biotransformations can be mediated by electron transfer from a short redox transfer chain which links to the dephosphorylated form of pyridine nucleotide, NADH. This system includes NADH-Cyt b_5 reductase, a flavoprotein with a single FAD prosthetic group, and the heme protein Cyt b_5 . Both the NADPH and NADH flavoprotein reductases as well as Cyt b_5 have been purified to homogeneity from a variety of mammalian microsomal fractions and have received intensive study during the past decade (20, 23, 24). Both of these proteins of the NADH linked chain are amphipathic (15) in nature, consisting of two definite domains, a water soluble catalytic portion and a hydrophobic fragment that binds the protein to the microsomal membrane. The catalytic domain can be separated from the holoenzyme by limited proteolytic digestion of the microsomal fraction (9, 21). Alternately, the intact enzyme can be obtained by detergent solubilization (16, 20, 24). Biophysical and biochemical characterizations of the mammalian proteins involved in these mixed function oxidative biotransformations have utilized numerous spectroscopic and structural probes, the primary amino acid sequence of the NADPH Cyt P-450 reductase, NADH-Cyt b_5 reductase, and numerous Cyt b_5

have been determined, and a high resolution x-ray structure of the soluble fragment of bovine Cyt b_5 is available (14). In terms of molecular biological characterization of these electron transport systems, a clone of the gene coding for rat liver NADPH Cyt P-450 reductase and Cyt b_5 have been obtained, but no success at cloning the complete NADH Cyt b_5 reductase gene has been realized. A totally synthetic gene coding for rat liver Cyt b_5 has been constructed in our laboratory and this protein expressed as 10% of total *Escherichia coli* cell protein (22).

Unfortunately, much less is known about the corresponding electron transport and P-450 monooxygenase systems from plant tissue. Galle *et al.* (5) have recently purified an NADH-ferricyanide reductase from potato tuber that is apparently involved in the desaturation of oleate to form linoleic acid in higher plants. Two mitochondrial forms of a reductase have also been isolated by Klein and Burke (8), the first of which contained two peptide fragments and was specific for NADH whereas the second was apparently comprised of five polypeptides, displayed a broad pH maxima, and utilized both NADH and NADPH. Efforts at the isolation of a Cyt P-450 reductase from the microsomal fraction of higher plants began with the partial purification from *Catharanthus roseus* nearly 10 years ago (12) and include the recent report by Fujita and Ashi (4) of the purification of an NADPH-dependent P-450 reductase from sweet potato tuber, and the purification from Jerusalem artichoke tubers by Durst and co-workers (1). To date, the only reported microsomal Cyt b_5 characterizations are a roughly 350-fold purification from potato tubers (2), a very recent purification to 30% homogeneity from *C. roseus* microsomes (13), and in the elegant work of Galle and Kader (6) using HPLC.

As an integral part of our on-going concerted effort to establish and characterize a reconstituted system of plant microsomal electron transport and P-450 monooxygenase activities, we report herein the isolation and purification to absolute homogeneity of both Cyt b_5 and NADH Cyt b_5 reductase from detergent solubilized pea seedling microsomes. These two pure protein fractions were reconstituted *in vitro* to generate an efficient NADH dehydrogenase-ferricyanide reductase activity and a Cyt b_5 -dependent Cyt c reductase reactivity. In addition, the existence of a functional 1:1 complex between NADH-Cyt b_5 reductase and Cyt b_5 has been demonstrated, and the flavoprotein reduction rates by NADH as well as the rates for transfer of reducing equivalents between the reductase and Cyt b_5 in the di-protein complex have been determined. Various physical and biochemical properties of the purified, homogeneous proteins are also reported, including molecular mass, pH and ionic strength dependence of the ferricyanide reductase activity, and the kinetic parameters of the Cyt b_5 -dependent Cyt c reductase activity. This report thus represents the first documentation of primary biochemical data, *in vitro* reconstitution of an NADH-specific plant electron transport chain, and biophysical characterizations of NADH-Cyt b_5 reductase and Cyt b_5 from higher plant tissue.

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MATERIALS AND METHODS

Isolation of the Microsomal Fraction from *Pisum sativum*. Pea seedlings were soaked in distilled water overnight, subsequently planted in vermiculite, and grown in the dark. At a height of 5 to 7 cm (typically 72 h of growth) the shoots were carefully separated from the seedling with a razor blade and homogenized in a Waring Blender with 3 volumes of 0.1 M K-phosphate (pH 7.5) containing 20 mM mercaptoethanol, 25% sucrose, 1 mM PMSF, 1 mM EDTA, and 0.5% (w/v) polyclar-AT. Cell debris was separated by a centrifugation at 10,000g for 20 min and the microsomes pelleted by ultracentrifugation at 150,000g for 1 h at 4°C. The microsomal pellet was resuspended using a Potter-Elvehem homogenizer in a buffer containing 20 mM Tris-HCl (pH 8.0), 10% glycerol, 0.5 mM DTT, and 1.0 mM EDTA. Typically, 20 mg of a wet microsomal fraction was obtained from 100 g dry weight of tissue.

