Studies on the b-Type Cytochromes from Mung Bean Seedlings

III. THE STRUCTURE OF CYTOCHROME b 555*

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In the previous papers of this series (1, 2), some of the properties of purified mung bean cytochrome b-555 have been described. The present investigation was undertaken to gain some information on the chemical structure of this hemoprotein. The size, amino acid composition, and terminal amino acids of cytochrome b-555 have been examined; the nature of the binding between heme and protein has also been studied.

EXPERIMENTAL PROCEDURE

The methods used for purification of mung bean cytochrome b-555 from etiolated seedlings of Phaseolus aureus were described earlier (1). The sample used for this study was homogeneous in the ultracentrifuge and migrated as a single band on electrophoresis in a starch bed. The ratio of the absorbancy at 280 mμ to that of the reduced a-band at 555 mμ was approximately 1. It was reported previously (2) that the purified preparation contained some ultraviolet-absorbing material that did not sediment with the protein in the ultracentrifuge. This was subsequently found to originate from the dialysis tubing (Visking) used during the purification; the material could be removed from the tubing by boiling in 0.1 N acetic acid for 30 minutes, followed by washing with water. The methods used for the preparation of the apoprotein of cytochrome b-555 and the reconstitution of the pigment were described earlier (2).

Absorption spectra were recorded with a Cary 14 spectrophotometer; a Beckman DU spectrophotometer equipped with photomultiplier attachment was used for some of the absorbancy measurements. Molar extinction coefficients of heme chloride were determined in a solution of protoheme IX (Eastman Organic Chemicals) in acid-acetone (0.5 ml of concentrated HCl in 100 ml of acetone), and values somewhat lower than those calculated from the data of Lewis (3) were obtained. The value of ε664 = 7.84 × 104 at 539 mμ was used to determine the concentration of protoheme.

The procedure used for the preparation of the pyridine hemochrome of cytochrome b-555 was essentially the same as that described previously (2), except that it was carried out under anaerobic conditions in a Thunberg cuvette. This precaution was taken because the absorbancy of the pyridine hemochrome at 556 mμ changed slowly after reduction by Na2S2O4 in the presence of air (4). The molar extinction coefficient of pyridine hemochrome at 556 mμ was determined to be 34 × 104, which agreed closely with previously reported values (5).

The amino acid hemochromes were prepared by adding 5 × 10^{-5} M protoheme in acid-acetone to 0.01 M solutions of the amino acids, buffered at various pH values. For the preparation of the histidine hemochrome, the amino acid solution was adjusted to pH 7.5 with concentrated NaOH, and 3 ml of a 0.68 M histidine solution was mixed with 0.35 ml of 1.6 × 10^{-4} M protoheme in acid-acetone. In each case, the absorption spectrum was recorded 5 minutes after the protoheme and amino acid were mixed.

Preparation of Dimethyl Ester of Protoheme IX—Recrystallized protoheme, 0.5 g, was methylated with HCl-saturated methanol (6), and approximately 0.1 g of the final product was obtained after two recrystallizations from a chloroform-methanol mixture. The product was examined by paper chromatography with three different solvent systems, by the methods described by Chu and Chu (7) and Morrison and Stotz (8). In every case, only one spot was detected on the chromatograms after they were sprayed with benzidine reagent. The Rf values were 1.0 with 2,6-lutidine-water (55:45) and 0.0 in either water-propanol-pyridine (55:1:4) or 0.05% formic acid-hexane-chloroform (2:10:88). The recrystallized protoheme dimethyl ester showed absorption bands at 695, 574, 540, 505, and 405 mμ in chloroform and at 642, 604, 557, 415, and 369 mμ in acid-acetone.

Preparation of Deuteroheme—Deuteroheme was prepared by the method of Fischer and Hummel (9), and a yield of 0.34 g of twice recrystallized deuteroheme was obtained from 0.5 g of protoheme. On chromatography with a mixture of pyridine-propanol-H2O (4:1:55) and silicone-impregnated Whatman No. 1 filter paper (8), the deuteroheme migrated as a single spot with an Rf of 0.81. In the same solvent, protoheme migrated with an Rf of 0.43. The oxidized form of the deuteroheme in 0.1 N NaOH showed absorption bands at 381, 485, and 596 mμ; when deuteroheme was reduced with Na2S2O4, the bands shifted to 404, 514, and 543 mμ. In acid-acetone, the absorption bands were found at 451, 500, 529, 525, and 628 mμ.

