# Gene Synthesis, Expression, and Mutagenesis of Zucchini Mavicyanin: The Fourth Ligand of Blue Copper Center Is Gln.

# Gene Synthesis, Expression, and Mutagenesis of Zucchini Mavicyanin: The Fourth Ligand of Blue Copper Center is Gln

Kunishige Kataoka*, Masami Nakai, Kazuya Yamaguchi, and Shinnichiro Suzuki

Department of Chemistry, Graduate School of Science, Osaka University, 1-16 Machikaneyama, Toyonaka, Osaka 560-0043, Japan

The gene coding for the 109 amino acid, non-glycosylated form of mavicyanin was synthesized and expressed in *Escherichia coli*. The recombinant protein refolded from *E. coli* inclusion bodies was purified and characterized. Its spectroscopic properties are fully identical to those of mavicyanin isolated from zucchini, even in the absence of its carbohydrate moiety. The blue cooper center of mavicyanin strongly binds three ligands (2His and Cys) as well as many blue copper proteins. To disclose the fourth ligand of mavicyanin, Met was substituted for Gln95 by site-directed mutagenesis. The replacement changes from a rhombic EPR signal to an axial one and exhibits the quite similar absorption and CD spectra to those of plastocyanin. The midpoint potential of Gln95→Met mavicyanin shows the positive shift of 187 mV compared with the recombinant protein, being close to the values of plastocyanins. The differences of the spectroscopic and electrochemical properties between mavicyanin and its mutant demonstrate that the fourth ligand of mavicyanin is Gln95.

*Key words:* Mavicyanin; Gene synthesis; Phytocyanin; Blue copper protein; Site-directed mutagenesis.

Mavicyanin isolated from zucchini peelings is a member of the family known as cupredoxins or blue copper proteins (1, 2). Proteins in this family are characterized by an intense electronic absorption band near 600 nm which gives them their characteristic blue to green-blue color, and an unusual small hyperfine coupling constants in EPR spectra (3, 4). The Cu(II) sites in cupredoxins have similar distorted trigonal-pyramidal or tetrahedral geometry in which the Cu atom forms normal bonds to the N atoms of two His residues, a short bond to the S atom of a Cys residue, and a long bond to the S atom of a Met residue; in the case of a trigonal pyramidal geometry, the fifth ligand is the O atom of a main chain carbonyl group. On the basis of sequence similarity, cupredoxins may be categorized into four main structural groups: 1) plastocyanin-

related proteins involving amycyanin and pseudoazurin, 2) azurins, 3) soluble $Cu_A$ domains derived from cytochrome oxidases, 4) phytocyanins, small blue copper proteins from the non-photosynthetic part of plants (5). The amino acid sequence of mavicyanin has recently reported by Scinina *et. al* (6). The sequence similarities indicate that mavicyanin belongs to the phytocyanins, together with a cucumber basic protein (CBP) (7), stellacyanin (8), umecyanin (9), a cucumber peeling cupredoxin (10), a putative blue copper protein in pea pods (11), and a blue copper protein from *Arabidopsis thaliana* (12). From the crystal structures of CBP and cucumber stellacyanin, the distinctive features of phytocyanins are the presence of a disulfide bridge close to the Cu center (13, 14). However, the roles of all these phytocyanins are still unknown.

Nersissian and his co-workers have recently cloned and characterized the cDNA encoding 182 amino acid long precursor stellacyanin from *Cucumis sativas*, and expressed 109 amino acid non-glycosylated Cu binding domain of that in *E. coli* (15). It is the only example of phytocyanin gene cloning. Comparison of the primary structure of mavicyanin with those of other cupredoxins shows that three copper ligands (two His and one Cys residues) are conserved, while the fourth ligand is probably the amido oxygen atom of Gln residue instead of the S atom of Met, like stellacyanin (6, 8).

There are some studies on the replacement of the Cu ligands to shed light on the copper site of stellacyanin (16-19). In the case of azurin, axial ligand substitution (Met121→Gln) causes significant change of the spectroscopic and electrochemical characters (16). In this study, to elucidate the copper center of mavicyanin, we have synthesized and expressed a gene coding for zucchini mavicyanin, and have prepared the mutant that the putative ligand (Gln95) is replaced with Met by site-directed mutagenesis. The spectroscopic and electrochemical properties of the recombinant and mutant proteins were compared to those of wild-type mavicyanin.