Microsomal Solubilization. The microsomal fraction was placed on ice, the protein concentration adjusted to 12.5 mg/ml using the microsomal pellet resuspension buffer, and solubilized with 1% Triton X-100, added dropwise with constant stirring for 1 h. The solubilized protein fraction was then clarified by ultracentrifugation at 150,000g for 1 h at 4°C.

Enzymatic assays. Cyt *b*₅ reductase was assayed according to the method of Mihara and Sato (16) with 0.1 M K-phosphate (pH 7.4) containing 1.0 mM potassium ferricyanide, and the reaction was initiated by the addition of 0.3 mM NADH. The reductase of potassium ferricyanide was monitored by the absorbance change at 420 nm, and activities were calculated from the initial rate using an extinction coefficient of 1020 M⁻¹ cm⁻¹.

Cyt *b*₅ was quantitated using a standard reduced-oxidized spectral assay, wherein the reduced protein was produced by the addition of 0.1 units of purified Cyt *b*₅ reductase and 15 nmol of NADH (7) using an extinction coefficient of 183,000 M⁻¹ cm⁻¹ between 429 nm and 408 nm as described by Estabrook and Werringer (3).

All chemicals were reagent grade obtained from standard suppliers. Chromatography materials including DEAE-Trisacryl, and Aca44 were from LKB. DEAE Sephacel, Sepharose CL6B, and NAD-bound agarose were purchased from Pharmacia. Spectral assays were performed on a Hewlett Packard 8450A diode array spectrophotometer. Kinetic studies were accomplished using a Varian DMS-100 spectrophotometer. Protein determinations were performed using the method of Lowry *et al.* (10).

RESULTS

Purification of Cyt *b*₅. A solubilized microsomal fraction as described in "Materials and Methods" was diluted with two volumes cold, double-distilled water and applied to a DEAE Trisacryl column (2.6 × 8.9 cm), equilibrated in 20 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol, 0.5 mM DTT, 0.4% Triton X-100, 1 mM EDTA. After loading was complete, the column was washed with two bed volumes of the equilibration buffer then eluted with potassium chloride gradient increasing from 0 to 0.4 M (Fig. 1). This step provided clean fractionation of Cyt *b*₅, NADH-specific Cyt *b*₅ reductase, NADPH Cyt P-450 reductase, and a second uncharacterized *b*-type Cyt. Only one of these *b*-type Cyt is reducible by NADH in the presence of purified NADH-Cyt *b*₅ reductase, as characterized by maxima and minima at 424 and 408 nm in a reduced-oxidized difference spectra, and is identified as the authentic Cyt *b*₅ component of this microsomal redox transfer chain. The second *b*-type Cyt is characterized by difference spectra maxima and minima at 426 and 409 nm, respectively, is only reduced by dithionite but not by purified plant or mammalian *b*₅ reductase, and has an unknown physiological function. The protein fraction containing authentic Cyt *b*₅ was found to elute between 0.12 M KCl and

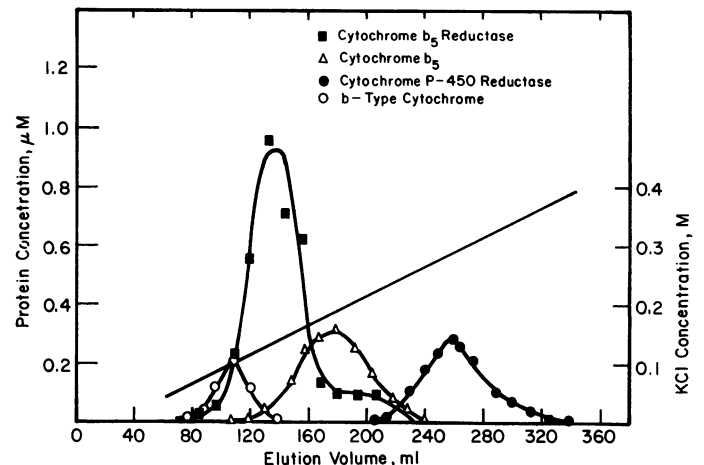


FIG. 1. Elution profile of the first DEAE trisacryl column. The absolute protein concentration in micromolar of Cyt *b*₅ (Δ), NADH-Cyt *b*₅ reductase (■), NADPH-Cyt P-450 reductase (●), and an unknown *b*-type Cyt (○) are plotted versus column elution volume. See text.

