Photoreduction of Cytochrome c₁

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SUMMARY

1. Ferricytochrome c₁ solution was reduced completely between pH 7 and 10 by illumination under anaerobic conditions. Photoreduction was not affected by the ionic strength of the medium. However, it did not take place at pH lower than 6 or higher than 10, or in the presence of p-hydroxymercuric benzoate. The ferricyanide-reoxidized photoreduced c₁ was not further reduced upon illumination. The reductant was most probably a specific sulfhydryl group in the subunit containing the heme of the cytochrome since this subunit contained one less p-HMB-titratable group in the 3-dimensional structures for both the oxidized and reduced c₁.

2. The photoreduced cytochrome c₁ showed the same spectra as the native cytochrome, and was not reactive with carbon monoxide. The equilibrium constant of the reaction

\[ c₁^{2+} + e^{+} ⇌ c₁^{3+} + e^{2+} \]

for the photoreduced c₁ was found to be slightly lower \((K_{eq} = 2.6)\) than that for the native c₁ \((K_{eq} = 3.5)\). The antimycin A-sensitive electron acceptor activity of ferricyanide-reoxidized photoreduced c₁⁺⁺ catalyzed by succinate-cytochrome c reductase was about 80% of that of the native c₁.

3. A somewhat simplified method for isolation of cytochrome c₁ was developed. Anaerobic ammonium sulfate fractionation and calcium phosphate gel chromatography were still used in order to achieve the purity level of about 25 nmol of heme/mg of protein. The cytochrome c₁ prepared by this procedure showed the same properties as that isolated by the p-mercaptoethanol method (Yu, C. A., Yu, L., and King, T. E. (1972) J. Biol. Chem. 247, 1012-1019).

Knowledge of cytochrome c₁ at the molecular level is meager mainly due to the lack of supply of the purified material. However, in order to understand electron transfer and energy transduction in mitochondria, it is important to study as many properties as possible for all purified respiratory components. This is especially true for cytochrome c₁ because it is the immediate electron donor of cytochrome c. The latter happens to have been studied exhaustively by practically all available methods and the 3-dimensional structures for both the oxidized and reduced c₁ as well as many other properties are known. On the other hand, the mechanism of electron transport involving cytochrome c₁ is still far from clear. This fact is generally attributed to the poor understanding of its electron donor, cytochrome c₁₃, and acceptor, cytochrome oxidase.

Recent availability of pure cytochrome c₁ (2) has made possible the study of some of its reactions. This paper reports the photoreduction of cytochrome c₁.

EXPERIMENTAL PROCEDURE

Preparations of Cytochrome c₁—Cytochrome c₁ from beef heart was prepared according to the method developed in this laboratory (2). The sample contained 25 nmol of heme/mg of protein and was in the reduced form. The oxidized cytochrome was prepared by oxidation with a small excess of ferricyanide and the excess reagent removed by Sephadex gel filtration.

An alternative procedure1 for the preparation of cytochrome c₁ without employing p-mercaptoethanol was also used. Succinate-cytochrome c reductase or the soluble cytochrome b₅₆ complex (8) was mixed with 20% sodium cholate and neutralized saturated ammonium sulfate solution to give final concentrations of 2% cholate, 20% saturation of ammonium sulfate, and about 10 mg of protein/ml in 50 mM phosphate buffer, pH 7.4. The mixture was allowed to stand at room temperature for 3 hours. The precipitate of cytochrome b was removed by centrifugation at 48,000 X g for 10 min. The supernatant solution was dialyzed against 50 mM phosphate buffer, pH 7.4, for 16 hours with two changes of buffer. The small amount of precipitate formed during dialysis was removed by centrifugation. The dialyzed solution was brought to 50% ammonium sulfate saturation and the precipitate was collected by centrifugation. The crude cytochrome c₁ obtained was dissolved in 50 mM phosphate buffer, pH 7.4. The further purification by anaerobic ammonium sulfate fractionation and column chromatography with calcium phosphate was carried out in the same manner as that used in the p-mercaptoethanol method (2).

The cytochrome c₁ isolated by this alternative method showed the same properties as that isolated by the p-mercaptoethanol method; the properties tested including the amino acid composition, the molecular weights of the subunits estimated on the sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns in the presence of β-mercaptoethanol, and the photoreduction as described in this paper.