**MATERIALS AND METHODS**

*Materials*.    The oligonucleotides were purchased from BEX (Japan). They were purified by reverse-phase high performance liquid chromatography. The vector pET-15b and host *Escherichia coli* BL21(DE3) (*hsdS gal* [λcI*ts*857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene*1*]) strain were purchased from Novagen. *Taq* DNA polymerase, DNA ligation kit, restriction endonucleases and other modifying enzymes were obtained from TaKaRa Shuzo and TOYOBO. All other chemicals were of analytical grade. Wild-type mavicyanin was prepared as described previously (2).

*Gene design and synthesis.*    The sequence of the synthetic mavicyanin gene was optimized for the expression system in *E. coli* cell. Seven overlapping oligonucleotides were used to construct the synthetic gene by a recursive PCR technique (20, 21). According to Dyson's method, gene assembly and amplification were carried out in two separate PCR reactions in MJ Research MiniCycler. The first recursive assembly PCR mixture contained 4 pmol each of the seven oligonucleotides and forward primer, 2.5 units of *Taq* DNA polymerase, and 0.25 mM of each deoxynucleotide triphosphate (dNTP) in a final volume of 100 μl PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$). The reaction involved a hot start at 90 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 59 °C, and 1 min 10 s at 72 °C. The reaction was completed with a 5 min incubation at 72 °C. Ten microliters of the first PCR reaction mixture were used for the second PCR. The reaction mixture of the amplification PCR contained 100 pmol of each of the forward and the reverse primers, 2.5 units of *Taq* DNA polymerase, and 0.25 mM of each dNTP in a final volume of 100 μl PCR buffer. The thermal cycling reaction was for 30 s at 95 °C, 1 min at 52 °C, and 1 min at 72 °C. Thirty cycles were repeated and followed 5 min incubation at 72 °C. The PCR products were digested with *Nco*I and *Bam*HI and cloned into expression plasmid pET-15b (Novagen) to yield pMAV1-1. The inserted synthesized gene was confirmed by DNA sequencing with an Applied Biosystems 373A DNA sequencer.

*Expression and purification of   recombinant mavicyanin.    Escherichia coli* BL21(DE3) cells carrying pMAV1-1 were grown at 37 °C in 1 l of LB broth containing 50 mg/ml ampicillin until $OD_{600} = 0.5 - 1.0$, and induced with 1 mM IPTG. After another 5 h of incubation at the same temperature, the cells were harvested by centrifugation (3000 x g, 10 min) and washed twice with 30 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl (buffer A).

The cells (wet weight, 4 g) suspended in 20 ml of buffer A were disrupted well with KUBOTA insonater 201M ultrasonic oscillator. After centrifugation, the pellets (inclusion bodies) were washed with 1 M glucose (20 ml) and suspended in 2% Triton X-100, 10 mM EDTA solution (100 ml), being left overnight at 4 °C. The washed pellets were solubilized with 5 ml of buffer B (30 mM Tris-HCl pH 7.5, 30 mM NaCl, 1 mM DTT) supplemented 8 M urea, and dialyzed against buffer B containing 4 M urea at 4 °C for 12 h. After another 24 h dialysis against buffer B, solubilized crude recombinant apoprotein was reconstituted by dialysis against   20 mM Tris-HCl (pH 7.5) containing 1 mM $CuSO_4$ for overnight at 4 °C. Reconstituted protein was dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and applied onto a CM-Sephadex column (φ2.5 x 12.5 cm) pre-equilibrated with the same buffer. After the unabsorbed proteins were washed out with the 10 mM buffer, the recombinant mavicyanin was eluted with 20 mM buffer. The fractions containing mavicyanin identified by SDS-

PAGE analysis were pooled and the recombinant protein thus purified was used for subsequent experiments.

*Mutagenesis*.   Site-directed mutagenesis were performed by the two-step PCR method reported by Higuchi (22). The two DNA fragments overlapping 20 bp each other and containing the same mutation (Gln-95 to Met, underlines) were amplified separately using the oligonucleotide primers shown below and pMAV1-1 as a template (1st PCR).