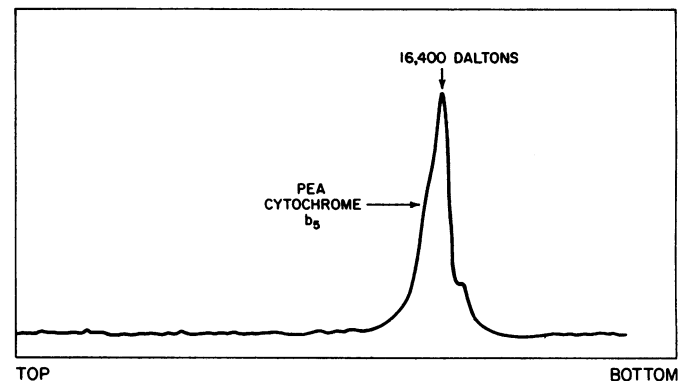


FIG. 2. SDS-PAGE of purified Cyt *b*₅. A densitometric scan of a 14% polyacrylamide gel of the purified Cyt *b*₅ from *P. sativum* is indicated.

0.18 M KCl in the salt gradient, was pooled (about 40 ml) and concentrated by ultrafiltration using an Amicon YM10 filter. This fraction, typically at 30 μM Cyt *b*₅, was then applied to an Aca44 gel filtration column (2.5 × 51 cm) which had been previously calibrated with molecular mass standards. Cyt *b*₅ eluted as an apparent tetramer with a molecular mass of 66,000 D when a buffer of 50 mM Tris-HCl (pH 8.0), containing 0.2% Triton X-100, 1.0 mM EDTA, and 0.5 mM DTT was used. Pooled fractions containing plant Cyt *b*₅ were subsequently applied to a second DEAE Sephacel column (1.2 × 4.4 cm) equilibrated in a buffer identical to the one used for the first Aca44 column. The loaded column was washed with two bed volumes of equilibration buffer and then eluted using an increasing gradient of potassium thiocyanate from 0 to 0.25 M. A second Aca44 gel filtration column, developed in the same buffer as above, except that the Triton X-100 is substituted with 0.5% (w/v) deoxycholate afforded pure, homogeneous Cyt *b*₅ eluting as an apparent dimer with a molecular mass of 33,000 D. Final sample integrity was judged by SDS-PAGE (Fig. 2), isoelectric focusing, N-terminal sequence analysis, and optical spectroscopy (Fig. 3). These steps in the purification of cytochrome *b*₅ are summarized in Table I.

Purification of NADH Cyt *b*₅ Reductase. Fractions eluting from the initial DEAE Trisacryl column between 0.08 and 0.12 M KCl were found to contain NADH-Cyt *b*₅ reductase activity. The pooled fractions from this first ion exchange column were titrated to pH = 6.5 using 1.0 M phosphoric acid then diluted

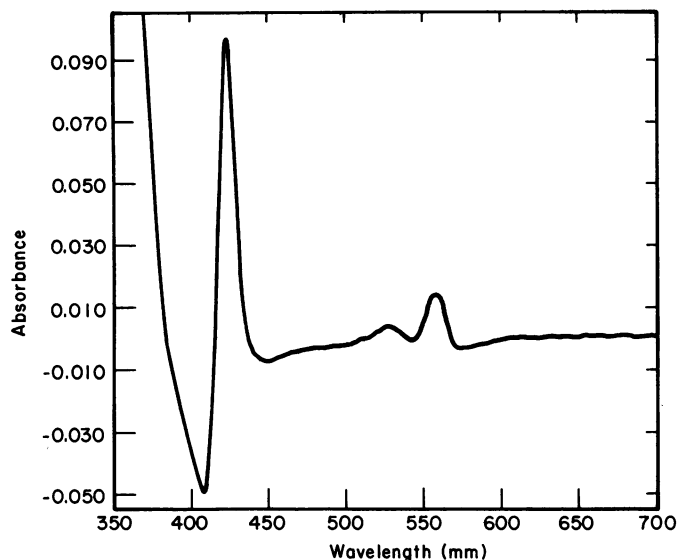


FIG. 3. Reduced-oxidized spectrum of Cyt *b*₅. Reduced protein was obtained using NADH and catalytic concentrations of Cyt *b*₅ reductase as described in "Materials and Methods."

