Purification and Some Properties of the Small Subunit of Cytochrome \(b_{558}\) from Human Neutrophils*

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We have attempted to purify the heme moiety of cytochrome \(b_{558}\) from human neutrophils. Cytochrome \(b_{558}\) was solubilized from the crude membrane fraction which was pretreated with both 1 M potassium phosphate buffer and 1% octyl glucoside at low ionic strength. The solubilization of cytochrome \(b_{558}\) was carried out efficiently with 1.6% octyl glucoside in the presence of 100 mM potassium phosphate buffer. Solubilized cytochrome \(b_{558}\) was purified by hydroxylapatite, DEAE-Sephaloc, and Mono Q fast protein liquid chromatography. The specific content of purified cytochrome \(b_{558}\) was 37 nmol/mg of protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified cytochrome \(b_{558}\) revealed a single band of 20,000 Da. The large subunit of cytochrome \(b_{558}\), which has been reported by others, could not be found in purified cytochrome \(b_{558}\) even with silver staining. The amino acid composition of the heme-containing moiety of cytochrome \(b_{558}\) was abundant in hydrophobic amino acids. The circular dichroism spectra of both oxidized and reduced \(b_{558}\)-type cytochromes exhibited bilateral bands with wavelengths of crossover points closely corresponding to those of the maxima in the optical absorbance spectra at the Soret region. Furthermore, there were some differences in the shoulders and peak widths of CD spectra between oxidized and reduced \(b_{558}\)-type cytochromes. These results indicate that this method provides the purification of the small subunit of human cytochrome \(b_{558}\) which is the heme-carrying subunit of cytochrome \(b_{558}\), and suggest that cytochrome \(b_{558}\) has heme-heme interaction and some conformational changes in the alternation of the redox state.

The neutrophil oxygen radical-generating system (an NADPH oxidase) is an important enzyme in host-defense mechanisms (1, 2). The NADPH oxidase is dormant in resting neutrophils and is activated to generate \(O_2^-\) and \(H_2O_2\) with a phagocytic stimulus or with the addition of soluble stimulants. The activated NADPH oxidase, which is located in the plasma membrane, is able to produce \(O_2^-\) coupled with the oxidation of NADPH (3, 4). Flavin and a b-type cytochrome (cytochrome \(b_{558}\)) have been postulated as electron-transporting groups of the NADPH oxidase (5-7). The heme protein has been extensively studied in connection with its participation in catalysis by the NADPH oxidase because frequent deficiencies of cytochrome \(b_{558}\) have been reported in neutrophils from many chronic granulomatous patients (5, 8). These phagocytes fail to make \(O_2^-\). Some investigators reported (9, 10) that cytochrome \(b_{558}\) copurifies with the NADPH oxidase. Furthermore, Iszakata et al. (11) reported that a high concentration of pyridine reversibly inhibited the NADPH oxidase. However, there is no direct evidence for the postulation that cytochrome \(b_{558}\) is a component of the NADPH oxidase.

Many attempts have been made to purify cytochrome \(b_{558}\) (12-15). However, the molecular weights of the various cytochrome \(b_{558}\) preparations as estimated by SDS-PAGE analysis have been markedly different from each other: 11,000-14,000 for bovine leukocytes (12), 32,000 for porcine neutrophils (13), and 68,000-127,000 for human leukocytes (14, 15). Recently, Segal (16) and Parkos et al. (17) reported that cytochrome \(b_{558}\) might be composed of two subunits: a large glycoprotein subunit of 76-92 kDa and a small subunit of 22-23 kDa. Teahan et al. (18) showed that the N-terminal sequence of the large subunit of cytochrome \(b_{558}\) was identical to the sequence of the gene found to be defective in X-linked cytochrome-negative chronic granulomatous disease (19). Furthermore, a close association between the large and small subunits of cytochrome \(b_{558}\) was demonstrated in human neutrophils by using a cross-linker (17). However, properties of the heme subunit of cytochrome \(b_{558}\) remain unclear.