Q95M-sense: 5'-GCCAGCTGGGT<u>ATG</u>AAAGTGGAG-3'
Q95M-antisense: 5'-CTCCACTTTC<u>AT</u>ACCCAGCTGGC-3'

After the second PCR using primers T7 promoter and T7 terminator (Novagen), the amplified fragment was digested with *Nco*I and *Bam*HI and cloned into pET-15b to yield pMAV-Q95M. The mutant mavicyanin gene was sequenced and expressed in *E. coli* BL21(DE3) as wild-type mavicyanin.

*Spectroscopy and cyclic voltammetry*.   UV-visible absorption and CD spectra of recombinant wild-type and mutant mavicyanin were measured at room temperature with a Shimadzu UV-2200 recording spectrophotometer and a JASCO J-500 spectropolarimeter equipped with a JASCO DP-501 data processor, respectively. The EPR spectra were recorded with a JEOL JES-FE1X X-band spectrometer at 77K.
    Cyclic voltammetry was performed using a Bioanalytical Systems Model CV-50W voltammetric analyzer. Modification of the gold electrode with bis-(4-pyridyl) disulfide was carried out, as descried previously (23).

**RESULTS AND DISCUSSION**

*Design and assembly of the synthetic gene*.   In order to bypass the difficulties of cDNA cloning, we have synthesized a gene coding for 109 amino acid, non-glycosylated zucchini mavicyanin. A gene coding for mavicyanin was designed with the guideline described in (20). Only the two most common codons for each amino acid were selected. Further high expression level of the designed gene requires the absence of palindromic sequences that can form hairpin loops and low overall G + C content. Long sequence repeats were also not permitted to prevent mispriming event during the PCR reactions. The optimized sequence of the synthetic mavicyanin gene is shown in Fig. 1. The seven oligonucleotides representing four fragments of the coding and three of the non-coding strand were chemically synthesized. Their lengths varied between 54 and 74 nucleotides. Neighboring oligonucleotides were overlapped by 20 or 21 bases.

4

Both ends were flanked by restriction sites for *Nco*I (5' end) and *Bam*HI or *Hin*dIII (3' end) for insertion in the desired linearized expression vector.

The full-length gene was assembled from the overlapping oligonucleotides by a recursive PCR method (20, 21). Gene assembly and amplification were carried out in two different PCR reactions, as described in (21). The final product shows a single band with the expected molecular weight on an agarose gel (data not shown), being inserted into the linearized pET-15b to yield pMAV1-1 (6.0 kbp). The insert of pMAV1-1 had the correct nucleotide sequence over the entire length of the synthetic gene.

*Protein expression, purification, and reconstitution.*    When *E. coli* BL21 (DE3) cells harboring pMAV1-1 was cultivated in the presence of    IPTG, distinct amount of recombinant apo-mavicyanin was produced as insoluble inclusion bodies. The inclusion bodies were solubilized by 8 M urea and the recombinant protein was purified with cation-exchange chromatography to a single protein band on SDS-PAGE (Fig. 2). Approximately 10 mg of the recombinant protein was obtained from 4 g of wet cells (1 l culture medium). The amino-terminal sequence was determined by means of automated Edman degradation with an Applied Biosystems 470A gas-liquid-phase protein sequencer. The result indicated that the initiator methionine residue is removed during purification (see Fig.1). The molecular mass of the recombinant apo-protein (calcd. 11,747 Da) was obtained to be 11,745.0 Da and 11,745.6 Da by ESI and MALDI-TOF mass spectroscopies, respectively (24). These data suggest that the primary structure of the recombinant protein is identical to non-glycosylated wild-type mavicyanin. The recombinant apo-mavicyanin was completely reconstituted with Cu (II) ion.

*Characterization of recombinant mavicyanin.*    The electronic absorption, CD, and EPR spectra of recombinant mavicyanin are compared to the wild-type protein isolated from zucchini in Figs. 3, 4, and 5, respectively. The spectroscopic properties of recombinant mavicyanin are identical to those of the wild-type protein. The visible absorption spectra (Fig. 3, solid and dotted lines) show three peaks at 448, 559, and 830 nm, which are assigned as S(Cys)-Cu(II) charge transfer transitions. The visible CD spectra display a positive extremum at 590 nm and two troughs at 444 and 780 nm (Fig. 4, solid and dotted lines). The 77-K EPR signals of these proteins are almost identical, showing a rhombic symmetry (Fig. 5, a and b). It is interesting that the characteristic spectroscopic properties of mavicyanin are retained even when the carbohydrate moiety is removed.