Photoreduction—In a Thunberg cuvette were placed 2.5 ml of about 10 μM ferricytochrome c₁ in 50 mM phosphate buffer. It was evacuated and flushed with argon for five times. The cuvette was suspended in the center of a Dewar flask of 10 cm in diameter and

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1 This method was first developed in collaboration with Doctors S. Takehori and W. Steele in 1962. However, the final purity of about 20 nmol of heme/mg was not achieved until the completion of the present study. Indeed, a sample prepared by this method, higher than 15 nmol/mg of protein, was used by Dutton et al. (7) for the determination of midpoint potential. They have found that \(Eₘ\) of the isolated cytochrome c₁ is the same as that in mitochondria.
30 cm in height with a flat window opening of 7 cm in diameter. The Dewar was filled with water. A slide projector with a 500-watt lamp with an aperture of 1 cm was placed about 1 cm from the Dewar window. An air current rapidly flowed between the projector and the Dewar to prevent heat accumulation. The temperature in the Dewar was maintained at 23 ± 1°C. The reduction was followed from time to time by scanning the sample in a Cary spectrophotometer, model 14. Control experiments were run under the same conditions, except no light was applied.

The equilibrium constant between photoreduced cytochrome c₁ and oxidized cytochrome c was determined according to the method reported previously (3).

14C Incorporation from Labeled p-Hydroxymercuric Benzoate into Cytochrome c₁. Subunits—The sample, native cytochrome c₁, or photoreduced c₁, was incubated with an excess of p-[14C]HMB (specific activity 1 mCi/mM) for 30 min at 23°C and then treated with 2.5% sodium dodecyl sulfate for 2 hours at 40°C. Aliquots of the sample were applied to polyacrylamide gel and then subjected to electrophoresis as usual (9) but without β-mercaptoethanol in the medium. The protein bands were cut out and their radioactivity was counted. The numbers of the sulphydryls or p-HMB-titratable groups in the subunits were calculated from the number of -SH groups directly determined in the dissociated cytochrome and from radioactive incorporation data.

Other Material and Methods—Cytochrome c₁, type III, and p-HMB were purchased from Sigma, p-[14C]HMB from Mann, and Chelex 100 from Bio-Rad. Other chemicals were procured commercially at the highest purity available. The water used was redistilled from the deionized, ordinarily distilled water in an all-Pyrex glass apparatus and stored in Teflon carboys.

The concentration of cytochrome c₁ and c was determined spectrophotometrically (3). Protein was determined by the biuret method in the presence of hydrogen peroxide (10). The radioactivity of the samples was measured in a Beckman liquid scintillation counter. Titration of the sulphydryl group with p-HMB was conducted spectrophotometrically (11).

Subunits of cytochrome c₁ in milligram quantities were prepared as follows. Purified cytochrome c₁ was treated with sodium dodecyl sulfate at 5 mg of the detergent per mg of protein in the presence of 1.0% of β-mercaptoethanol at 37°C for 1 hour. The dissociated subunits were then separated on a Sephadex G-150 column, which was equilibrated with 0.1% sodium dodecyl sulfate and 0.1% of the thiol, and eluted with the same medium. The sodium dodecyl sulfate in the fractions containing the subunits was removed by Dowex 1-X2 ion exchange resin in the presence of 4 M urea; the area was removed by dialysis (cf. Ref. 12). The purity of the subunits was ascertained by polyacrylamide gel electrophoresis (9).

Amino acid analysis and performic acid oxidation were done according to conventional methods (13, 14).

The Chelex-treated cytochrome c₁ was prepared by passing a c₁ solution through a chefluting resin column. Chelex 100 (100 to 200 mesh) in sodium form was suspended in water and adjusted to pH 7.4 with 2 N HCl. The resin was then transferred to 50 mm glycylglycine buffer and packed into a column of 1.5 × 15 cm and equilibrated with the same buffer. About 2 ml of cytochrome c₁ solution in 20 mM glycylglycine were then applied to the column and eluted with the same buffer. Cytochrome c₁ in glycylglycine buffer was prepared from the usual cytochrome c₁ solution in phosphate buffer. The latter solution was allowed to react with a slight excess of potassium ferriyanaide and then passed through a Sephadex G-25 column, equilibrated with 50 mm glycylglycine buffer, pH 7.4, to remove the excess oxidant and the phosphate buffer.

