Effect of Inorganic Sulfur and Selenium on \( O_2 \)-Uptake and Aniline Hydroxylation by Dithiothreitol-Iron Complex in Comparison with Adrenodoxin and Se-Adrenodoxin

MOTOKO KUNISHIMA, YUKIO SUGIURA, MAYUMI TAKIMOTO, and HISASHI TANAKA

Faculty of Pharmaceutical Sciences, Kyoto University

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The additional coordination of inorganic sulfur or selenium to dithiothreitol (DTT)-iron complex induced a dramatic change of iron spin state from \( g = 4.4 \) (high) to \( g = 2.0 \) (low). The average \( g \)-values of DTT-Fe-S(1.99) and DTT-Fe-Se(2.02) complexes were larger than those of native S-adrenodoxin(1.97) and Se-adrenodoxin(2.00). The ability of \( O_2 \)-uptake and activity of aniline hydroxylation increased in the order, DTT-Fe-Se > DTT-Fe-S > DTT-Fe complexes. In addition, the optimum pH regions for \( O_2 \)-uptake and aniline hydroxylation by the model complexes were approximately parallel with those for complex formation in these DTT-iron systems. On the other hand, the hydroxylation activity of reduced S-adrenodoxin (yield of \( \beta \)-aminophenol: 10.90% per 90 min.) was clearly larger than that of reduced Se-adrenodoxin (7.64%). The effect of DTT-Fe-S and DTT-Fe-Se complexes on the activity of aniline hydroxylation, was compared with that of adrenodoxin and its selenium derivative.

The active site of iron-sulfur proteins is grouped into three classes: (1) Fe\( S_4 \) type, \( \text{e.g., rubredoxins} \); (2) Fe\( S_2 S^* \) type, \( \text{e.g., adrenodoxin and green plant ferredoxins} \); (3) Fe\( S^* \) type, \( \text{e.g., bacterial ferredoxins and high-potential iron proteins} \). Except for rubredoxins, all of these proteins contain inorganic sulfur or labile sulfur atoms which are denoted by \( S^* \). The presence of the inorganic sulfur in the iron-sulfur clusters has attracted the attention of us. Therefore, the incorporation of inorganic sulfur by model iron-sulfur complexes, the reconstitution of native Fe\( S_2 S^* \) typed adrenodoxin from artificial Fe\( S_4 \) typed adrenodoxin and the metal replacement for iron in adrenodoxin have been investigated to clarify the structural and functional roles of the inorganic sulfur. On the other hand, the labile sulfur atoms in the Fe\( S_2 S^* \) typed proteins were replaced with selenium atoms and the physico-chemical properties of the selenium derivatives have been compared with those of native proteins.

In this paper, \( O_2 \)-uptake and aniline hydroxylation by dithiothreitol (DTT)-Fe, DTT-Fe-S and DTT-Fe-Se complexes are investigated and the effect of the inorganic sulfur or selenium is discussed in comparison with native adrenodoxin and its selenium derivative. Recently, 2-mercaptopbenzoic acid and cysteine-iron complexes have been reported as a model for microsomal mixed function oxygenases.

1) Location: Yoshida, Shimoadachi-cho, Sakyo-ku, Kyoto, 606, Japan.
Experimental

Dithiothreitol (DTT) and $^{14}$C(U)-aniline hydrochloride (10.0 mCi/m mole) were obtained from Sigma and The Radiochemical Centre, respectively. Bovine adrenal ferredoxin (adrenodoxin) and the selenium derivative were prepared by a method similar to that of Kimura, et al.$^{50}$ A solution (2 ml) containing 20 mg of the apoprotein in 0.5 m Tris buffer (pH 7.4) was placed in a reaction vessel; a 20-fold molar excess of DTT over the protein was added to the apoprotein solution and urea was added to make a 6 m solution. After 1 hr. at 4°, a 3-fold molar excess of Na$_2$S (or H$_2$SeO$_3$) and a 3-fold molar excess of FeCl$_2$ in 0.5 ml of H$_2$O were added to the mixture. The reaction mixture was then kept for 1 hr at 4°. Then the reaction mixture was placed on a small DEAE-cellulose column (0.7 x 8 cm) and subsequently washed with 100 ml of 0.01 m phosphate buffer (pH 7.4) containing 0.17 m KCl. Adrenodoxin (or the selenium-substituted protein) was eluted with 0.01 m phosphate buffer (pH 7.4) containing 0.50 m KCl. The concentration of adrenodoxin and its selenium derivative obtained was calculated from the molar extinction coefficients, 8900 (at 414 nm) and 9000 (at 438 nm), respectively.$^{40}$ All other reagents used were of commercial reagent grade.