The cyclic voltammogram of recombinant mavicyanin shows a well-defined response with the midpoint potential of $E_{1/2}$ = +213 mV *versus* NHE and the peak-to peak separation of $\Delta Ep$ = 68 mV at pH 7.0 (data not shown). The midpoint potential is

close to the potential (+184 mV) of stellacyanin (25). The midpoint potential of wild-type mavicyanin was reported to be +285 mV (pH 7.0) by reductive titration methods (1). The difference between the midpoint potentials may be due to the carbohydrate moiety.

*Characterization of mutant mavicyanin.*    The Gln95→Met (Q95M) mutation was introduced in the synthetic mavicyanin gene by PCR mutagenesis. The mutant was expressed and purified with same procedures as the recombinant protein.

The electronic absorption spectrum of Q95M mavicyanin is presented with that of the wild type (Fig. 3). The absorption bands of wild-type mavicyanin at 599 and 448 nm are slightly shifted to higher wavelengths by the replacement of Gln95. Both the 602- and 454-nm bands of Q95M mavicyanin decrease the intensities compared to the wild-type protein. The broad band around 830 nm in the wild type is moved to 750 nm in the mutant. These three absorption bands are all indicative of S(Cys)-Cu(II) charge transfer transitions (26, 27).

In the CD spectrum of Q95M mavicyanin, the absorption band near 400 nm probably arising from a S(Met)-Cu(II) charge transfer transition (Fig. 4, broken line) appears, as observed in the CD spectra of the Met-coordinate blue copper proteins like azurin and plastocyanin (28, 29). On the other hand, the Gln-coordinate blue copper proteins like mavicyanin and stellacyanin show no extremum at this region (2).

The X-band EPR spectra for wild-type and Q95M mavicyanins are exhibited in Fig. 5. The replacement of Gln with Met changes from a rhombic signal to an axial signal which is very similar to those of azurin and plastocyanin (28, 29). The rhombicity of the EPR signal is correlated with the displacement of the Cu atom from the trigonal plane composed of two His and one Cys ligands in the direction of the fourth ligand (30). Namely, Cu proteins with a relatively strong axial ligand and a Cu ion that is about 0.3 Å above the NNS plane defined by 2N (His) and S (Cys) have rhombic EPR signals (pseudoazurin and stellacyanin) (13, 15), while Cu proteins with a weak axial ligand and a Cu ion that is about 0.1 Å out of the NNS plane show axial EPR signals (azurin and plastocyanin) (28, 29).

The midpoint potential of Q95M mavicyanin ($E_{1/2}$ = +400 mV, $\Delta Ep$ = 63 mV) shows the positive shift of 187 mV compared with the recombinant protein and close to the values of plastocyanin ($E_{1/2}$ = +370 - +390 mV) (31). The binding of Met to Cu ion instead of Gln stabilizes the reduced state of the active center. The negatively shifted potential of mavicyanin compared with the values of the Met-coordinate blue copper proteins would be related with its biological roles.

Recently, the crystal structure of cucumber stellacyanin has been reported (14). The tetrahedral copper ion of stellacyanin is coordinated by His46, Cys89, His94 and Gln99. Despite of low homology score (overall similarity is 50%), all these ligands in

stellacyanin are conserved in mavicyanin. In this work, the spectroscopic and electrochemical properties of Q95M mutant indicate that Gln95 is the fourth ligand of the blue copper center in mavicyanin.

## ACKNOWLEDGMENTS

## REFERENCES

1. Marchesini, A., Minelli, M., Merkle, H., and Kroneck, P. M. (1979) *Eur. J. Biochem.* **101,** 77-84.

2. Maritano, S., Marchesini, A., and Suzuki, S. (1997) *J. Biol. Inorg. Chem.* **2,** 177-181.

3. Adman, E. T. (1985) in: Topics in molecular and structural biology (Harrison P.M., Ed), Metalloproteins, vol 6, pp. 1-42, MacMillan, London.

