Reduction of Cytochrome c by Ferrous Ions and Ethylenediaminetetraacetic Acid in Acid Solution*

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SUMMARY
Cytochrome c was reduced by ferrous EDTA over the pH range 0.7 to 5.5, but not by ferrous ions alone. A typical low spin type hemochromogen of the low spin type was observed between pH 2.2 and 5.5, but spectra of the high spin type were seen between pH 0.7 and 2.1.

CO-Ferrocytochrome c was obtained over the pH range of 1.6 to 3.2 by this method. The spectrum was similar to that of alkaline CO-ferrocytochrome c and two bands in the Soret region were observed at 392 and 413 μm. α-Porphyrin, α',α''-dipyridyl, 8-hydroxyquinoline, or amino acids could not be substituted for EDTA for the reduction of cytochrome c at acid pH.

At neutral pH, cytochrome c was fully reduced by a 2-fold molar excess of ferrous ions in the absence of EDTA.

EXPERIMENTAL PROCEDURE
Horse heart cytochrome c type III Sigma (1 to 2 × 10⁻⁵ M) was dissolved in different buffer solutions. For pH 0.7 to 3.6, 0.05 M KCl-HCl buffer, 0.05 M glycine-HCl buffer solution, or acetic acid (5 to 22%); for pH 3.6 to 5.6, 0.1 M acetate buffer; for pH 5.6 to 8.3, 0.1 M Tris-acetate or Tris-HCl buffer solution; for pH 8.4 to 10.6, 0.1 M Tris-acetate buffer solution was used. Reduction of cytochrome c in acid condition was initiated by the addition of ferrous ammonium sulfate, ferrous acetate, or ferrous sulfate (7.5 to 15 × 10⁻⁴ M), and EDTA (3.5 to 7 × 10⁻³ M) to the cuvette (1-cm light path) under nitrogen and the reaction was followed by recording absorbance from 350 to 700 μm with a Shimadzu multipurpose recording spectrophotometer type MPS-50 (Shimadzu Seisakusho, Ltd., Kyoto, Japan). Care was taken to avoid oxidation of added ferrous ions by bubbling nitrogen through all solutions. The pH was determined after each recording of the absorption spectrum. In neutral and alkaline solutions, EDTA was omitted from the reaction mixture.

RESULTS AND DISCUSSION
Reduction of Cytochrome c by Ferrous EDTA at Acid pH—Cytochrome c was not reduced by ferrous ions alone but was reduced by ferrous ions and EDTA over the pH range of 0.7 to 5.5 and a typical low spin type hemochromogen was observed between pH 2.2 and 5.5. A typical experiment is as follows. The absorption spectrum of ferricytochrome c (1 to 2 × 10⁻⁵ M) in 5% acetic acid (final pH 2.4) revealed a typical high spin spectrum (3). The addition of more than 75-fold molar excess of ferrous ammonium sulfate or 350-fold molar excess of EDTA to ferricytochrome c gave a slightly different spectrum showing a wide band from 495 to 530 μm with a slight shift of the Soret to 39 μm and a decrease of optical density at the Soret band. However, upon addition of a 75-fold molar excess of ferrous ions plus a 350-fold molar excess of EDTA to ferricytochrome c in 5% acetic acid, a prompt reduction of cytochrome c was observed. Typical low spin absorption spectra were obtained in the reduced form (Fig. 1). The molar extinction coefficients in the reduced form at the low pH were slightly different from those of reduced cytochrome c at neutral pH.

The ferricytochrome c solution thus obtained was optically clear and was not oxidized under acid conditions for at least 1 hour.

Between pH 0.7 and 2.1, ferrocyanohemochrome c spectra of the high spin type were observed. Such a pH effect on the absorption spectra was also studied under nitrogen by varying the acetic acid concentration from 5 to 22% at fixed concentration of cytochrome c (2.5 × 10⁻⁴ M), ferrous ions (1.9 × 10⁻³ M), and EDTA (8.7 × 10⁻³ M). The optical density at 520 and 550 μm decreased with the increased acetic acid concentration in the range of 14 to 18% and the spectra changed from the low to high spin type when the acid concentration was increased to 22% (pH 0.7).

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2.1). Upon dilution with water to 5 to 10% of acetic acid, hemochromogen spectra reappeared. The ferrous state of cytochrome c in 22% acetic acid was confirmed by the formation of CO-ferrocytochrome c.

The effects of various iron-chelating agents such as o-phenanthroline, α,α'-dipyridyl, 8-hydroxyquinoline, or amino acids such as glycine, lysine, glutamic acid, histidine, methionine, acetyl-

methionine, or tryptophan upon the reduction of cytochrome c in the presence of ferrous ions in acid condition were tested. None of these could be substituted for EDTA.

Reduction of Cytochrome c by Ferrous Ions at Neutral and Alkaline pH—The addition of 2-fold molar excess of ferrous ions to ferricytochrome c at pH 7.0 resulted in complete reduction within 15 min. The addition of even equimolar amounts of ferrous ions gave an 84% reduction. EDTA added to the reduced preparation did not affect the absorption spectrum. These results showed that ferrous ions reduced cytochrome c even in the absence of EDTA at neutral pH. The Fe⁺⁺-α,α'-dipyridyl complex did not reduce cytochrome c, but Fe⁺⁺-8-hydroxyquinoline did so at neutral pH.