RESULTS

Photoreduction of Cytochrome c₁.—The spectral change of cytochrome c₁ in the course of photoreduction at pH 7.4 is given in Fig. 1. It takes about 30 hours (see Fig. 2A) to complete the reduction at pH 7.4 under the conditions described above (5).

The abbreviation used is: p-HMB, p-hydroxymercuric benzoate.

Separation of subunits of cytochrome c₁ could be accomplished also by polyacrylamide gel electrophoresis in a medium containing sodium dodecyl sulfate and p-HMB in the absence of β-mercaptoethanol. However, in the absence of either of these latter two compounds, the separation of subunits on the gel was not possible.

A small amount of copper, apparently of extraneous origin, was detected by sensitive electron paramagnetic resonance spectroscopy at near-helium temperature in two samples of concentrated (1 mM) cytochrome c₁ solution (unpublished observation from an experiment done collaboratively with Dr. J. Peisach and W. E. Blumberg). The removal or nonremoval of copper or other metals was not further checked in the Chelex-treated c₁ by atomic absorption or chemical methods, mainly because it does not seem worthwhile to sacrifice large amounts of the sample and the solutions available were far too diluted for EPR detection of copper.

Experimental Procedure.—As shown in Fig. 3, under the same conditions, the rate and extent of photoreduction were much greater in cytochrome c₁ than in cytochrome c. When photoreduction was conducted in the presence of oxygen, the reduction rate of cytochrome c₁ was much slower and maximal reduction was found to be only about 40% of that under anaerobic conditions. In all cases the concentration of ascorbate-reducible cytochrome c₁ (measured at 552.5 nm) present in the cuvette after reaction was used as the basis (i.e. 100%) for the calculation of the degree of reduction.

Effect of pH on Photoreduction of Cytochrome c₁—Fig. 2 shows the time course of photoreduction as a function of pH. The rate of reduction increased as the pH of the solution was raised from 7.4 to 10.5. The degree of reduction was found to be ultimately the same between these pH values although denaturation occurred after prolonged exposure of the cytochrome c₁ solution at pH higher than 10 in the presence or absence of illumination (cf. Fig. 2B). Very little photoreduction was observed when the pH was at 6 or lower or higher than 11.0. At pH 11, cytochrome c₁ became denatured almost immediately. The denaturation was judged by the loss of its ascorbate reducibility and the denatured cytochrome c₁ was autoxidizable.

Effect of Ionic Strength and Buffer Systems on Photoreduction of Cytochrome c₁—No significant difference in the rate and extent of photoreduction was observed in the systems of ionic strengths between 50 and 500 mM phosphate buffer. Even addition of KCl to a final concentration of 4 M did not alter the reduction rate. High concentrations of ammonium sulfate up to 20% also showed no effect.

The photoreduction of cytochrome c₁ in glycylglycine buffer was also compared with the preparation in the usual phosphate buffer. The time required to complete the reduction was found to be about 30% shorter in glycylglycine buffer than in phosphate. The Chelex-treated cytochrome c₁ was about another 25 to 30% faster than the cytochrome c₁ in glycylglycine buffer for complete photoreduction.
FIG. 2. Effect of pH on photoreduction of cytochrome c. A, photoreduction as a function of time at five pH values. Ferricytochrome c, 10 \mu M in 50 mM phosphate buffer, was photoreduced under the conditions as described in the text at pH 6 (O--O); 7.4 (X--X); 8.4 (A--A); 10.5 (O--O); and 11.0 (A--A). B, photoreduction as a function of pH. Percent of reduction was plotted against pH at illumination times of 3 (O--O); 5 (A--A); 7 X--X); 12 X--X); 18 (O--O) hours.