In this study, we have attempted to purify the small subunit of human neutrophil cytochrome \(b_{558}\) to clarify the properties of the heme-carrying subunit. Several chromatography procedures provided highly purified cytochrome \(b_{558}\) that migrated as a single band on SDS-PAGE. We discuss here some properties of the heme-carrying subunit of cytochrome \(b_{558}\).

**EXPERIMENTAL PROCEDURES**

**Materials**—Hydroxylapatite Ultro Gel, DEAE-Sephaloc, a Mono Q packed column for FPLC, dextran T500, and heparin-Sepharose 6B (Pharmacia LKB Biotechnology Inc.); octyl glucoside (Bio-Rad and Dojindo Laboratories); and leupeptin, antipain, diisopropylfluorophosphate, N\(^\prime\)-tosyl-L-lysine chloromethyl ketone (TLCK), and phenylmethylsulfonyl fluoride (Sigma) were purchased from the suppliers indicated. Other reagents were of analytical grade.

**Solubilization of Neutrophil Cytochrome \(b_{558}\)**—Human neutrophils were prepared by the dextran sedimentation method as reported previously (23); suspended in Tris/sucrose buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 0.1 mM TLCK, 2 mM leupeptin, 2 mM antipain, 0.5 mM dithiothreitol, and 0.015% hexamethyldisilazane.

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phosphoramidase (an antidenaturating agent) (21); and stored at −80 °C. Two-hundred ml of pooled neutrophil suspending containing about 6 × 10^9 cells was thawed, mixed with 1 mM (final concentration) diisopropylfluorophosphate, and sonicated at 0 °C (10 s at 40 watts) to disrupt the cells. The following procedures were done at 0–4 °C. Two ml of 0.1 mM phenylmethylsulfonyl fluoride was added to the sonicates. After centrifugation at 450 × g for 10 min, the supernatant was centrifuged at 45,000 × rpm for 60 min in Beckman Ti-60 rotor. The precipitate (particulate fraction) was suspended in 60 ml of the Tris/sucrose buffer with a Potter-Elvejhem homogenizer, mixed with 120 ml of 1.5 M (final concentration of 1.0 M) potassium phosphate buffer (pH 7.3), and then sonicated at 0 °C (5 s at 40 watts). The sonicate was centrifuged at 45,000 × rpm for 60 min using a Beckman Ti-60 rotor, and the slightly brown precipitate (KPN-washed fraction) was suspended in 60 ml of the Tris/sucrose buffer. Octyl glucoside (final concentration of 1.0%) was added to the suspension, which was then stirred at 0 °C for 15 min, and finally centrifuged at 45,000 × rpm for 60 min using a Beckman Ti-60 rotor. The resulting pellet was resuspended in 60 ml of 100 mM potassium phosphate buffer (pH 7.3) containing 5% glycerol, 0.5 mM dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml antipain, 0.1 mM TLCK, and 0.015% hexamethylphosphoramidase (solubilizing buffer). This suspension usually contained 1.5–2.2 μM cytochrome bs68. Cytochrome bs68 was solubilized by the addition of octyl glucoside (final concentration of 1.6%) followed by stirring at 0 °C for 20 min. The insoluble material was removed by centrifugation at 50,000 × rpm for 60 min using a Beckman Ti-75 rotor. The solubilized fraction contained 1.1–1.74 nmol of cytochrome bs68/mg of protein (octyl glucoside-solubilized fraction).