with two volumes of cold distilled water and applied to an NAD⁺ affinity column (1.4 × 3.2 cm) preequilibrated with 30.0 mM K-phosphate (pH 6.5), containing 20% glycerol, 0.5 mM DTT, 1.0 mM EDTA, and 0.2% Triton X-100. After protein loading was complete the column was washed with 15 column volumes of the equilibration buffer described above and then eluted by the addition of 2 mM NADH to the loading buffer. Fractions containing NADH-Cyt *b*₅ reductase activity were subsequently concentrated on an Amicon YM10 ultrafiltration membrane and applied to a CL6B Sepharose column (1.5 × 27 cm) and eluted in 50 mM Tris-HCl buffer (pH 8.0), containing 0.5% deoxycholate, 20% glycerol, 1.0 mM EDTA, and 0.5 mM DTT. The Cyt *b*₅ reductase elutes from this column as an apparent tetramer with a molecular mass of 135,000 D, regardless of whether Triton X-100 or deoxycholate are used as detergent. This final chromatographic step afforded pure, homogeneous Cyt *b*₅ reductase as judged by gel electrophoresis and isoelectric focusing. These steps in the purification of Cyt *b*₅ reductase are summarized in Table II.

NADH-Cyt *b*₅ Reductase Activity Determination. The activity of purified reductase from *Pisum sativum* can be quantitated by two distinct assay systems. In one, the potassium ferricyanide-dependent reductase activity is determined in a system contain-

ing NADH and flavoprotein. Figure 4 presents the ionic strength dependence and Figure 5 the pH dependence of the absolute rates for this flavoprotein-dependent NADH reduction of potassium ferricyanide. No systematic variation in the observed rate was noted. A turnover number for the reduction of potassium ferricyanide by NADH Cyt *b*₅ reductase of 22,000 min⁻¹ was obtained. This compares to a value of 29,000 min⁻¹ determined by Strittmatter for the protease solubilized beef liver reductase (21) and 23,000 min⁻¹ for the detergent solubilized beef liver reductase (19). Identical concentrations of purified protein were determined using this value of 22,000 min⁻¹ turnover and the optical absorption at 484 nm with an extinction of 8,500 M⁻¹ cm⁻¹ (21).

Molecular Mass Determination of Cyt *b*₅ and Reductase. Mol wt for the purified proteins were determined by SDS-PAGE (Fig. 6). Cyt *b*₅ from *P. sativum* was found to have a molecular mass of 16,400 D and its reductase a molecular mass of 34,500 D. The validity of these monomer molecular masses were supported by calibrated gel filtration chromatography.

N-Terminal Amino Acid Sequence of Purified Cyt *b*₅. The purified fraction of Cyt *b*₅ described above was subjected to final HPLC purification by reverse phase chromatography on an Anspec C-4 column eluted with acetonitrile containing 0.10% trifluoroacetic acid. Five nmol of highly purified Cyt *b*₅ from this step was subjected to sequential Edmund degradation with an Applied Biosystems 480A analyzer. Scheme A presents the derived amino terminus sequence of the amino acids obtained at each step.

Reconstitution of Cyt *b*₅ Dependent Cyt *c* Reductase Activity. Cyt *b*₅ and NADH-Cyt *b*₅ reductase components, purified as described above, can also be assayed in a reconstituted system with Cyt *c* as a terminal electron acceptor. Inasmuch as the purified flavoprotein is unable to reduce Cyt *c* without Cyt *b*₅ as an obligatory electron transfer intermediate (21), this assay directly probes redox transfer at the physiological interface between flavoprotein and Cyt *b*₅. The specific conditions of the assay are presented in the figure legends.

Complex Formation between NADH-*b*₅ Reductase and Cyt *b*₅. Figure 4 documents the ionic strength profile for this Cyt *b*₅-dependent activity. In order to probe the effect of detergent on the formation of a kinetically competent complex various levels of Triton X-100 or deoxycholate were added to the assay mixture. Figure 7 presents the effect of increasing detergent content on the maximal velocity of NADH-dependent reduction of Cyt *b*₅ by the purified reductase. In order to further quantitate the association between flavoprotein and Cyt *b*₅, a standard iterative Hanes-Woolf analysis as described in the Legend was performed wherein the free concentration of both protein components was

Table I. Cyt *b*₅ Purification Summary

Step	Cyt <i>b</i> ₅ Concentration	Volume	Total Content	Yield	Total Protein	Specific Content	Purification
	μM	ml	μmol	%	mg/ml	nmol/mg	fold
Resuspended microsomes	3.55	46	163	100	12.5	0.283	1.0
Solubilized microsomes (1% Triton X-100)	1.03	100	103	63	2.30	0.448	1.58
DEAE-Tris-acryl m ion exchange chromatography	1.69	38.5	65.1	40	4.7	1.0	3.53
First Aca 44 gel filtration chromatography	0.366	54	19.7	12	0.114	3.2	11.34
DEAE-Sepharose chromatography	2.77	6.6	18.3	11	0.543	5.1	18.0
Second Aca 44 gel filtration chromatography	19.9	0.76	15.12	9.3	0.44	46	173