TABLE I

**p-HMB-titratable sulfhydryls and half-cystine residues in cytochrome c1 and its subunits**

The values are given in moles per mol of protein. The sulfhydryls in intact cytochrome samples were directly determined spectrophotometrically (11) and also by \(^{14}C\) incorporation with radioactive p-HMB. The number of sulfhydryls in the subunits was calculated from \(^{14}C\) incorporation experiments (as shown in Table II). Half-cystine was determined from amino acid analysis after performic acid oxidation of the samples. N.D., not determined.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Intact cytochrome</th>
<th>Heme-containing subunit</th>
<th>Heme not-containing subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−SH Half-cystine</td>
<td>−SH Half-cystine</td>
<td>−SH Half-cystine</td>
</tr>
<tr>
<td>Native c1</td>
<td>4.7</td>
<td>11.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Photoreduced c1</td>
<td>3.7</td>
<td>11.5</td>
<td>1.9</td>
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</tbody>
</table>

TABLE II

**Incorporation of \(^{14}C\)HMB into subunits of cytochrome c1**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Home-containing subunit</th>
<th>Home not-containing subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native c1</td>
<td>3251</td>
<td>2160</td>
</tr>
<tr>
<td>Photoreduced c1</td>
<td>3005</td>
<td>2821</td>
</tr>
</tbody>
</table>

See the text for details of the operation of the experiment.

Fig. 3. Comparison of photoreduction rates between cytochromes c and c1. Cytochromes c and c1 were photoreduced under anaerobic conditions as described in the text at pH 7.4. The concentration of cytochrome c or cytochrome c1 is 10 \mu M. Cytochrome c1 (O--O); cytochrome c (A--A).

Effect of Wavelength and Intensity of Light for Photoreduction—The effective wavelengths and the intensity of the light on the rate of photoreduction are not known. Present facilities available do not easily allow us to obtain action spectra with precision. However, an experiment was performed by placing two filters between the light source and the Chelex-treated cytochrome c solution. These filters consisted of a Corning filter No. 5113 (which cut off the light of the wavelength above 460 nm and below 360 nm) and a 0.5-cm light path cuvette with 12.5 mM potassium ferricyanide in water (which possessed an absorption maximally between 400 and 420 nm). A control containing the same Chelex-treated c1 solution was used of which the light passed through the Corning filter and a 0.5-cm water filter instead of ferricyanide. The control was photoreduced completely within 6 hours, whereas the experimental sample with the potassium ferricyanide filter showed only less than 20% reduction. This result indicates that light around 400 to 420 nm, which is in the Soret region of c1 absorption, with a maximum of 408 nm, is essential for reduction by the light under the conditions used, i.e. between 400 and 360 nm. (At the end of the experiment the concentration of the ferricyanide solution in the filter was found to be 10.5 mM by absorption.)

Effect of p-HMB on Photoreduction of Cytochrome c1—Addition of sulfhydryl agents, such as p-HMB, abolished photoreduction. At the very beginning in less than 10 min about 10% apparent reduction was observed. However, this reduction was found to be light-insensitive. When the illumination proceeded, the now reduced cytochrome c1 became reoxidized.

Cysteine Content of Purified Cytochrome c1—By amino acid analysis it was found that 11 (or 12) mol of half-cystine existed per mol of cytochrome c1. Seven (or eight) half-cystine residues were found in the subunit with heme and four in the subunit without heme. Among these half-cystine residues 2 are evidently bound to the heme in the thio-ether linkage (2, 6). Direct titration with p-HMB revealed that 5 sulfhydryl groups existed in free form in the native c1. Therefore, the other 4 half-cystine residues might be present as disulfides.

Possible Electron Donor for Photoreduction of Cytochrome c1—Since no obvious external electron donor was added or present in the system, the source of the reductant for the photoreduction of cytochrome c1 came likely from the molecule itself. One attractive candidate was the sulfhydryl groups of the protein moiety of the cytochrome itself. It was found that 1 mol of p-HMB-titratable −SH group disappeared per mol of cytochrome c1 photoreduced as shown in Table I, but amino acid analysis of the photoreduced c1 gave the same number of half-cystine residues per mol of heme as the cytochrome before photoreduction. This stoichiometric relationship was substantiated by the result obtained from \(^{14}C\) incorporation experiments using radioactive p-HMB as summarized in Tables I and II.
When the photoreduced cytochrome c₁ was reoxidized by ferricyanide and then illuminated again under the same anaerobic conditions as the original photoreduction, no further reduction was observed. The reoxidized samples were also ascorbate- and dithionite-reducible. That the failure of further reduction might be due to the presence of trace amounts of ferricyanide in the sample was ruled out because the ferricyanide treatment was followed by either extensive dialysis or by Sephadex G-25 column chromatography. Moreover, the original photoreduction which was conducted on native cytochrome c₁ also reacted with ferricyanide in the same manner. These results suggest that only one specific sulfhydryl group among five p-HMB titratable groups of the protein could serve as the electron donor in the photoreduction (cf. Table I).