**Column Chromatography of Cytochrome bs68**—Solubilized cytochrome bs68 was applied to hydroxylapatite (first hydroxylapatite eluate fraction) equilibrated with solubilizing buffer containing 0.5% octyl glucoside. After washing with equilibration buffer, cytochrome bs68 was eluted with a gradient of 0.1–1.0 M potassium phosphate buffer (pH 7.3) containing 0.5% octyl glucoside, 5% glycerol, 0.5 mM dithiothreitol, protease inhibitors (2 μg/ml antipain, 2 μg/ml leupeptin, and 0.1 mM TLCK), and 0.015% hexamethylphosphoramidase (Fig. 1). Buffers referred to subsequently contained protease inhibitors and hexamethylphosphoramidase. The peak fractions of cytochrome bs68 were pooled and dialyzed against 0.1 M potassium phosphate buffer. The slightly cloudy cytochrome bs68 solution (dialysate) was clarified with octyl glucoside (final concentration of 1.5%) and then rechromatographed on hydroxylapatite (second hydroxylapatite eluate fraction). The peak fractions of cytochrome bs68 were pooled and dialyzed against 50 mM potassium phosphate buffer (pH 7.3). The dialysates were supplemented with octyl glucoside (final concentration of 1.5%) and then applied to DEAE-Sephalac equilibrated with 50 mM potassium phosphate buffer (pH 7.3) containing 0.5% octyl glucoside (50 mM DEAE effluent fraction). The effluent fraction was dialyzed against 10 mM potassium phosphate buffer (pH 7.3). The dialysate was supplemented with octyl glucoside (final concentration of 1.5%) and applied to a Mono Q FPLC column equilibrated with 10 mM phosphate buffer (pH 7.3) containing 0.5% octyl glucoside. Cytochrome bs68 was eluted with a gradient of 0.01–1.5 M potassium phosphate buffer containing 0.5% octyl glucoside (Fig. 2). The peak fractions of cytochrome bs68 were collected for characterization and dialyzed against 10 mM phosphate buffer (pH 7.3).

In some experiments, the 50 mM DEAE effluent fraction was concentrated after dialysis using DEAE-Sephalac equilibrated with 10 mM phosphate buffer (pH 7.3) and analyzed by CD spectrophotometry. For amino acid composition, the DEAE-concentrated samples were further purified by SDS-PAGE.

**Binding Affinity of Cytochrome bs68 to Heparin-Sepharose**—Cytochrome bs68 fractions from various purification steps were pooled and dialyzed against 25 mM potassium phosphate buffer (pH 7.3). The cytochrome bs68-containing dialysate was supplemented with octyl glucoside (final concentration of 1.5%) and protease inhibitors and then applied to heparin-Sepharose equilibrated with 25 mM phosphate buffer (pH 7.3) containing 0.5% octyl glucoside, protease inhibitors, and 0.015% hexamethylphosphoramidase.

**Spectrophotometric Assay**—The content of cytochrome bs68 was determined by reduced-minus-oxidized difference spectroscopy at 558–540 nm using an extinction coefficient of 21.6 mM−1 cm−1 (22). Difference spectra were measured in a Hitachi Model 557 spectrophotometer. For the measurement of CO difference spectra, samples were reduced by the addition of a few grains of dithionite, and then the reduced spectra were measured and stored in the data processor memory. After CO gas was bubbled through the reduced sample, the CO difference spectra were recorded using the subtraction mode.

The CD spectra of the DEAE-concentrated samples of 14–18 μM cytochrome bs68 in 50 mM potassium phosphate buffer (pH 7.3) containing 5% glycerol, protease inhibitors, 0.015% hexamethylphosphoramidase, and 2% octyl glucoside were recorded at 20 °C using a Jasco J-500 spectropolarimeter. The Δε values of the CD spectra were calculated on the basis of the concentration of cytochrome bs68 determined spectrophotometrically in the same samples.

**SDS-Polyacrylamide Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis was performed according to the Lasemml method (23) using a 15% running gel.

**Amino Acid Analysis of Cytochrome bs68**—To obtain highly purified cytochrome bs68 in quantities sufficient for amino acid analysis, DEAE-concentrated cytochrome bs68 was applied to SDS-PAGE and then transferred to a nitrocellulose membrane (Fig. 3). The 20-kDa band was cut out and extracted with 0.1 M ammonium acetate buffer (pH 8.9) in 45% acetonitrile (24). The extracted band was lyophilized and then subjected to hydrolysis in 6 N HCl in a deoxygenated tube at 110 °C for 48 h. Amino acid analysis was performed in a Hitachi Model 835 auto-amino acid analyzer.
Black staining. The main 20-kDa band was extracted with PAGE on a transferred to a nitrocellulose filter and then visualized with Amido DEAE-Sephacel-concentrated ter. To obtain highly purified cytochrome b558 in quantities sufficient for amino acid analysis, cytochrome b558 was purified as follows.