Photooxidation—Photooxidation of the cytochrome was carried out as follows: 0.2 ml of 0.02% methylene blue in distilled water was added to 2 ml of cytochrome b-555 (5.7 × 10^{-4} M) either in distilled water or in 0.1 M phosphate buffer, pH 7.5. Test tubes containing this solution were placed in a water bath at 31° and irradiated with two 250-watt white lamps at a distance...
of approximately 1 cm. At appropriate intervals, samples were removed and cooled to room temperature. In order to eliminate the methylene blue, a small amount of activated charcoal was added to the sample and the solution was centrifuged; absorption spectra of the supernatant solution, both before and after reduction by hydroxylamine, were then recorded. A similar procedure was used to study the photooxidation of the apoprotein prepared from cytochrome b-555. Proteoheme was added to the irradiated apoprotein, the spectrum was recorded, and the amount of reconstitution was estimated.

**Amino Acid Analyses**—Cytochrome b-555, 0.8 mg, was hydrolyzed with 1 ml of 6N HCl in a sealed tube at 110° for 30 hours, and the amino acid composition of the hydrolysate was determined with a Spinco model MS automatic amino acid analyzer. The tryptophan content of the protein was estimated both by a colorimetric method (10) and by a spectral method (11). The cysteine content was determined by the method of Boyer (12): to 5-ml aliquots of 1 X 10⁻⁴ M cytochrome b-555 in 0.1 M phosphate buffer (pH 7.5) containing 8 M urea, known volumes of 3.08 X 10⁻⁴ M p-hydroxymercuribenzoate were added and the increase in absorbancy at 255 mp was recorded after 10 minutes.

To reduce the disulfide linkages, a few grains of NaBH₄ were added to the urea-containing solution of the cytochrome, which was then incubated at 51° for 1 hour (13). After the excess borohydride was removed by adding a few drops of 0.1 N HCl, the solution was neutralized with 0.1 N NaOH and the number of sulfhydryl groups was determined by titration with p-hydroxymercuribenzoate. The histidine content was determined colorimetrically by the method of Hunter (14) after the tyrosine had been destroyed with KMnO₄ (15).

The NH₂-terminal amino acid was determined by treating cytochrome b-555 with dinitrofluorobenzene and separating the dinitrophenol derivatives in the acid hydrolysate by two-dimensional chromatography, as described by Fraenkel-Conrat, Harris, and Levy (16). The COOH-terminal amino acids were determined by identifying the free amino acids present after hydrazinolysis of the cytochrome (16); the amount of each acid formed during the hydrazinolysis was determined with the automatic amino acid analyzer.

### RESULTS

**Amino Acid Composition**—The results of the quantitative amino acid analyses are shown in Table I. In order to compare the amounts of the different acids, the concentrations are expressed as mole ratios, with a value of 1 mole of tryptophan assumed per mole of cytochrome b-555. This value is based on the results of the colorimetric determination, which gave a value of 0.76 mole of tryptophan per mole of protein. The less reliable spectral method gave values ranging from 1 to 2 moles of tryptophan. The total number of amino acids per mole of protein was 122, and there were an additional 12 moles of ammonia formed on hydrolysis.

The results of the titration of cytochrome b 555 with p-hydroxymercuribenzoate are shown in Fig. 1; no free, reactive sulfhydryl groups could be detected either in the native or in the urea-treated sample. This was confirmed by the demonstration that these samples gave a negative reaction with the nitroprusside reagent. After the protein had been reduced by NaBH₄ in the presence of 8 M urea (13), however, 6.1 sulfhydryl groups could be titrated per mole of cytochrome b-555; this indicates that there are three disulfide linkages in the native protein.

After the treatment of cytochrome b-555 with dinitrofluorobenzene, only one DNP⁻¹ derivative could be identified clearly in the hydrolysate and this was found to be DNP-alanine; several other faint yellow spots could be seen on the paper chromatograms. Spectrophotometric analysis showed that 0.03 μmole of DNP-alanine was obtained from 0.08 μmole of cytochrome. This corresponds to a yield of 37.5% of the theoretical, uncorrected for destruction during hydrolysis, losses during isolation, etc. The corrected yield is estimated to be

<table>
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<th>Amino acid</th>
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<th>Mole ratio</th>
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<tr>
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<td>14</td>
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<tr>
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<tr>
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<td>8</td>
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* See the text.
somewhat over 50%. After the hydrazinolysis of 0.054 μmole of cytochrome b-555, 0.052 μmole of serine, 0.048 μmole of glycine, and 0.044 μmole of alanine were isolated.