4. Adman, E. T. (1991) *Adv. Protein Chem.* **42,** 145-197.

5. Ryden, L. G. and Hunt, L. T. (1993) *J. Mol. Evol.* **36,** 41-66.

6. Schinina, M. E., Maritano, S., Barra, D., Mondovi, B., and Marchesini, A. (1996) *Biochim. Biophys. Acta* **1297,** 28-32.

7. Murata, M., Begg, G. S., Lambrou, F., Leslie, B., Simpson, R. J., Freeman, H. C., and Morgan, F. J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79,** 6434-6437.

8. Bergman, C., Gandvick, E.-K., Nyman, P. O., and Strid, L. (1977) *Biochem. Biophys. Res. Commun.* **77,** 1052-1059.

9. Van Driessche, G., Dennison, D., Sykes, A. G., and Van Beeumen, J. (1995) *Protein Sci.* **4,** 209-227.

10. Mann, K., Schafer, W., Thoenes, U., Messerschmidt, A.,. Mehrabianm, Z., and Nalbandyan, R. (1992) *FEBS Letters* **314,** 220-223.

11. Drew, J. E. and Gatehouse, J. A. (1994) *J. Exp. Bot.* **45,** 1873-1884.

12. Van Gysel, A., Van Montagu, M., and Inze, D. (1993) *Gene* **136,** 79-85.

13. Guss, J. M., Merritt, E. A., Phizackerley, R. P., and Freeman, H. C. (1996) *J. Mol. Biol.* **262,** 686-705.

14. Hart, P. J., Nersissian, A. M., Herrmann, R. G., Nalbandyan, R. M., Valentine, J. S., and Eisenberg, D. (1996) *Protein Sci.* **5,** 2175-2183.

15. Nersissian, A. M., Mehrabian, Z. B., Nalbandyan, R. M., Hart, P. J., Fraczkiewicz, G., Czernuszewicz, R. S., Bender, C. J., Peisach, J., Herrmann, R. G., and Valentine, J. S. (1996) *Protein Sci.* **5,** 2184-2192.

16. Romero, A., Hoitink, C. W. G., Nar, H., Huber, R., Messerschmidt, R., and Canters, G. W. (1993) *J. Mol. Biol.* **229,** 1007-1021.

17. Chang, T. K., Iverson, S. A., Rodrigues, C. G., Kiser, C. N., Lew, A. Y., Germanas, J. P., and Richards, J. H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88,** 1325-1329.

18. Suzuki, S., Kataoka, K., Nakai, M., Kondo, A., Yamaguchi, K. in preparation.

19. Hibino, T., Lee, B.H., Takabe, T., and Takabe, T. (1995) *J. Biochem. (Tokyo)* **117,** 101-106.

20. Casimiro, D. R., Toy-Palmer, A., Blake, R. C., and Dyson, H. J. (1995) *Biochemistry* **34,** 6640-6648.

21. Prytulla, S., Dyson, H. J., and Wright, P. E. (1996) *FEBS Letters* **399,** 283-289.

22. Higuchi, R. (1989) in: PCR technology (Erlich, H.A., Ed), pp. 61-88, Stockton Press, New York.

23. Kohzuma, T., Shidara, S., and Suzuki, S. (1994) *Bull. Chem. Soc. Jpn* **67,** 138-143.

24. Fukuo, T., Kubota, N., Kataoka, K., Nakai, M., Suzuki, S., and Arakawa, R. to be published.

25. Reinhammar, B. R. M. (1972) *Biochem. Biophys. Acta* **275,** 245-259.

26. Solomon, E. I., Hare, J. W., Dooley, D. M., Dawson, J. H., Stephens, P. J., and Gray, H. B. (1980) *J. Am. Chem. Soc.* **102,** 168-178.

27. Gewirth, A. A., Cohen, S. L., Schugar, H. J., and Solomon, E. I. (1987) *Inorg. Chem.* **26,** 1133-1146.

28. Yamaguchi, K., Deligeer, Nakamura, N., Shidara, S., Iwasaki, H., and Suzuki, S. (1995) *Chem. Letters* 353-354.

29. Suzuki, S., Sawada, S., Nakahara, A., and Nakajima, T. (1986) *Biochem. Biophys. Res. Commun.* **136,** 610-615.

30. Andrew, C. R., Yeom, H., Valentine, J. S., Karlsson, B. G., Bonander, N., Pouderoyen, G. von, Canters, G. W., Loehr, T. M., and Loehr, J. S. (1994) *J. Am. Chem. Soc.* **116,** 11489-11498.