In order to ascertain which subunit of cytochrome c₁ contained the photooxidizable sulfhydryl group, we examined the ¹⁴C incorporation from the ¹⁴C-labeled p-HMB into the subunits of cytochrome c₁. As shown in Tables I and II, the heme-containing subunit of photoreduced cytochrome c₁ possesses one sulfhydryl less than that of the native c₁, whereas the number of p-HMB-titratable groups in the subunit without heme remains the same after photoreduction. Therefore, the photoactive sulfhydryl group is evidently from the heme-containing subunit.

**Properties of Photoreduced Cytochrome c₁**—Some properties of the photoreduced cytochrome c₁ were compared with a control or native c₁. No difference in spectral properties was observed. Neither the reduced nor the oxidized form was reactive to carbon monoxide. Similar to the untreated sample, photoreduced cytochrome c₁ did not react with cyanide.

Making use of available reported data of kinetic rates and equilibrium constants of electron transfer between cytochromes c₁ and c, the photoreduced cytochrome c₁ was allowed to react with cytochrome c under the same conditions described previously (3). The \( K_{eq} \) of Reaction 1 was found to be 2.6, whereas the nonphotoreduced c₁ gave the \( K_{eq} \) of 3.5.

\[
c_{1}^{\circ} + c^3+ \rightleftharpoons c_{1}^{3+} + c^2+ \tag{1}
\]

Native ferrocytochrome c₁ as prepared shows very little autoxidizability, which was even less than that of cytochrome c (2). The photoreduced cytochrome c₁ was found to be slightly more autooxidizable than the native cytochrome c₁. The difference was small but reproducible.

It has been shown that purified cytochrome c₁ is capable of serving as oxidant, i.e. an electron acceptor (2), for the oxidation of succinate catalyzed by succinate-cytochrome c reductase. The reduction of cytochrome c₁ was antimycin A-sensitive. We found that the ferricyanide reoxidized photoreduced cytochrome c₁ was also capable of serving as an electron acceptor from succinate catalyzed by succinate-cytochrome c reductase. The reaction was also antimycin A-sensitive. However, the rate of reduction is about 20% lower than that of the untreated sample.

**DISCUSSION**

Some salient conclusions may be deduced from the results presented. The obvious point is the specificity of the sulfhydryl which can serve as the reductant for the photoreduction. This sulfhydryl is most probably of intramolecular origin and structurally is very proximate to the heme. The intermolecular origin of the sulfhydryl seems improbable, evidence from several different lines (4) suggests that the heme group in cytochrome c₁ is more covered up than that in cytochrome c. The requirement of light for reduction cannot be unequivocally answered at present. It is possible that irradiation may reduce the activation energy barrier for reduction. It is known that light may turn porphyrin to triplet or other excited states. The excited state of cytochrome c₁ may be capable of extracting an electron from the proximate sulfhydryl as porphyrin extracting electrons from amines upon illumination (15). Another possibility, among others, is that electron (hydrogen) transfer can be effected from the nearby sulfhydryl to the heme prosthetic group upon a conformational change resulted from illumination. The product of the oxidation of the specific sulfhydryl was not examined, however.

Since cytochrome c does not possess any sulfhydryl groups at all, it is more difficult to explain the photoreduction of cytochrome c₁ although reduction occurs to a much lesser extent (about 25%) even after prolonged illumination. The reduction may possibly be due to the presence of some impurities or the reductant originated from the medium or the sample. In this connection, we are tempted to speculate that the photoreduction of c₁ may serve as a model of physiological electron transfer in mitochondria mainly from reasoning that the heme is almost completely covered by the protein moiety. Needless to say, substantive experiments of more convincing nature remain to be done.

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**REFERENCES**


* Unpublished results from this laboratory.