**Molecular Weight and Extinction Coefficients**—In the previous paper (2), the minimal molecular weight of cytochrome b-555 was estimated to be approximately 12,000 on the basis of its sedimentation coefficient. From the amino acid composition described above, the minimal molecular weight is calculated to be approximately 13,500. A third estimate of the size can be made if the amount of pyridine hemochrome that is formed from a known weight of the cytochrome is known. A solution of cytochrome b-555 was dried at 110° to a constant weight. On treatment with alkaline pyridine, 1 ml of a solution containing 0.180 mg, dry weight, gave 0.0124 μmole of the pyridine hemochrome. This indicates a minimal molecular weight of approximately 14,500. The fairly good agreement of this value with the other estimates indicates that there is only 1 heme per mole of cytochrome and that there is little contaminating nonprotein material.

By assuming a molecular weight of 14,000, the molar extinction coefficients of cytochrome b-555 can be calculated, and these are listed in Table II. These values were used to determine the amount of protoheme required to reconstitute a given amount of cytochrome b-555. Fig. 2 shows that the amount reconstituted was directly proportional to the amount of protoheme added, and that exactly 1 mole of b-555 was formed on addition of 1 mole of protoheme. This confirmed the fact that there was only 1 heme per mole of protein. Since the reconstituted pigment showed the characteristic asymmetry of the reduced α-band, this property clearly was not due to the presence of more than one prosthetic group.

**Heme to Protein Binding**—In order to investigate the nature of the linkage between heme and protein, some of the structural requirements for the reconstitution of the cytochrome from apoprotein and protoheme were examined. To determine whether the two free carboxyl groups of protoheme IX were essential, the dimethyl ester was prepared. When this was added to the apoprotein, the spectral changes indicated that the reconstitution was only 6% of that obtained with unesterified protoheme. This result strongly suggests that the carboxyl groups of protoheme are involved in the binding.

The role of the vinyl groups of protoheme was tested with the use of deuteroheme, in which the two vinyl groups are replaced by hydrogens. When deuteroheme was added to apocytochrome b-555, a complex having a spectrum similar to, but not identical with, that of cytochrome b-555 formed immediately (Fig. 3). In the oxidized state, the Soret peak was at 401 μm and there were two broad bands at approximately 530 and 560 μm; on reduction with hydrosulfite, sharp bands appeared at 409, 516, 542, and 546 μm. The last two peaks made up a double headed α-band similar to the α-band of cytochrome b-555 at -190° (1). All of the bands of this deuteroheme-protein complex were at wave lengths approximately 13 μm lower than for the corresponding bands in b-555. Since the absorption bands of deuteroheme itself were shifted to wave lengths approximately 10 μm lower than those of protoheme, this, rather than any difference in heme-protein interaction, might account for the spectral shifts. These results suggest that the vinyl groups of protoheme are not directly involved in the binding to the apoprotein of cytochrome b-555.

**Protoporphyrin IX**, which lacks the central iron atom, did not replace protoheme in the reconstitution of cytochrome b-555. When it was added to the apoprotein, a complex with weak absorption bands at 500, 533, 565, and 620 μm and with a Soret band at 402 μm was formed. This spectrum differed strikingly

| Table II: Molar extinction coefficients of cytochrome b-555 |
|-----------------|-----------------|
| Absorption band | μm              | X 10⁴ |
| Oxidized:       |                 |       |
| 560             |                 | 0.85  |
| 429             |                 | 1.02  |
| 413             |                 | 10.5  |
| Reduced:        |                 |       |
| 555             |                 | 2.27  |
| 527             |                 | 1.33  |
| 423             |                 | 15.9  |
from that of cytochrome b-555, suggesting that iron was involved in the binding of heme to protein in the cytochrome. Normal reconstitution takes place with protoheme when the iron is in either the ferric or ferrous state.

It is well known that protoheme forms complexes with a variety of nitrogen-containing compounds, including amino acids (17). The interaction of protoheme with several basic amino acids was examined in the hope that this might suggest which groups on the protein are involved in binding the heme. At pH values lower than 6, all of the amino acids combined with protoheme to form hemochromes with a weak Soret band around 418 mp. At pH 7.0, the only amino acids that formed an appreciable amount of hemochrome were tryptophan and histidine, and only in the latter case did the spectrum resemble that of a cytochrome. Fig. 4 shows the remarkable similarity of the absorption spectra of histidine hemochrome and cytochrome b-555, in both the oxidized and reduced forms. Since the cytochrome contains 4 histidine residues, this amino acid could be involved in binding the heme.