31. Sanderson, D. G., Anderson, L. B., and Gross, E. L. (1986) *Biochem. Biophys. Acta* **852,** 269-278.

**FOOTNOTES**

*To whom correspondence should be addressed. Telephone: +81-6-850-5768, Fax: +81-6-850-5785, E-mail: kataoka@ch.wani.osaka-u.ac.jp

Abbreviations: CBP, cucumber basic protein; EPR, electron paramagnetic resonance; ESI, electrospray ionization; dNTP, deoxyribonucleotide; IPTG, isopropyl-1-thio-β-D-galactoside; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; NHE, normal hydrogen electrode; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate

**FIGURE LEGENDS**

FIG. 1. Sequence of the seven overlapping oligonucleotides used to assemble the mavicyanin gene. The short sequences at termini (lower case) correspond to the amplification primers used in the second PCR. The amino acid sequence is shown below (6) the coding region of the DNA. The N-terminal sequence of the recombinant protein is underlined and four ligand residues of Cu are boxed. The restriction sites designed are also shown.

FIG. 2. SDS-PAGE analyses of mavicyanin expression and purification. Lanes 1 and 5: Standard proteins (Bio-Rad). Lane 2: Total cell lysate of *E. coli* BL21(DE3) harboring pMAV1-1. Lane 3: Insoluble fraction of the lysate. Lane 4: Purified recombinant mavicyanin.

FIG. 3. Electronic absorption spectra of wild-type (solid line), recombinant (dotted line), and Q95M (broken line) mavicyanins in 0.1 M potassium phosphate buffer (pH 7.0) at room temperature.

FIG. 4. CD spectra of wild-type (solid line), recombinant (dotted line), and Q95M (broken line) mavicyanins in 0.1 M potassium phosphate buffer (pH 7.0) at room temperature.

FIG. 5. X-band EPR spectra of wild-type (a), recombinant (b), and Q95M (c) mavicyanins in 0.1 M potassium phosphate buffer (pH 7.0) at 77K.