Table II. NADH Cyt *b*₅ Reductase Purification Summary

Step	Activity	Volume	Total Activity	Yield	Total Protein	Specific Activity	Purification
	<i>units/ml</i>	<i>ml</i>	<i>units</i>	<i>%</i>	<i>mg/ml</i>	<i>units/mg</i>	<i>-fold</i>
Resuspended microsomes	36.1	32	1150	100	5.0	7.2	1.0
Solubilized microsomes (1% Triton X-100)	12.0	64	829	72.0	1.55	8.32	1.15
DEAE-Tris-acryl M ion exchange chromatography	11.6	66	766	66.4	0.44	26.3	3.66
NAD-affinity chromatography	16.0	15	240	21.0	0.19	84.2	11.69
Sephacrose CL-6B gel filtration chromatography	18.0	8.50	153	13.0	0.10	180.0	25.0

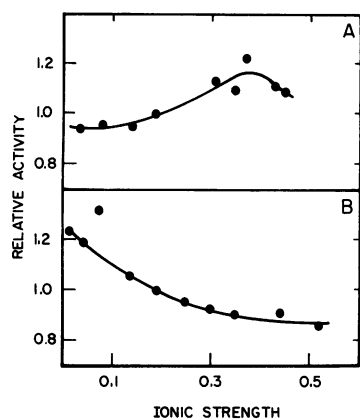


FIG. 4. Ionic strength dependence of NADH-Cyt *b*₅ reductase activities. The lower curve (B) represents the Cyt *b*₅-dependent reduction of Cyt *c* by NADH-Cyt *b*₅ reductase. Cyt *b*₅ and reductase are in a 1:1 stoichiometric limiting concentration of 12.2 nM with the electron acceptor Cyt *c* in saturating excess at 40 μ M. The NADH concentration was 300 μ M, detergent concentrations were 0.0002% (v/v) Triton X-100, 0.0012% (w/v) deoxycholate, and dithiothreitol was at 10 μ M. Activities are normalized to that observed at an ionic strength of 0.19 M, 16.0 nmol Cyt *c* reduced/min. The top curve (A) displays the relative activity of the reductase using ferricyanide as electron acceptor. The reductase is at 4.5 nM and the ferricyanide saturating at 1 mM and the NADH concentration was 300 μ M. Activities are relative to an ionic strength of 0.19 M, 12.0 nmol ferricyanide reduce/min. Ionic strengths were varied by the addition of potassium phosphate and the pH of all buffers were maintained at 7.4.

systematically varied (Fig. 8). Completely consistent results were obtained regardless of whether *b*₅ or flavoprotein concentration was altered. A Michaelis constant of 7 nM was derived which describes the association of these two proteins in a 1:1 electron transfer complex. A maximal velocity of 0.44 nmol Cyt *b*₅ reduced per minute was obtained from this iterative Hanes-Woolf analysis.

DISCUSSION

This manuscript presents the purification and determination of the physical and kinetic parameters of two important electron transfer proteins, Cyt *b*₅ and Cyt *b*₅ reductase, from the microsomes of *P. sativum*.

The purification of Cyt *b*₅ was designed to take advantage of the difference in protein polymerization between Triton X-100 and deoxycholic acid solubilization. In 0.2% Triton X-100, the protein exists as an apparent tetramer with a molecular mass of 66,000 D, while in deoxycholate it is an apparent dimer with a molecular mass of 33,000 D. This alteration in apparent molec-

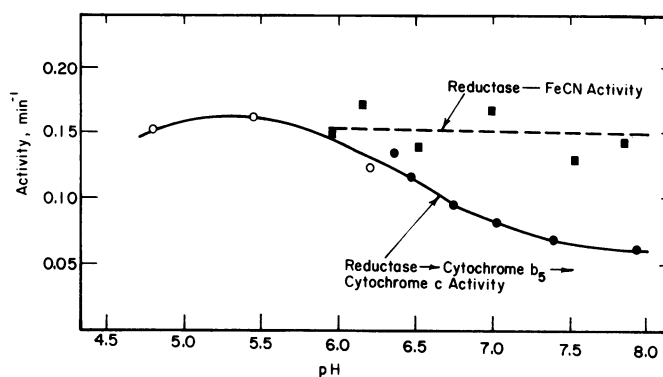


FIG. 5. pH dependence of NADH-Cyt *b*₅ reductase activity in the reconstituted system. The Cyt *b*₅-dependent reduction of Cyt *c* is depicted by the solid line with filled circles representing data obtained with 50 mM potassium phosphate buffer and open circles with sodium acetate buffer adjusted to equal ionic strength of 0.075 M. The dashed line and solid squares illustrate the pH dependence of ferricyanide reduction by the reductase. All activities are expressed in nmol of electron acceptor reduced per nmol reductase per min. Assay conditions are as described in the legend to Figure 4.