More direct evidence for the role of histidine in the binding of heme was obtained from studies on the photooxidation of the protein in the presence of methylene blue; this treatment is known to destroy the histidine residues in a variety of proteins (18). When cytochrome b-555 was irradiated for 30 minutes, there was little change in the absorption spectrum, even though the histidine content, as determined colorimetrically after hydrolysis of the protein, did decrease (Fig. 5). When apocytochrome b-555 was irradiated, it rapidly lost its ability to recombine with protoheme and reconstitute the cytochrome with its characteristic reduced a-band at 555 mp (Fig. 5). The irradiated apoprotein did combine with protoheme to form a complex with a Soret band, indicating that some nonspecific heme binding can take place.

Effects of Concentrated Urea Solutions - In an attempt to examine the relationships between the three-dimensional structure of the protein and the absorption spectrum, a sample of cytochrome b-555 in 0.1 m phosphate buffer, pH 7.5, was mixed with an equal volume of 12 m urea and allowed to stand at room temperature. As shown in Fig. 6, there was an immediate 24% decrease in the height of the Soret peak; thereafter, there was no significant change for 20 hours. The urea-treated cytochrome did not combine with CO. The spectral change caused by urea was only partially reversible; after dialysis against water for 20 hours at 5°C, the absorbancy at 413 mp was 0.40, whereas that of the undialyzed, urea-containing control was 0.34 (initial absorbancy at 413 mp = 0.50). A more drastic effect was obtained when the disulfide linkages in the cytochrome were reduced by NaBH₄ in the presence of urea (13); under these conditions, the absorption spectrum disappeared completely and did not reappear on aeration. When a solution of horse heart cytochrome c (Sigma) of comparable concentration was mixed with an equal volume of 12 m urea, there was no decrease in the height of the Soret peak, but rather a slight increase.

A solution of the apoprotein of cytochrome b-555 was treated with an equal volume of 12 m urea and allowed to stand at room temperature for 1 hour. When protoheme was added to an aliquot of the solution, there was no evidence of any reconstitution of the cytochrome. Dialysis of the urea-treated apoprotein
Although only one NH₂-terminal amino acid, alanine, was identified, the yield was too low to make it certain that there is only one such end group. The presence of traces of other DNP-derivatives in the hydrolysates could be due either to low molecular weight contaminants or to partial degradation of the cytochrome during the isolation. After hydrazinolysis, three free amino acids were obtained in good yield. Although this suggests that there is more than one COOH-terminal group in the protein, the possibility of cleavage of peptide bonds during hydrazinolysis cannot be ruled out. Definitive characterization of the COOH-terminal acid must await analysis by another method, such as digestion with carboxypeptidase.

Several conclusions can be drawn with respect to the linkages that bind the heme in cytochrome b 555. The vinyl groups of protoheme do not appear to be essential for the binding, although the absence of these groups does slightly alter the spectrum, as expected. It is of interest that horseradish peroxidase having full activity can be reconstituted from its apoprotein and deuteroheme (23). One or both of the carboxyl groups of the propionic acid side chains of protoheme appear to be essential for the binding in cytochrome b-555, since the dimethyl ester does not combine with the apoprotein. A salt linkage probably is formed between the carboxyl group and some basic amino acid in the protein. A similar type of heme to protein binding is found in horseradish peroxidase (23, 24) and in the complex formed between heme and serum albumin (25). In hemoglobin and myoglobin, on the other hand, the carboxyl groups are not essential: artificial hemoglobin (26) and myoglobin (27) can be prepared from hemes without any free carboxyl groups.

The iron of protoheme probably is involved in binding it to the protein to make cytochrome b-555. The complex formed between protoporphyrin IX and the apoprotein shows spectral characteristics that are strikingly different from those of the cytochrome. In some hemoproteins, such as hemoglobin, the iron is known to be linked to the protein. In the case of peroxidase, however, reconstitution of a complex with the characteristic Soret band can be carried out with the apoprotein and either protoheme or protoporphyrin (24). The iron in protoheme can combine with a variety of ligands, and one possibility in proteins is the imidazole group of histidine. The general features of the absorption spectra of histidine hemochromes are strikingly similar to those of cytochrome b-555. Heme binding in hemoglobin (28) and also in cytochrome b₅ (22) is known to involve this amino acid.