Fig. 1

```
    1        Nco I                                                              90
5' CAGACCATGGCGACCGTGCACAAAGTGGGTGATAGTACCGGTTGGACCACGCTGGTGCCATACGATTACG 3'
5' cagaccatggcgaccgtgcac 3'(reverse)                 3' GCGACCACGGTATGCTAATGCGCTTCACCCGCTCGTCGTTG

     MetAlaThrValHisLysValGlyAspSerThrGlyTrpThrThrLeuValProTyrAspTyrAlaLysTrpAlaSerSerAsn


    91                                                    Pst I                  180
     5' ACGTGGGTGATAGCCTGCTGTTTAACTACAACAATAAATTTCACAACGTGCTGCAGGTGGATCAGGAACAG 3'
   TTTAAAGTGCACCCACTATCGGACGAC 5'              3' GACGTCCACCTAGTCCTTGTCAAATTTTCGACG
                                                        44
   LysPheHisValGlyAspSerLeuLeuPheAsnTyrAsnAsnLysPhe His AsnValLeuGlnValAspGlnGluGlnPheLysSerCys


    181                                             Kpn I                        270
           5' GAGCTACACCAGCGGTGCCGATAGCATCCCGCTGAAGCGCCCAGGTACCTTTTACTTTCTGTGCGGTATC 3'
