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Communication

Primary Structure of Cytochrome b_5 from Cauliflower (*Brassica oleracea* L.) Deduced from Peptide and cDNA Sequences[1]

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ABSTRACT

Cytochrome b_5 is a microsomal protein that functions as an intermediate electron donor in fatty acid desaturation and other oxidation/reduction reactions. cDNA clones were isolated from cauliflower (*Brassica oleracea* L.) by using oligonucleotides based on the partial amino acid sequence of the protein. The deduced amino acid sequence of the polypeptide exhibited approximately 30% sequence identity with the homologous protein from vertebrates.

Cyt b_5 is an integral membrane protein that is associated with the microsomal membranes of higher plants (3, 17) and animals (14). In animals, the protein has been implicated as an intermediate electron donor in fatty acid desaturation (9, 21), Cyt P-450 monooxygenase activity (13), cholesterol biosynthesis (16), fatty acid elongation (8), and plasmalogen biosynthesis (15). In higher plants, Cyt b_5 has recently been shown to function as an intermediate electron donor in the desaturation of fatty acids (7, 20) and has been suggested as a possible participant in β-oxidation in glyoxysomes (3). Considering the diversity of functions in animals, it seems possible that Cyt b_5 also has a role in other oxidation/reduction reactions in higher plants.

Previous investigations of the structure of Cyt b_5 from plants have resulted in purification of the protein to varying degrees from microsomal preparations of potato tubers (2), etiolated seedlings of *Catharanthus roseus* (10), etiolated pea stems (5), and cauliflower florets (6, 7). We report here the isolation and characterization of the first cDNA clone for Cyt b_5 from a plant. The availability of the cDNA clones should provide novel opportunities to experimentally examine the role of Cyt b_5 in other oxidation/reduction reactions in higher plants.

MATERIALS AND METHODS

Genetic Materials

Escherichia coli strain XL1-Blue was obtained from Stratagene (La Jolla, CA). A λUNI-ZAP XR cDNA library prepared from the meristematic surface of cauliflower floret (*Brassica oleracea* var *cauliflora*) was obtained from June Medford (12).

Peptide Sequence Analysis

Cyt b_5 was purified from cauliflower floret microsomes as previously described (7) and digested to completion with trypsin. The resulting peptides were resolved by HPLC on a C_{18} reversed-phase column and subjected to automated Edman degradation on a gas-phase sequenator.

Cloning Procedures

As a matter of convenience, a partial cDNA clone was first isolated from canola (*Brassica napus* L.), then used as a probe to isolate a full-length cDNA clone from *B. oleracea*. It was assumed, and subsequently verified empirically, that Cyt b_5 from the two species would share sufficient amino acid sequence identity so that mixed oligonucleotide probes based on *B. oleracea* peptide sequences would hybridize to cDNA from *B. napus*.

Single-stranded cDNA was prepared from 1 μg of total RNA from developing seeds of canola by priming the reverse transcriptase reaction with the mixed oligonucleotide 5′-TC(A/G)AT(C/T)TC(A/T)CC(A/G)ATGTAGTA. The cDNA was then used as the template in a polymerase chain reaction (4) that was primed at the 3′ side with the mixed oligonucleotide above, and at the 5′ side with the mixed oligonucleotide 5′-GGNTT(C/T)GA(A/G)GA(A/G)GTNTC. The oligonucleotides were designed to encode the *B. oleracea* Cyt b_5 peptide sequences YYIGEID and GFEEVS, respectively. The relative orientation of these two peptides within the Cyt b_5 polypeptide was determined beforehand by alignment with animal

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Cyt b_5 sequences (14). The 0.2 kilobase product of the polymerase chain reaction was cloned into the *Hin*dIII site of pUC119 and used to probe nitrocellulose filter replicas of a λUNI-ZAP XR cDNA library from cauliflower florets (11). The filters were hybridized at 65°C in 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA [pH 7.4]), 0.5% (w/v) nonfat dry milk, 0.5% (w/v) SDS, 5% (w/v) dextran sulfate, and washed twice at room temperature and once at 65°C in 2 × SSPE, 0.1% SDS. The positive clones were converted to pBluescript SK^- form by coinfection with an M13 helper phage.

The DNA sequence of the cDNA inserts was determined by manual dideoxy sequencing of double-stranded plasmid DNA using synthetic oligonucleotides as primers.

Structural Analyses

Hydropathy plots were calculated using the algorithm of Rose and Roy (18) as implemented in the Hitachi PROSIS software package for microcomputers.

