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 turn over 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). Biological 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 coworkers (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 Galie and Kader (6) using HPLC.

As an integral part of our past 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% (v/v) polyacryl-AT. Cell debris was separated by a centrifugation at 10,000 × g for 20 min and the microsomes pelleted by ultracentrifugation at 150,000 × g for 1 h at 4°C. The microsomal pellet was resuspended using a Potter-Elveshem 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 suspension 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 150,000 × g for 1 h at 4°C.

Enzymatic assays. Cyt b5 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 b5 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 b5 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 Werringloer (3).

All chemicals were reagent grade obtained from standard suppliers. Chromatography materials including DEAE-Trisacryl, and Aca44 were from LKB. DEAE Sephadex, 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 b5. 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 fractionization of Cyt b5, NADH-specific Cyt b5 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 b5 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 b5 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 b5 reductase, and has an unknown physiological function. The protein fraction containing authentic Cyt b5 was found to elute between 0.12 m KCl and 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 µg Cyt b5, was then applied to an Aca44 gel filtration column (2.5 × 51 cm) which had been previously calibrated with molecular mass standards. Cyt b5 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 b5 were subsequently applied to a second DEAE Sephadex 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% (v/v) deoxycholate afforded pure, homogeneous Cyt b5 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 b5 are summarized in Table I.

Purification of NADH Cyt b5 Reductase. Fractions eluting from the initial DEAE Trisacryl column between 0.08 and 0.12 m KCl were found to contain NADH-Cyt b5 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

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with two volumes of cold distilled water and applied to an NADH affinity column (1.4 × 3.2 cm) prequilibrated with 30.0 mM potassium 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 b₅ 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 b₅ 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 b₅ reductase as judged by gel electrophoresis and isoelectric focusing. These steps in the purification of Cyt b₅ reductase are summarized in Table II.

NADH-Cyt b₅ 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 containing 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 b₅ 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 b₅ and Reductase. Mol wt for the purified proteins were determined by SDS-PAGE (Fig. 6). Cyt b₅ 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 chromotography.

N-Terminal Amino Acid Sequence of Purified Cyt b₅. The purified fraction of Cyt b₅ 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 b₅ 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 b₅ Dependent Cyt c Reductase Activity. Cyt b₅ and NADH-Cyt b₅ 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 b₅ as an obligatory electron transfer intermediate (21), this assay directly probes redox transfer at the physiological interface between flavoprotein and Cyt b₅. The specific conditions of the assay are presented in the figure legends.

Complex Formation between NADH-b₅ Reductase and Cyt b₅. Figure 4 documents the ionic strength profile for this Cyt b₅-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 b₅ by the purified reductase. In order to further quantitate the association between flavoprotein and Cyt b₅, 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 b₅ Purification Summary

<table>
<thead>
<tr>
<th>Step</th>
<th>Cyt b₅ Concentration</th>
<th>Volume</th>
<th>Total Content</th>
<th>Yield</th>
<th>Total Protein</th>
<th>Specific Content</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>ml</td>
<td>μmol</td>
<td>%</td>
<td>mg/ml</td>
<td>nmol/mg</td>
<td>-fold</td>
</tr>
<tr>
<td>Resuspended microsomes</td>
<td>3.55</td>
<td>46</td>
<td>163</td>
<td>100</td>
<td>12.5</td>
<td>0.283</td>
<td>1.0</td>
</tr>
<tr>
<td>Solubilized microsomes (1% Triton X-100)</td>
<td>1.03</td>
<td>100</td>
<td>103</td>
<td>63</td>
<td>2.30</td>
<td>0.448</td>
<td>1.58</td>
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<tr>
<td>DEAE-Tris-acryl m ion exchange chromatography</td>
<td>1.69</td>
<td>38.5</td>
<td>65.1</td>
<td>40</td>
<td>4.7</td>
<td>1.0</td>
<td>3.53</td>
</tr>
<tr>
<td>First AcA 44 gel filtration chromatography</td>
<td>0.366</td>
<td>54</td>
<td>19.7</td>
<td>12</td>
<td>0.114</td>
<td>3.2</td>
<td>11.34</td>
</tr>
<tr>
<td>DEAE-Sephacel chromatography</td>
<td>2.77</td>
<td>6.6</td>
<td>18.3</td>
<td>11</td>
<td>0.543</td>
<td>5.1</td>
<td>18.0</td>
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<tr>
<td>Second AcA 44 gel filtration chromatography</td>
<td>19.9</td>
<td>0.76</td>
<td>15.12</td>
<td>9.3</td>
<td>0.44</td>
<td>46</td>
<td>173</td>
</tr>
</tbody>
</table>
The deoxycholate of the 66,000 D, molecular describes the transfer was altered. Systematically at the association of the reductase and dithiothrietol, and dithionoester was at 10 μM. Activities are reported 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 μM. Activities are relative to an ionic strength of 0.19 M, 12.0 nmol ferricyanide reduce/min. These activities 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 b5 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 b5 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 b5 and Cyt b5 reductase, from the microsomes of *P. sativum*.