   TTGTCGAGATCGGGTCGCCGCTCGATGTGGTCGCCACGGCT 5'           3' GAAAGACACGCCATAG
                                                                        85
   AsnSerSerSerProAlaAlaSerTyrThrSerGlyAlaAspSerIleProLeuLysArgProGlyThrPheTyrPheLeu Cys GlyIle


    271 Sma I                                         BamHI  HindIII  353
   CCGG 3'                    5' GGAGATCAAGGTTGATCCGGGTAGCAGCAGCGCGTAAGGATCCAAGCTTGGCAC 3'
   GGCCGGTGACGGTCGACCCAGTCTTTCACCTCTAGTTCCAACTAGGCC 5'      3'cgcattcctaggttcgaaccgtg 5'(forward)
              90                95
   ProGly His CysGlnLeuGly Gln LysValGluIleLysValAspProGlySerSerSerAla***
```
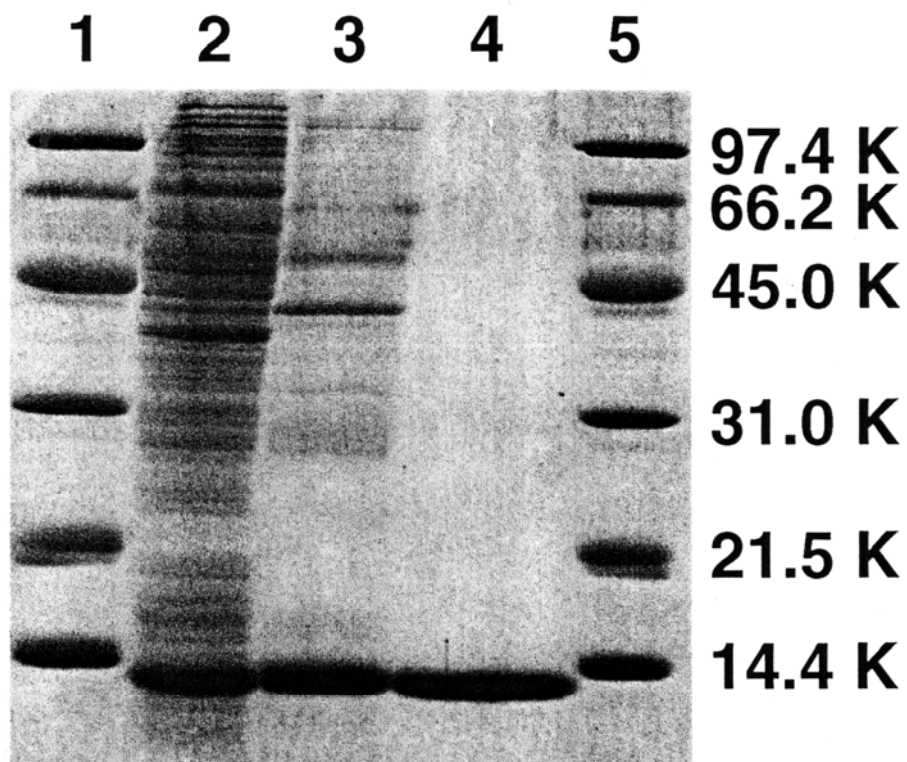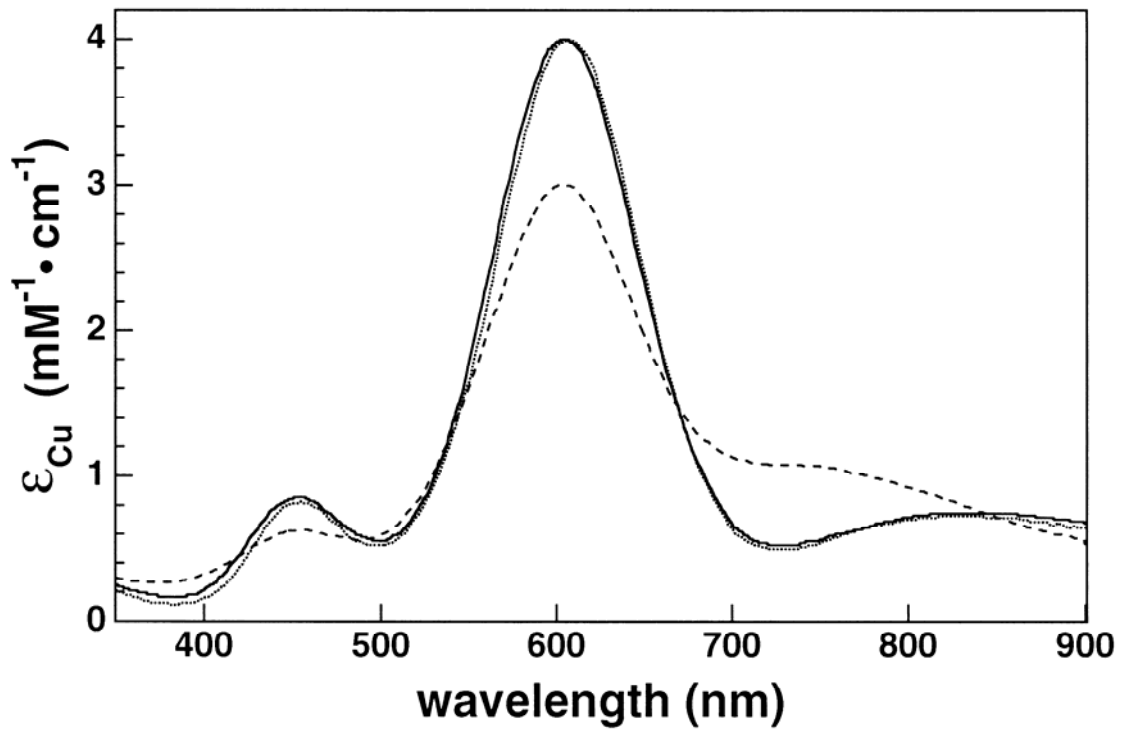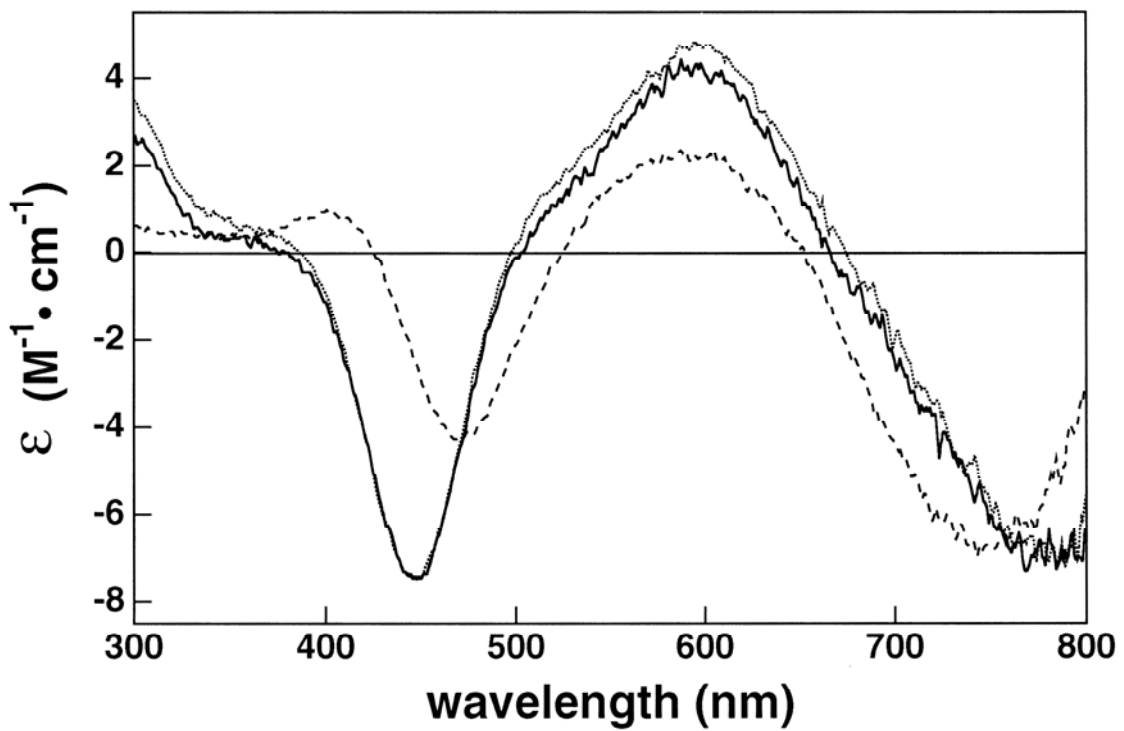
1

Fig. 2



| | 1 | 2 | 3 | 4 | 5 | |
|---|---|---|---|---|---|---|
| | | | | | | 97.4 K |
| | | | | | | 66.2 K |
| | | | | | | 45.0 K |
| | | | | | | 31.0 K |
| | | | | | | 21.5 K |
| | | | | | | 14.4 K |

Fig. 3



Fig. 4

Fig. 5



magnetic flux density (T)