RESULTS AND DISCUSSION

The extra-membrane domain of Cyt b_5 from microsomal membranes of cauliflower florets was solubilized by treating the membranes with trypsin, and purified by column chromatography (7). The amino acid sequence of the amino terminus and several tryptic peptides was determined by gas-phase sequencing (Fig. 1). Degenerate oligonucleotides based on the partial amino acid sequence were then used in the polymerase chain reaction (4) to amplify a 220-base pair DNA fragment from *Brassica napus* cDNA. The product of this reaction was used as a hybridization probe to identify Cyt b_5 cDNA clones from a cauliflower cDNA library in λZAP. The DNA sequence of four of the largest cDNA clones was completely determined on both strands. The four sequences were in perfect agreement in the region of overlap except that one of the clones had a G-to-T transversion at nucleotide 110 (Fig. 1) that resulted in a lysine-to-asparagine change in the deduced amino acid sequence. One cDNA extended from nucleotide 1 to 585. Two cDNAs extended from nucleotide 68 to 675. These three cDNAs contained poly(A) tails of 19 A-residues at the 3′ end. The fourth cDNA extended from nucleotide 74 to 738 and lacked a poly(A) tail.

The cDNA clones encoded a polypeptide of 134 amino acids with a predicted molecular mass of 15 kD. For the peptides where amino acid sequence was obtained (underlined regions of Fig. 1), the deduced amino acid sequence was in perfect agreement with the directly determined sequences. The first five N-terminal amino acids of the deduced amino acid sequence were not present on the trypsin-solubilized polypeptide, suggesting that the peptide bond between the fifth and sixth residues was susceptible to cleavage by trypsin. Trypsin cleavage of Cyt b_5 from the ER of cauliflower florets was previously shown to result in a polypeptide of apparent molecular mass of about 12 kD on SDS-PAGE (7). This suggests that the trypsin cleavage occurred adjacent to lysine-110, which, in addition to cleavage at lysine-5, would produce a polypeptide of predicted molecular mass of 11 kD. However, in view of the potential error in molecular

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   1                                                    AGTTCTCAAGTAGGTGA