Lowry et al. (25) or Bradford (26) with bovine serum albumin as standard.

RESULTS

Purification of Human Cytochrome b558—A typical purification of cytochrome b558 is summarized in Table I. The cytochrome content of the particulate fraction was 0.154 nmol/mg of protein, a value about twice that of the whole cells. Pretreatment with both high ionic strength phosphate buffer and 1% octyl glucoside at low ionic strength was found to be effective for the solubilization of cytochrome b558 by octyl glucoside in the presence of 100 mM potassium phosphate (pH 7.3). Cytochrome b558 was solubilized in a 87–95% yield from the pretreated particulate fraction. The first hydroxylapatite chromatography step resulted in a marked increase in the specific content of cytochrome b558. On the other hand, the major portion of the flavin (mainly FAD) in the solubilized fraction was recovered in pass-through fractions. The Mono Q eluate fraction contained 37 nmol of cytochrome b558/mg of protein; this value was higher than those reported previously (12–17). Furthermore, the retained content of cytochrome b558 was calculated to be 255-fold (about 500-fold from the whole cells)

Figure 4 shows the SDS-PAGE protein pattern of the sample from each purification step of cytochrome b558. The eluate from Mono Q FPLC exhibited a single band whose molecular mass was calculated to be about 20 kDa. It was noted that the Mono Q eluate fraction did not contain the high molecular mass protein which was described by Segal (16) and Parkos et al. (17). Harper et al. (14) and Parkos et al. (17) both reported that human cytochrome b558 was retained by heparin-Sepharose or wheat germ agglutinin-Sepharose, suggesting that cytochrome b558 may bind to the resin through the glycosylated large molecular mass subunit. We examined the ability of cytochrome b558 from the different purification steps to bind to heparin-Sepharose (Fig. 5). The major portion of cytochrome b558 in the first hydroxylapatite eluate fraction was retained by heparin-Sepharose, as observed by others (14, 17). Furthermore, the retained cytochrome b558 could be eluted with 0.75 M potassium phosphate buffer. However, little of the cytochrome b558 in the Mono Q eluate fraction was retained by heparin-Sepharose. Fig. 6 shows the SDS-PAGE analysis of the heparin-Sepharose eluate material and the material purified by our procedure.

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific content</th>
<th>Total amount</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg protein</td>
<td>nmol</td>
<td>%</td>
</tr>
<tr>
<td>Particulate</td>
<td>0.154</td>
<td>475*</td>
<td>100</td>
</tr>
<tr>
<td>KF-washed</td>
<td>0.310</td>
<td>360</td>
<td>75</td>
</tr>
<tr>
<td>Octyl glucoside-solubilized</td>
<td>1.74</td>
<td>228</td>
<td>48</td>
</tr>
<tr>
<td>1st HTP eluate</td>
<td>12.3</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>2nd HTP eluate</td>
<td>20.3</td>
<td>73.3</td>
<td>15.4</td>
</tr>
<tr>
<td>50 mM DEAE effluent</td>
<td>30.1</td>
<td>58.9</td>
<td>12.4</td>
</tr>
<tr>
<td>Mono Q FPLC eluate</td>
<td>37.0</td>
<td>18.9</td>
<td>3.9 (×180)</td>
</tr>
</tbody>
</table>

* The total amount of cytochrome b558 was obtained from ~6 × 10^10 human neutrophils.

b HTP, hydroxylapatite.

Fig. 2. Column chromatography of cytochrome b558 on Mono Q FPLC. ○, cytochrome b558; — —, absorbance at 280 nm; — —, gradient of 0.01–1.5 M potassium phosphate buffer (pH 7.3).