The purification of Cyt b5 was designed to take advantage of the difference in protein polymerization between Triton X-100 and deoxycholate 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 molecular

**Table II. NADH Cyt b5 Reductase Purification Summary**

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
<th>Volume</th>
<th>Total Activity</th>
<th>Yield</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resuspended microsomes</td>
<td>36.1</td>
<td>32</td>
<td>1150</td>
<td>100</td>
<td>5.0</td>
<td>7.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Solubilized microsomes (1% Trit-</td>
<td>12.0</td>
<td>64</td>
<td>829</td>
<td>72.0</td>
<td>1.55</td>
<td>8.52</td>
<td>1.15</td>
</tr>
<tr>
<td>ton X-100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Tris-acryl m ion exchange</td>
<td>11.6</td>
<td>66</td>
<td>766</td>
<td>66.4</td>
<td>0.44</td>
<td>26.3</td>
<td>3.66</td>
</tr>
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<td>chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD-affinity chromatography</td>
<td>16.0</td>
<td>15</td>
<td>240</td>
<td>21.0</td>
<td>0.19</td>
<td>84.2</td>
<td>11.69</td>
</tr>
<tr>
<td>Sepharose CL-6B gel filtration</td>
<td>18.0</td>
<td>8.50</td>
<td>153</td>
<td>13.0</td>
<td>0.10</td>
<td>180.0</td>
<td>25.0</td>
</tr>
<tr>
<td>chromatography</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Fig. 4.** Ionic strength dependence of NADH-Cyt b5 reductase activities. The lower curve (B) represents the Cyt b5-dependent reduction of Cyt c by NADH-Cyt b5 reductase. Cyt b5 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 μM. The NADH concentration was 300 μM, detergent concentrations were 0.0002% (v/v) Triton X-100, 0.0012% (v/v) deoxycholate, and dithionoester was at 10 μM. 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 μM. Activities are relative to an ionic strength of 0.19 M, 12.0 nmol ferricyanide reduce/min. These activities were varied by the addition of potassium phosphate and the pH of all buffers were maintained at 7.4.

**Fig. 5.** pH dependence of NADH-Cyt b5 reductase activity in the reconstituted system. The Cyt b5-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.

Purification afforded pure Cyt b5 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 b5 (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 b5 reported herein, including isoelectric points, and optical spectra are also very similar to the Cyt b5 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 b5 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 b5 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 b5 reductase (21), although highly purified detergent solubilized enzyme yielded a
and Cyt bs
Cyt
the
acrylamide
Cyt
of electron
turnover
kinetics of
use
of rate
b5
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.

–ASP–ASP–ASP–GLY–

SCHEME A. NH2-Terminal Amino Acid Sequence of Plant Cyt b5

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 b5 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 b5 reductase and Cyt b5, since
the Cyt c concentration is saturating the 40 μM and since the
kinetics of electron transfer between Cyt b5 and Cyt c in the
di-protein complex is extremely fast (11). With the Cyt b5 reductase
and Cyt b5 in equal concentration at 12.2 nM, the effect of ion

strength on the rate of Cyt c reduction was investigated (Fig. 4).
The electron transfer rate clearly increases with decreasing ion strength. The potassium ferricyanide reductase activity, however,
demonstrated a lesser ion strength effect and in the opposite
direction of higher absolute activity at higher ion strength. This
difference can, in all probability, be explained with the ion
character of the respective protein-protein interactions. Strittmatt
(9) has suggested that the complex between the soluble
reductase and Cyt b5 is stabilized by ionic interaction as deduced
to chemical modification of the reactive lysyl groups on
the surface of the reductase. The strong increase in rate with
decreasing ion strength demonstrated by these studies suggests
a highly ionic character for the protein-protein complex, since
higher ion 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 b5-

Fig. 6. Apparent molecular mass of NADH-Cyt b reductase and Cyt
b5 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.

Fig. 7. Effect of detergent concentration on the activity of NADH-
Cyt b2 reductase. Cyt c was used as an electron acceptor, saturating a 1:1
complex of reductase and Cyt b5 (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.

Fig. 8. Iterative Hanes-Woolf analysis of NADH-Cyt c reductase
activity. (●), velocity of Cyt c reduction observed with varying
concentrations of free Cyt b5 at fixed reductase concentration of 5 nM; (○), the
same assay when Cyt b5 is held fixed at 5 nM and the free NADH Cyt b5
reductase concentration is varied. The existence of a 1:1 complex between
Cyt b5 and its reductase is indicated with a Km of 7 nM and a maximal
velocity of electron transfer of 0.44 nmol Cyt b5 reduced/min. In these
assays the Cyt c and NADH were saturating at 40 and 300 μM, respectivley.
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 STOTAL = S + [E·S] where
E represents the other protein, either reductase or Cyt. Thus, the linear-
ized form of the Hanes-Woolf equation ([S]/vmax = (S + Ks)/Vmax +
1) was first fit using the total substrate concentration for S, and the derived values
of Ks and Vmax for this iteration used to calculate a new S for all data
points via Snew = S - (ν/VmaxSTOTAL). Convergence of the correlation
coefficient occurred in all cases with less than four iterations.
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 reductase and Cyt b5 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 to this effort is the availability of primary sequence data on the purified proteins. The data for Cyt b5 presented in Scheme A represent the first amino acid sequence data available for microsomal electron transport proteins in higher plants.

**REFERENCES**

1. **BENVENSTE I, B GABRIEL, F DURST 1986 Purification and characterization of the NADPH-cytochrome P-450 (cytochrome c) reductase from higher-plant microsomal fraction. Biophys J 55: 365-377.**


4. **FUJITA M, T ASAHI 1985 Purification and properties of sweet potato NADPH-cytochrome c (P-450) reductase. Plant Cell Physiol 26: 397-405.**


12. **MADYASTHA K, C COSCIA 1979 Detergent solubilization NADPH-cytochrome c (P-450) reductase from the higher plant, *Catharanthus roseus*. J Biol Chem 254: 2419-2427.**