The studies on the photobradylation of cytochrome b 555 suggest that histidine may in fact be involved in binding of the heme to this protein. Irradiation of the apoprotein in the presence of methylene blue rapidly destroys its ability to reconstitute the cytochrome. Although histidine is not the only amino acid destroyed by this treatment, it is the most susceptible to photobradylation (18, 29). Since irradiation of the whole cytochrome b-555 does not markedly alter its absorption spectrum, even though some histidine is destroyed, 1 or more of the histidine residues may be protected or stabilized by binding to the protoheme. Conversely, the presence of the heme may stabilize the protein structure, since concentrated urea solutions do not markedly alter the spectrum of the whole cytochrome but do rapidly destroy the ability of the apoprotein to recombine with protoheme.

Fig. 6. The effect of 6 M urea on the absolute absorption spectrum of cytochrome b-555. ——, control; —-—, 3 minutes after urea treatment.

**DISCUSSION**

Our results indicate that cytochrome b-555 has a molecular weight close to 14,000 and that it contains 1 mole of protoheme per mole of protein. In size, it is rather similar to both cytochromes c and b₅, which have molecular weights between 12,000 and 13,000. The partially purified mammalian cytochrome b is larger, and appears to be made up of monomeric units of approximately 28,000 (19). Cytochrome b₅ is much larger, with a molecular weight of 75,000 (20); the fact that this is a flavohemoprotein suggests that it is considerably more complex.

Cytochrome b-555 is an acidic protein, containing 12 glutamic and 14 aspartic acid residues; some of these must exist in the amide form in the protein, since 12 moles of ammonia were also present in the hydrolysate. In contrast to the basic cytochrome c, which has 19 lysines (21), b-555 has only 9 lysine residues. Cytochrome b-555 has 9 serines, whereas this amino acid is not present in horse heart cytochrome c. There is good evidence that cytochrome b-555 contains three disulfide linkages, and in this respect too it differs markedly from cytochrome c, in which the only cysteine sulfhydryl groups are involved in binding of the heme to the protein. The mung bean cytochrome also differs in composition from those b-type cytochromes that have been analyzed: yeast cytochrome b2 lacks methionine (20), and liver microsomal b₅ has only 3 tyrosine residues (22). Except for the differences noted, there is a general similarity in the amino acid compositions of cytochromes b-555, b₅, and c.

The terminal amino acid analyses gave ambiguous results. Although only one NH₂-terminal amino acid, alanine, was identified, the yield was too low to make it certain that there is only one such end group. The presence of traces of other DNP-derivatives in the hydrolysates could be due either to low molecular weight contaminants or to partial degradation of the cytochrome during the isolation. After hydrazinolysis, three free amino acids were obtained in good yield. Although this suggests that there is more than one COOH-terminal group in the protein, the possibility of cleavage of peptide bonds during hydrazinolysis cannot be ruled out. Definitive characterization of the COOH-terminal acid must await analysis by another method, such as digestion with carboxypeptidase.
sulfhydryl groups are ruled out. The sudden change in the absorption spectrum of cytochrome b-555 above pH 10 (1) suggests the possible involvement of a group with a high pKₐ. Strittmatter (22) has suggested that in cytochrome bs an amino group may participate in the heme binding.

Disulfide linkages play an important role in maintaining the tertiary structure of many proteins, and this may also be true for cytochrome b-555. Treatment of the cytochrome with urea and NaBH₄ unmasks six sulfhydryl groups and at the same time destroys its characteristic absorption spectrum. Urea alone causes a smaller spectral change, presumably by disrupting hydrogen bonds in the native protein. In interpreting these results, it must be borne in mind that the absorption spectrum alone is not necessarily a good indicator of changes in the three-dimensional configuration, as shown recently with catalase and peroxidase (30).

**SUMMARY**

On the basis of amino acid composition, sedimentation characteristics, and pyridine hemochrome analysis, the molecular weight of cytochrome b-555 is estimated to be approximately 13,500. It contains 1 mole of protoheme. On hydrolysis, it yields 122 amino acids and 12 moles of ammonia per mole of protein; there is a preponderance of acidic over basic amino acids.

The native cytochrome b-555 has no free, titratable sulfhydryl groups, but six such groups can be identified after borohydride reduction. Some evidence that disulfide linkages are involved in maintaining the tertiary structure of the protein is presented. Alanine probably is an NH₄-terminal amino acid. After hydrazinolysis, alanine, glycine, and serine were obtained in good yield.

The nature of the linkage between protoheme and apocytochrome b-555 has been investigated by testing the ability of a modified prosthetic group or apoprotein to reconstitute the cytochrome. Reconstitution is prevented by removal of the iron or by methylation of the carboxyl groups of protoheme; it is also prevented by photooxidation or urea treatment of the apoprotein. The results suggest that protoheme iron, the carboxyl groups of protoheme, and histidine in the protein all are involved in the binding.

**REFERENCES**

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