Fig. 3. Blotting of cytochrome b558 onto nitrocellulose filter. To obtain highly purified cytochrome b558 in quantities sufficient for amino acid analysis, cytochrome b558 was purified as follows. DEAE-Sephacel-concentrated cytochrome b558 was subjected to SDS-PAGE on a 15% gel. After electrophoresis, the separated bands were transferred to a nitrocellulose filter and then visualized with Amido Black staining. The main 20-kDa band was extracted with 45% acetonitrile in 0.1 M ammonium acetate buffer (pH 8.9). Numbers indicate the molecular masses of marker proteins.

Protein Determination—Protein was determined by the method of Lowry et al. (25) or Bradford (26) with bovine serum albumin as standard.

97.4K
66.2K
42K
31K
21.5K
14.4K

Fig. 4. SDS-PAGE pattern of purified and crude b558-type cytochromes. Lane 1, 1.0 M potassium phosphate buffer-washed particulate fraction; lane 2, 1.8% octyl glucoside-solubilized fraction; lane 3, DEAE-Sephacel effluent fraction; lane 4, Mono Q FPLC eluate fraction. Each lane contained about 0.5 µg of protein. The gel was stained with Coomassie Blue R-250. Numbers indicate the molecular masses of marker proteins.
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Fig. 5. Heparin-Sepharose column chromatography of crude and purified b\textsubscript{558}-type cytochromes. \textit{a}, chromatography pattern of the sample from the first hydroxylapatite eluate fraction; \textit{b}, chromatography pattern of a sample from the Mono Q FPLC eluate fraction. ○, cytochrome b\textsubscript{558} concentration; ●, protein concentration.

Fig. 6. SDS-PAGE pattern of purified and heparin-Sepharose eluate fractions. \textit{Lanes} 1 and 3, Mono Q eluate fraction; \textit{lanes} 2 and 4, heparin-Sepharose eluate fraction described for Fig. 5a; \textit{lanes} 1 and 2, Coomassie Blue staining pattern; \textit{lanes} 3 and 4, silver staining pattern.

The heparin eluate fraction contained 20- and 60–90-kDa proteins, the latter one presumably identical to the large subunit of cytochrome b\textsubscript{558} (16, 17), whereas the Mono Q-purified material showed no large subunit even with silver staining.

Characterization of Cytochrome b\textsubscript{558}—Fig. 7 shows the spectrophotometric data from purified cytochrome b\textsubscript{558}. The oxidized spectrum revealed the absorption maximum at 413.5 nm, and the reduced spectrum shows absorption maxima at 426, 529, and 558 nm, identical to previous reports (27, 28). The CO- and KCN-binding ability of purified cytochrome b\textsubscript{558} was also examined spectrophotometrically. Fig. 8 (upper) shows the CO and KCN difference spectra of cytochrome b\textsubscript{558}. Clearly, CO binds to our purified cytochrome b\textsubscript{558}, whereas KCN does not bind. Fig. 8 (lower) shows the absolute spectra of cytochrome b\textsubscript{558} in the presence of CO, indicating that when reduced, most of the purified cytochrome b\textsubscript{558} took up CO.

This finding is consistent with previous observations (12). On the contrary, the finding that cytochrome b\textsubscript{558} in the membrane fraction did not substantially interact with CO under any conditions examined (data not shown) is in agreement with the results of Iizuka et al. (29).

To obtain structural information about the heme region,
the CD spectra of cytochrome \(b_{568}\) were examined. The CD spectrum of oxidized cytochrome \(b_{568}\) in the DEAE-Sephasel-concentrated fraction measured at 20 °C. Spectrum A, CD spectrum of oxidized cytochrome \(b_{568}\); spectrum B, CD spectrum of dithionite-reduced cytochrome \(b_{568}\).