While the absorption spectrum of ferricytochrome c from pH 8.4 to 10.5 is indistinguishable from that at pH 6.8 to 7.0, at the alkaline pH, only a limited extent of reduction and no effect of EDTA were observed. This is most likely caused by the hydrolysis of the ferrous species in solution.

The data on the reduction of cytochrome c by ferrous ions and EDTA at various pH values are summarized in Table I.

Reduction of Other Hemopeptides by Ferrous EDTA—Hemin, c, hemin-octa-, and hemin-undecapeptides of cytochrome c were not reduced at various pH values by ferrous ions or by ferrous ions plus EDTA. The further addition of imidazole, histidine, acetylmethionine, or tryptophan to the reaction mixture also did not cause the reduction of these hemopeptides.

EDTA did not give low spin spectra with heme c or heme-octa-

peptide reduced by Na₂SO₄ over the pH range of 1.8 to 9.0. This means that the ligands which caused the hemochromogen to be in the low spin state between pH 2.2 and 5.5 were not EDTA.

FIG. 1. Comparison of spectrum of ferricytochrome c (---) with spectrum of cytochrome c reduced by ferrous ions and EDTA (----) and spectrum of CO-ferrocytochrome c (-----) in 5% acetic acid (pH 2.4).

TABLE I

<table>
<thead>
<tr>
<th>Buffer and pH value</th>
<th>Addition*</th>
<th>State of Cytochrome c</th>
<th>Absorption maxima (nm) (ε mm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 0.05 M KCl-HCl buffer</td>
<td>Fe⁺⁺ + EDTA</td>
<td>Oxidized</td>
<td>395 (149.5)</td>
</tr>
<tr>
<td>pH 1.5</td>
<td>Reduced</td>
<td>395 (147.4)</td>
<td>495 (6.9)</td>
</tr>
<tr>
<td>pH 1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. 5% Acetic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2.4</td>
<td>Oxidized</td>
<td>395 (140.5)</td>
<td>495 (6.9)</td>
</tr>
<tr>
<td>pH 2.4</td>
<td>Reduced</td>
<td>395 (113.0)</td>
<td>520 - 530 (6.9)</td>
</tr>
<tr>
<td>pH 2.4</td>
<td>Fe⁺⁺ + EDTA</td>
<td>415 (131.0)</td>
<td>520 (14.7)</td>
</tr>
<tr>
<td>pH 2.4</td>
<td>Fe⁺⁺ + EDTA + CO</td>
<td>392 (9.4)</td>
<td>413 (9.8)</td>
</tr>
<tr>
<td>C. 0.1 M Tris-acetate buffer</td>
<td>Fe⁺⁺</td>
<td>Oxidized</td>
<td>410 (106.0)</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>Reduced</td>
<td>415 (125.0)</td>
<td>520 (15.0)</td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. 0.1 M Tris</td>
<td>Fe⁺⁺</td>
<td>Oxidized</td>
<td>415 (139.0)</td>
</tr>
<tr>
<td>pH 10.5</td>
<td>Reduced</td>
<td>414 (114.5)</td>
<td>520 (14.9)</td>
</tr>
</tbody>
</table>

* Ferrous ammonium sulfate or ferrous sulfate (30-fold molar excess), EDTA (300-fold molar excess) added in A and B, ferrous ions (2-fold molar excess) added in C, and ferrous ions (30-fold molar excess) added in D.
resulting solution showed absorption spectrum with two bands in the Soret region (see Fig. 1 and Table I). The spectrum was similar to that of alkaline CO-ferrocytochrome c (4, 5). From pH 3.2 to 5.5, CO-ferrocytochrome c was not obtained.

Photoreduction of Cytochrome c in EDTA—Irradiation of cytochrome c in 0.1 M EDTA at pH 5.8 under strictly anaerobic conditions with white light obtained from a 1000-watt tungsten projection lamp with infrared absorbing glass according to the method of Mauzerall (6), produced the reduced form of cytochrome c in 10 min and the reduction was complete within 1 hour. The absorption spectrum was identical with that obtained by reduction with ferrous ions, sodium dithionite, or hydrogen over palladium at neutral pH. No photoreduction was observed with EDTA in either alkaline solution or very acidic solution. It was also observed that hydrogen was not introduced to methene bridges of heme.

Inasmuch as the reduction of cytochrome c by ferrous ions and EDTA in acid solution proceeded in the dark condition, the mechanism appears different from photoreduction (6). The ferrous EDTA complex at low pH is a very strong reducing agent with an oxidation-reduction potential \(E_m\) from 0.1824 to 0.1200 volt over the pH range of 2.29 to 3.68 and an \(E_m\) of 0.1172 volt over the pH range of 4.06 to 5.75 (7). These potentials are lower than that of cytochrome c (8), viz. 0.26 volt. The reduction of cytochrome c observed here is consistent with this difference in potentials.

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REFERENCES
Reduction of Cytochrome c by Ferrous Ions and Ethylenediaminetetraacetic Acid in Acid Solution
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