ular masses afforded pure Cyt *b*₅ with a minimal number of chromatographic steps, allowing rapid purification with minimal protein degradation. The apparent monomeric molecular mass, determined by electrophoresis under denaturing conditions (Fig. 6), was found to be 16,400 D which agrees closely with those values reported for mammalian Cyt *b*₅ (20). The determination of molecular masses of membrane proteins by SDS gels, however, is prone to larger error than that associated with soluble proteins. Other physical parameters of this plant Cyt *b*₅ reported herein, including isoelectric points, and optical spectra are also very similar to the Cyt *b*₅ purified from mammalian microsomes. These similarities lend credence to the evolutionary significance of this electron transport protein and to the conservation of surface recognition epitopes.

Our Cyt *b*₅ reductase purification procedure is designed to take advantage of the specific NADH binding site of the protein in the use of NAD-agarose affinity chromatography. Previous purifications of the corresponding mammalian protein have used reactive blue (5) or ADP-bound agarose (18) affinity chromatography. The monomeric molecular mass of the pea NADH-Cyt *b*₅ reductase determined by electrophoresis under denaturing conditions (Fig. 6) was 34,500 D. This agrees closely with its mammalian counterpart (15). Under our assay conditions ("Materials and Methods") the turnover number of the reductase with potassium ferricyanide was found to be 22,000 min⁻¹. The turnover number of 29,000 min⁻¹ was initially reported for the proteolytically solubilized mammalian Cyt *b*₅ reductase (21), although highly purified detergent solubilized enzyme yielded a

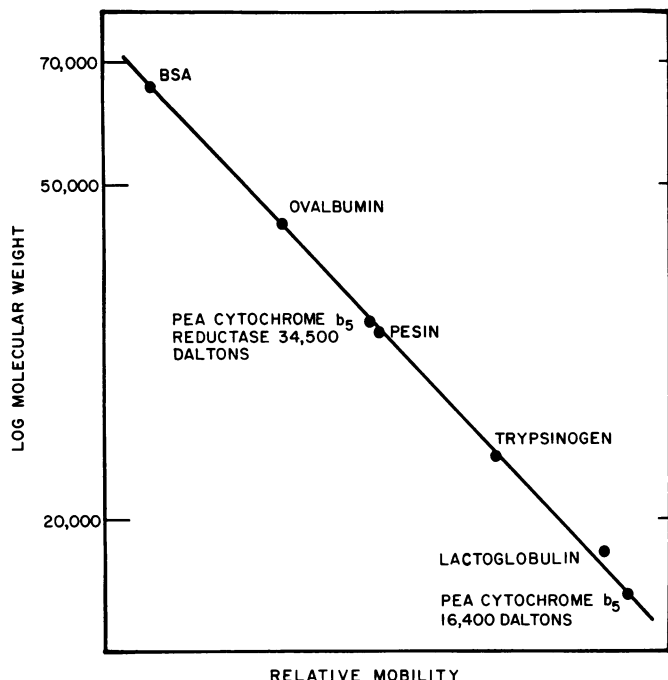
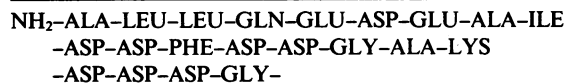


FIG. 6. Apparent molecular mass of NADH-Cyt *b*₅ reductase and Cyt *b*₅ from *P. sativum*. Polyacrylamide gel electrophoresis was performed according to the method of Neville (17), using a separation gel of 10% total acrylamide and 5% bis-acrylamide cross-linker and run at a pH of 8.64 in the presence of 2.0 M urea and 0.1% (w/v) SDS.



SCHEME A. NH₂-Terminal Amino Acid Sequence of Plant Cyt *b*₅

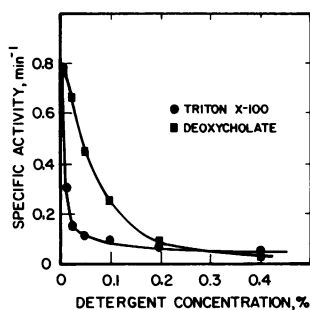


FIG. 7. Effect of detergent concentration on the activity of NADH-Cyt *b*₅ reductase. Cyt *c* was used as an electron acceptor, saturating a 1:1 complex of reductase and Cyt *b*₅ (12.2 nM) at a concentration of 40 μM. Activities are expressed as nmol Cyt *c* reduced/min/nmol reductase. Assay conditions were as described in the legend to Figure 4 with the ionic strength held at 0.075 M at a pH of 7.4.

turnover number of 23,000 min⁻¹ (19). The specificities of the plant and mammalian enzymes are similar in that both efficiently use NADH but not NADPH, and neither will reduce Cyt *c* without Cyt *b*₅ as a mediator.