**TABLE II**

Circular dichroism characteristics of cytochrome \(b_{568}\)

<table>
<thead>
<tr>
<th></th>
<th>(\lambda_{\text{max}})</th>
<th>(\Delta\varepsilon)</th>
<th>(\lambda_{\text{molar absorptivity}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized</td>
<td>n.m.</td>
<td>M(^{-1}) cm(^{-1})</td>
<td>nm</td>
</tr>
<tr>
<td>cytochrome (b_{568})</td>
<td>406</td>
<td>121</td>
<td>413</td>
</tr>
<tr>
<td>Reduced</td>
<td>426</td>
<td>-119</td>
<td></td>
</tr>
<tr>
<td>cytochrome (b_{568})</td>
<td>420</td>
<td>102</td>
<td>424.5</td>
</tr>
<tr>
<td></td>
<td>429</td>
<td>-150</td>
<td></td>
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</tbody>
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**TABLE III**

Amino acid composition of human cytochrome \(b_{568}\)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mol %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>12.9</td>
</tr>
<tr>
<td>Thr</td>
<td>1.7</td>
</tr>
<tr>
<td>Ser</td>
<td>4.4</td>
</tr>
<tr>
<td>Glu</td>
<td>12.9</td>
</tr>
<tr>
<td>Gly</td>
<td>12.0</td>
</tr>
<tr>
<td>Ala</td>
<td>6.8</td>
</tr>
<tr>
<td>Cys</td>
<td>1.03</td>
</tr>
<tr>
<td>Val</td>
<td>9.2</td>
</tr>
<tr>
<td>Met</td>
<td>0.27</td>
</tr>
<tr>
<td>Ile</td>
<td>5.1</td>
</tr>
<tr>
<td>Leu</td>
<td>10.7</td>
</tr>
<tr>
<td>Tyr</td>
<td>ND°</td>
</tr>
<tr>
<td>Phe</td>
<td>2.9</td>
</tr>
<tr>
<td>Lys</td>
<td>10.2</td>
</tr>
<tr>
<td>His</td>
<td>0.7</td>
</tr>
<tr>
<td>Arg</td>
<td>7.8</td>
</tr>
<tr>
<td>Pro</td>
<td>9.4</td>
</tr>
</tbody>
</table>

*Values are the average of two 48-h hydrolysates.
°ND, not detected.

The CD spectra of cytochrome \(b_{568}\) were examined. The CD spectrum of oxidized cytochrome \(b_{568}\) in the Scet region showed a bilobed band with a maximum at 406 nm (which is approximately identical to the absorption maximum of oxidized cytochrome \(b_{568}\) (Fig. 9), a minimum at 420 nm, and a crossover at 413 nm. The spectrum of reduced cytochrome \(b_{568}\) exhibited a similar bilobed shape. The bilobed spectrum of reduced cytochrome \(b_{568}\) is less symmetrical around the crossover point than that of the oxidized form, although the wavelength of the central crossover point (424.5 nm) closely corresponded to the maximum optical absorbance of reduced cytochrome \(b_{568}\). The negative peak of reduced cytochrome \(b_{568}\) was more sharp than that of the oxidized form. The positive band of reduced cytochrome \(b_{568}\) had a clearer shoulder around 406 nm than the corresponding band in the oxidized spectrum. These bilobed shapes of the CD spectra of oxidized and reduced \(b_{568}\) type cytochromes were very similar to those of the CD spectra of cytochrome \(b\) in the mitochondrial cytochrome \(b\), complex (30) or in Chromatium strain D cytochrome \(b_{568}\) (31). The major characteristics of the CD spectra of cytochrome \(b_{568}\) are summarized in Table II.

Data were derived from Fig. 8. The \(\Delta\varepsilon\) values are calculated on the basis of the concentration of cytochrome \(b_{568}\) as determined from optical absorbance.