  18  AGAGTTACCCCACCAGGTGCTATCGCAATTCTGAATCTGAAAGCAGA

   1                                                M   A   S   E
  79  AAGATCCCCCACCTTTGATTTGAGGAGAGAG ATG GCT TCA GAG

   5   K   K   V   L   G   F   E   E   V   S   Q   H
 108  AAG AAG GTT CTA GGT TTC GAA GAA GTT TCG CAG CAC

  17   N   K   T   K   D   C   W   L   I   I   S   G
 144  AAC AAA ACC AAG GAT TGT TGG CTT ATT ATC TCC GGC

  29   K   V   Y   D   V   T   P   F   M   D   D   H
 180  AAG GTC TAT GAT GTG ACC CCT TTC ATG GAT GAT CAT

  41   P   G   G   D   E   V   L   L   S   S   T   G
 216  CCC GGT GGC GAT GAA GTG TTA TTG TCC TCA ACA GGG

  53   K   D   A   T   N   D   F   E   D   V   G   H
 252  AAA GAT GCT ACG AAT GAC TTT GAA GAC GTT GGT CAC

  65   S   D   T   A   R   D   M   M   E   K   Y   Y
 288  AGC GAC ACC GCG AGG GAC ATG ATG GAG AAG TAC TAC

  77   I   G   E   I   D   S   S   T   V   P   A   T
 324  ATT GGC GAG ATC GAT TCG TCT ACT GTT CCA GCG ACA

  89   R   T   Y   V   A   P   V   Q   P   A   Y   N
 360  AGG ACA TAC GTT GCA CCA GTG CAA CCC GCG TAC AAC

 101   Q   D   K   T   P   E   F   M   I   K   I   L
 396  CAA GAC AAG ACA CCA GAA TTC ATG ATC AAG ATC CTT

 113   Q   F   L   V   P   I   L   I   L   G   L   A
 432  CAG TTC CTT GTT CCA ATC TTG ATC TTG GGT CTT GCT

 125   L   V   V   R   Q   Y   T   K   K   E
 468  CTC GTC GTC CGT CAG TAT ACT AAG AAA GAG TAGAAGC

 505  AGAGAGAGGCTTGCGGTATTGCTTCCCATTGGATAACTCTTTCTCAT

 552  ATCTCTGTATTTCGAAACCTTGGTTTGGTTCTAGATTGCACACCTCT

 599  GTTCTAAAAATTATTCTTGTTAGAACTACTATAAACCAATAATCAAT

 646  GTGTTGTTAGTGTTTTCGTTGGTGGTATCATCTGGTTTATTTTGTGT

 693  GTTGATTGAGATGTGTTCAACTTTGATGATCAAATAAAAGAGTGTT
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Figure 1. DNA sequence and deduced amino acid sequence of microsomal Cyt b_5 from cauliflower. The regions of the polypeptide for which amino acid sequence was obtained by direct sequencing of the protein are underlined. The peptide sequences were: VYDVTPFMDDHPGGDEVL, TKDCWLIISGK, YYIGEIDSSTVPATR, KVLGFEEVSQHNK. The regions of nucleotide sequence that hybridized to the oligonucleotide primers used in the polymerase chain reaction are indicated with a double underline. The nucleotide sequence has been assigned GenBank accession number M87514.

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Bo              MASEKKVLGFEEVSQ-HNKTK  20
Gg  MVGSSEAGGEAWRGRYYRLEEV-QKHNNSQ     29

Bo  DCWLIISGKVYDVTPFMDDHPGGDEVLLSS     50
Gg  STWIIVHHRIYDITKFLDEHPGGEEVLREQ     59

Bo  TGKDATND-FEDVGHSDT-ARDMMEKYYIG     78
Gg  AGGDAT-ENFEDVGHS-TDARALSETFIIG     87

Bo  EIDSSTVPATRTYVAPVQPAYNQDKTPEFM    108
Gg  ELHPDDRPKLQKPAETLITTVQSNSSSWSN    117

Bo  IKILQFLVPILILGLALVVRQYTKKE        134
Gg  WVIPAIAAIIVALMYRSYMSE            138
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Figure 2. Comparison of the deduced amino acid sequence of Cyt b_5 from cauliflower (Bo) and chicken (Gg). Amino acid sequence identity is indicated by a solid box and conservative amino acid substitutions by a stippled box. The presence of dashes in the sequences indicate discontinuities that were introduced to maximize the sequence alignment.

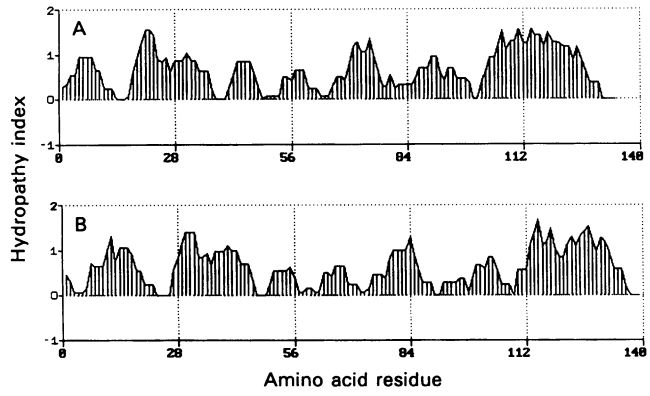

Figure 3. Rose hydropathy plots for Cyt b_5 from cauliflower (A) and chicken (B). The hydropathy index of each residue is a positive value, expressed in kcal/mol, which indicates the free energy of transfer of each amino acid residue from an aqueous to an organic solution. The most positive values indicate the most hydrophobic character.

mass estimates based on mobility in SDS-PAGE, cleavage at lysine-103 is also a possibility.

When aligned for maximum sequence identity but minimum displacement (Fig. 2), the *B. oleracea* polypeptide showed 31% sequence identity with the homologous protein from chicken (*Gallus gallus*) (22). This increased to 44% if conservative substitutions (*i.e.* Q/N, E/D, K/R, M/L/V/I, F/Y/W) were considered. The sequence identity was highest in the region of the protein surrounding the pairs of histidine residues (His-40 and His-64) that correspond to the axial ligands for heme binding by the protein from animals. Although the plant and animal amino acid sequences were highly divergent in the carboxy terminal third of the polypeptide, both polypeptides had a strikingly similar hydropathy plot (Fig. 3). This apparent conservation of a region of higher order structure rather than sequence *per se* was previously described as an example of a "topogenic determinant" (1). Both proteins contained a strongly hydrophobic carboxy-terminal domain to anchor the proteins in the membrane. The carboxy-terminal amino acid sequence of the *B. oleracea* protein (KKX) was reminiscent of the KKXX motif found on the cytoplasmically exposed carboxy terminus of certain proteins of the ER (19). This is consistent with the observation that the carboxy terminus of the vertebrate Cyt b_5 has been shown to be located on the cytoplasmic side of the ER (14). Surprisingly, there was no sequence identity of the cauliflower polypeptide with an amino-terminal amino acid sequence previously reported for a Cyt b_5 from pea (*Pisum sativum*) (5).