In the reconstitution of NADH Cyt *c* reductase activity, the rate of reduction of Cyt *c* is assumed to be indicative of the rate of electron transfer between Cyt *b*₅ reductase and Cyt *b*₅, since the Cyt *c* concentration is saturating the 40 μM and since the kinetics of electron transfer between Cyt *b*₅ and Cyt *c* in the di-protein complex is extremely fast (11). With the Cyt *b*₅ reductase and Cyt *b*₅ in equal concentration at 12.2 nM, the effect of ionic

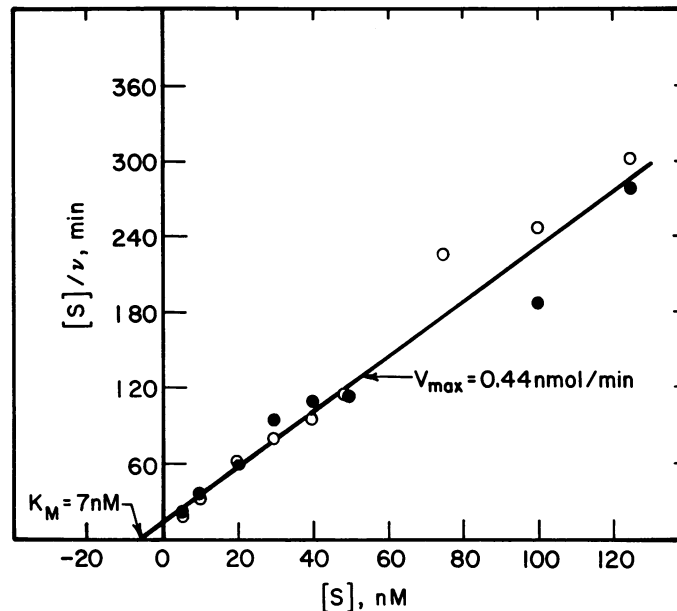


FIG. 8. Iterative Hanes-Woolf analysis of NADH-Cyt *c* reductase activity. (●), velocity of Cyt *c* reduction observed with varying concentrations of free Cyt *b*₅ at fixed reductase concentration of 5 nM; (○), the same assay when Cyt *b*₅ is held fixed at 5 nM and the free NADH Cyt *b*₅ reductase concentration is varied. The existence of a 1:1 complex between Cyt *b*₅ and its reductase is indicated with a K_m of 7 nM and a maximal velocity of electron transfer of 0.44 nmol Cyt *b*₅ reduced/min. In these assays the Cyt *c* and NADH were saturating at 40 and 300 μM, respectively. The ionic strength was maintained at 0.075 M (pH 7.4). The detergent concentrations were held constant at 0.005% (v/v) Triton X-100 and 0.01% (w/v) deoxycholate. The substrate concentrations indicated in the ordinate and abscissa are free protein concentrations determined from the total added by iteration using $S_{TOTAL} = S + [E \cdot S]$ where E represents the other protein, either reductase or Cyt. Thus, the linearized form of the Hanes-Woolf equation $(S/v) = (S_i + K_{Mi})/V_{Mi}$ is first fit using the total substrate concentration for S_i and the derived values of K_{Mi} and V_{Mi} for this iteration used to calculate a new S_i for all data points via $S_{i+1} = S_i - (v_i/V_{Mi}E_{TOTAL})$. Convergence of the correlation coefficient occurred in all cases with less than four iterations.

strength on the rate of Cyt *c* reduction was investigated (Fig. 4). The electron transfer rate clearly increases with decreasing ionic strength. The potassium ferricyanide reductase activity, however, demonstrated a lesser ionic strength effect and in the opposite direction of higher absolute activity at higher ionic strength. This difference can, in all probability, be explained with the ionic character of the respective protein-protein interactions. Strittmatter (9) has suggested that the complex between the soluble reductase and Cyt *b*₅ is stabilized by ionic interaction as deduced through chemical modification of the reactive lysyl groups on the surface of the reductase. The strong increase in rate with decreasing ionic strength demonstrated by these studies suggests a highly ionic character for the protein-protein complex, since higher ionic strength tends to shield the charges on the protein surface. The ionic character of these protein-protein interactions can also be visualized in the pH dependence of the pea proteins (Fig. 5), the reaction wherein the rate of Cyt *c* reduction exhibits a plateau at pH 5.5 with the midpoint of the titration curve at roughly pH 6.0. It thus appears that an ionizable group, perhaps a histidine residue, facilitates the electron transfer in the protonated state, either by improving the architecture of the protein complex or by providing a critical hydrogen bonding pathway for electron transfer.