**DISCUSSION**

In this study, we attempted to purify the heme-containing subunit of cytochrome \(b_{568}\). We found that purification by hydroxylapatite, DEAE-Sephasel, and Mono Q FPLC provided a highly purified cytochrome \(b_{568}\) which gave a single band on SDS-PAGE. The molecular mass of the purified cytochrome \(b_{568}\) was estimated to be about 20 kDa. The maximum heme content of purified cytochrome \(b_{568}\) was 37 nmol/mg of protein, a value higher than those reported by others (12-17). The specific content of cytochrome \(b_{568}\) increased 500-fold through our purification procedures as compared to that of whole cells. Recently, Segal and Parkos et al. (17) reported that human neutrophil cytochrome \(b_{568}\) was composed of two subunits: a large subunit of 76-92 kDa and a small one of 22-23 kDa. They utilized heparin-Sepharose or wheat germ agglutinin-Sepharose to purify cytochrome \(b_{568}\) respectively. These results suggest that our purified cytochrome \(b_{568}\) corresponds to the small subunit reported by Parkos et al. (17) and Segal (16).
the findings that the negative peak of reduced cytochrome \( b_{558} \) was somewhat sharper than that of oxidized cytochrome \( b_{558} \), and that a broad shoulder was seen in the upper edge without binding. In this study, purified or partially purified human cytochrome \( b_{558} \) may be able to transfer an electron to oxygen at the heme edge without binding. In this study, purified or partially purified human cytochrome \( b_{558} \) took up CO (Fig. 8, upper and lower), whereas cytochrome \( b_{558} \) in the membrane fraction could not bind to CO (data not shown). Presumably, the state of solubilized cytochrome \( b_{558} \) may differ from its state in the plasma membrane. This is supported by the finding that the redox potential of cytochrome \( b_{558} \) was altered from -247 to -205 mV during the purification procedures, similar to a previous report (14).\(^3\)

The CD spectra provide information about the heme-binding region (35). Oxygen-binding proto-IX heme protein such as hemoglobin (34) or peroxidase (35) exhibits a positive Cotton effect at the Soret region, whereas electron transfer-type cytochrome \( b \) (36, 37) shows a negative Cotton effect. The CD spectra of both oxidized and reduced \( b_{558} \)-type cytochromes exhibited a bilobed shape with crossover points whose wavelengths closely correspond to those of the maxima in the optical absorbance spectra at the Soret region. These S-shaped CD spectra were similar to those of cytochrome \( b \) in the mitochondrial cytochrome \( bc \) complex (30) or in Chro

matium strain D cytochrome \( b_{558} \), but differed markedly from other \( b \)-type cytochromes. In general, bilobed CD spectra are explained by splitting due to heme-heme interaction. Therefore, it seems likely that the bilobed shape of human cytochrome \( b_{558} \) at the Soret region may be attributed to heme-heme interaction. This postulation may be supported by the observation that the \( \alpha \)-band absorption peak of reduced cytochrome \( b_{558} \) was split in liquid nitrogen (29). On the other hand, the findings that the negative peak of reduced cytochrome \( b_{558} \) was somewhat sharper than that of oxidized cytochrome \( b_{558} \) and that a broad shoulder was seen in the positive peak suggest that there are some structural changes around the heme region that occur when cytochrome passes from the oxidized form to the reduced form. Furthermore, similar CD spectra were also observed with porcine cytochrome \( b_{558} \).\(^3\)

The amino acid composition of cytochrome \( b_{558} \) has already been reported by two groups (12, 15), but they used partially purified preparations. In this study, we analyzed the amino acid composition of the small subunit of human cytochrome \( b_{558} \) purified by means of extraction from a single blotted band in order to eliminate contamination by other proteins. Our purified cytochrome \( b_{558} \) contained predominantly hydrophobic amino acids, such as proline, leucine, isoleucine, and valine, and was very low in threonine, tyrosine, histidine, and methionine. N-terminal amino acid sequence analysis (Edman method) yielded no phenylthiohydantoin-derivative release with even more than 20 \( \mu \)g of purified protein (data not shown). It is highly probable that the \( NH_2 \) terminus of the small subunit of cytochrome \( b_{558} \) may be modified, as suggested by Pember et al. (12).

In conclusion, the small subunit of human cytochrome \( b_{558} \), with a molecular mass of about 20 kDa as determined by SDS-PAGE, was purified to homogeneity. The amino acid composition of the purified heme protein seemed to be abundant in hydrophobic and aromatic acids. From the CD spectra, human cytochrome \( b_{558} \) may possess a heme-heme interaction, and some structural changes may occur during the redox cycle.

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\(^3\) T. Yamaguchi and T. Hayakawa, manuscript in preparation.
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