The cDNA clone described here is the first Cyt b_5 gene characterized from a nonvertebrate species. The availability of the clone should create many new opportunities to investigate the involvement of this protein in microsomal oxidation and reduction reactions in higher plants.

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LITERATURE CITED

1. **Bendzko P, Prehn S, Pfeil W, Rapoport TA** (1982) Different modes of membrane interactions of the signal sequence of carp preproinsulin and of the insertion sequence of rabbit cytochrome b5. Eur J Biochem **123:** 121–126
2. **Bonnerot C, Galle AM, Jolliot A, Kader J-C** (1985) Purification and properties of plant cytochrome b5. Biochem J **226:** 331–334
3. **Fang TK, Donaldson RP, Vigil EL** (1987) Electron transport in purified glyoxysomal membranes from castor-bean endosperm. Planta **172:** 1–13
4. **Gould SJ, Subramani S, Scheffler IE** (1989) Use of the DNA polymerase chain reaction for homology probing: isolation of partial cDNA or genomic clones encoding the iron-sulfur protein of succinate dehydrogenase from several species. Proc Natl Acad Sci USA **86:** 1934–1938
5. **Jollie DR, Sligar SG, Schuler M** (1987) Purification and characterization of microsomal cytochrome b5 and NADH cytochrome b5 reductase from *Pisum sativum*. Plant Physiol **85:** 457–462
6. **Kearns EV** (1991) The structure of cytochrome b5 and its func-

tion in Δ12 oleate desaturation in the microsomes of developing safflower seeds. PhD thesis. Michigan State University, East Lansing

7. **Kearns EV, Hugly S, Somerville CR** (1991) The role of cytochrome b5 in Δ12 desaturation of oleic acid by microsomes of safflower (*Carthamus tinctorius* L.). Arch Biochem Biophys **284:** 431–436
8. **Keyes SR, Alfano JA, Jansson I, Cinti DL** (1979) Rat liver microsomal elongation of fatty acids. J Biol Chem **254:** 7778–7784
9. **Lee TC, Baker RC, Stephens N, Snyder F** (1977) Evidence for participation of cytochrome b5 in microsomal Δ6 desaturation of fatty acids. Biochim Biophys Acta **489:** 25–31
10. **Madyastha KM, Krishnamachary N** (1986) Purification and partial characterization of microsomal cytochrome b555 from the higher plant *Catharanthus roseus*. Biochem Biophys Res Commun **136:** 570–576
11. **Maniatis T, Fritsch EF, Sambrook J** (1984) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. **Medford JI, Elmer JS, Klee HJ** (1991) Molecular cloning and characterization of genes expressed in shoot apical meristems. Plant Cell **3:** 359–370
13. **Noshiro M, Omura T** (1978) Immunochemical study on the electron pathway from NADH to cytochrome P450 of liver microsomes. J Biochem **83:** 61–77
14. **Ozols J** (1989) Structure of cytochrome b5 and its topology in the microsomal membrane. Biochim Biophys Acta **997:** 121–130
15. **Paltauf F, Prough RA, Masters BSS, Johnston JM** (1974) Evidence for the participation of cytochrome b5 in plasmalogen biosynthesis. J Biol Chem **249:** 2661–2662
16. **Reddy VVR, Kupfer D, Caspi E** (1977) Mechanism of C-5 double bond introduction in the biosynthesis of cholesterol by rat liver microsomes. J Biol Chem **252:** 2797–2801
17. **Rich PR, Bendall DS** (1975) Cytochrome components of plant microsomes. Eur J Biochem **55:** 333–341
18. **Rose GD, Roy S** (1980) Hydrophobic basis of packing in globular proteins. Proc Natl Acad Sci USA **77:** 4643–4647
19. **Rothman JE, Orci L** (1992) Molecular dissection of the secretory pathway. Nature **355:** 409–415
20. **Smith MA, Cross AR, Jones OTG, Griffiths WT, Stymne S, Stobart K** (1990) Electron transport components of the 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine Δ^{12}-desaturase in microsomal preparations from developing safflower (*Carthamus tinctorius* L.) cotyledons. Biochem J **272:** 23–29
21. **Strittmatter P, Spatz L, Corcoran D, Rogers MJ, Setlow B, Redline R** (1974) Purification and properties of rat liver microsomal stearoyl coenzyme A desaturase. Proc Natl Acad Sci USA **71:** 4565–4569
22. **Zhang H, Somerville CR** (1990) Soluble and membrane-bound forms of cytochrome b_5 are the products of a single gene in chicken. Arch Biochem Biophys **280:** 412–415