In order to further define the existence and nature of Cyt *b*₅-

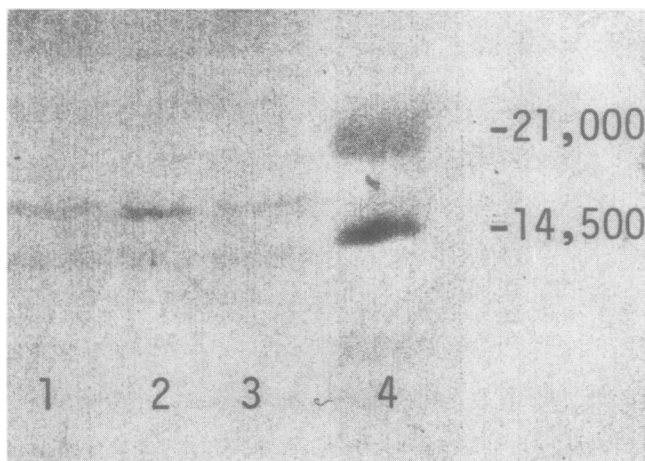


FIG. 9. Western blot of *P. sativum* total microsomal protein and purified Cyt b_5 . Antibody was raised in rabbits to the soluble core fragment of highly purified rat liver Cyt b_5 (22). Lanes 1 and 3 are total pea microsomal protein in the molecular mass range of 10,000 to 28,000 D. Lane 2 is purified pea Cyt b_5 as described in this manuscript. Lane 4 contains 21,500 D and 14,500 D molecular mass markers. Purified pea Cyt b_5 has a molecular mass of 16,500 by this Western blot, consistent with that shown in Figure 3.

Cyt c complex, the kinetics of electron transfer were examined in detail. Figure 8 illustrates a standard Hanes-Woolf analysis of the NADH Cyt c reductase activity. In these experiments one protein concentration is held constant while the other varied. The closed circles correspond to a constant Cyt b_5 reductase concentration of $5 \mu\text{M}$ and a varying Cyt b_5 concentration. The open circles reflect the converse experiment. Both of these experiments yield virtually identical kinetic data which clearly demonstrates the existence of a 1:1 complex between the two proteins. Linear regression analysis under a Michaelis constant of 7 nM and a maximum velocity (V_{max}) under these conditions of $440 \mu\text{M}/\text{min}$, corresponding to a turnover number of 100 min^{-1} . These figures contrast sharply with the values reported in mammalian systems earlier by Spatz and Strittmatter (19) of $K_m = 38 \mu\text{M}$ and a V_{max} of $14,100 \text{ min}^{-1}$. The differences between these two systems is quite striking, but may be accounted for by the fact that Spatz and Strittmatter observed the rate of NADH oxidation and not heme protein reduction and hence their kinetic data include any flavoprotein first order auto-oxidation and idling rates. But this cannot account for the difference of orders of magnitude observed between the mammalian and plant systems. Indeed, Mihara and Sato (15), using an assay system analogous to ours, observed kinetic constants similar to those found by Spatz and Strittmatter.

The primary amino terminal sequence of plant Cyt b_5 presented in Scheme A displays the initial alanine residue and richness in acidic residues observed in mammalian Cyt b_5 , but is far less homologous with the eight known b_5 's than expected. Figure 9 displays a Western blot of total microsomal protein and purified Cyt b_5 from *P. sativum* developed by alkaline phosphatase linked assay to antibody raised against the soluble core of rat liver Cyt b_5 . Clean, strong cross-reactivity is indicated. Control experiments demonstrated no reactivity in the absence of antibody or against a nonspecific bacterial cell lysate which does not contain Cyt b_5 . Thus, in spite of the many similarities between the mammalian and plant electron transport systems, many important differences in structure and multiprotein interactions during redox transfer are apparent.

SUMMARY

The microsomal electron transport systems of higher plants are involved in a variety of central metabolic transformations including fatty acid desaturation and the mixed function oxidase

activities of the Cyt P-450-dependent monooxygenases. The procedures documented in this manuscript provide reproducible protocols for the generation of milligram quantities of NADH Cyt b_5 reductase and Cyt b_5 from the microsomal fraction of *Pisum sativum*. These results present the first demonstration of microsomal Cyt purification from this tissue. A key advantage in the choice of *P. sativum* as a source for microsomal electron transfer and monooxygenase proteins is that stable and efficient transformation systems exist for propagation to progeny phenotypic traits of cloned native and mutant proteins. Our laboratory is currently involved in an intensive effort at the purification and biochemical characterization of the microsomal proteins responsible for these activities, and the ultimate cloning and expression of wild-type and mutant Cyt. Central of this effort is the availability of primary sequence data on the purified proteins. The data for Cyt b_5 presented in Scheme A represent the first amino acid sequence data available for microsomal electron transport proteins in higher plants.

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