The model complexes, DTT-Fe-S and DTT-Fe-Se complexes, were prepared by the addition of sulfide or selenide ion to the mixture of excess DTT and ferric chloride in an aqueous solution at pH 9, and were characterized by optical and ESR spectra. It has been found that the molar ratio of sulfide or selenide to iron in the complexes is 1:1 and that the order of the stability is DTT-Fe-S$\geq$DTT-Fe-Se$\geq$DTT-Fe complexes.$^{9}$ The optical and ESR spectra were measured with a Shimadzu recording spectrophotometer, model Double-40R and a Varian spectrometer, model E-4, respectively. Oxygen uptakes were measured at 20° with a Toa dissolved oxygen meter, model DO-1A, equipped with a Clark electrode. The standard incubation mixture contained the model complex (0.4 mm) and $^{14}$C-aniline (4 mm) in an aqueous acetone medium (pH 9.2 and 50% v/v). The reaction mixture was incubated at 20° for 90 minutes. The separation and identification of the reaction products were performed by thin-layer chromatography, using the solvent system of benzene: methanol: acetic acid = 45: 8: 4. The yield of $p$-aminophenol formed was determined by measurement of $^{14}$C-radioactivity with a Beckman liquid-scintillation counter, model LS-238 and an Aloka 2π thin-layer radiochromatoscanner. All values represent averages of three experiments.

Results and Discussion

Characteristics of DTT-Iron Complexes

The DTT-Fe complex exhibited an ESR signal at $g=4.4$, which should be assigned to high-spin ferric ion(S=5/2) under a rhombic environment. This signal is similar to that of rubredoxin (g=4.3), in which the iron atom is bound by the four cysteine residues in nearly tetrahedral

![Fig. 1. ESR Spectra of DTT-$^{56}$Fe, DTT-$^{56}$Fe-$^{32}$S, and DTT-$^{56}$Fe-$^{80}$Se Complexes](image)

The spectra were measured by mixing DTT (100 mm) and FeCl$_3$ (5 mm), or DTT (100 mm), Na$_2$S or H$_2$SeO$_3$ (5 mm) and FeCl$_3$ (5 mm) at pH 9.2.

Modulation amplitude, 5 G; microwave power, 5 mW; microwave frequency, 9.28 GHz; temperature, 77 K.

symmetry on the basis of X-ray crystallography. On the other hand, the DTT-Fe-S and DTT-Fe-Se complexes showed a characteristic ESR absorption near \( g = 2.0 \), which might be based on low-spin ferric ion \((S = 1/2)\) (see Fig. 1).

The average \( g \)-values of the DTT-Fe-S \((1.99)\) and DTT-Fe-Se \((2.02)\) complexes are larger than those of reduced adrenodoxin \((1.97)\) and its selenium derivative \((2.00)\). Table I summarizes the characteristics of these model complexes.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Abs. Max., nm</th>
<th>( g )-Value</th>
<th>Spin state</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT-Fe</td>
<td>480</td>
<td>4.4</td>
<td>high ((S = 5/2))</td>
</tr>
<tr>
<td>DTT-Fe-34S</td>
<td>395</td>
<td>2.00, 1.96</td>
<td>low ((S = 1/2))</td>
</tr>
<tr>
<td>DTT-Fe-34Se</td>
<td>420</td>
<td>2.05, 2.01</td>
<td>low ((S = 1/2))</td>
</tr>
</tbody>
</table>

Perhaps, the additional coordination of inorganic sulfur or selenium to the DTT-Fe complex enhances the ligand field around iron atom, and subsequently induces the change of spin state.

**O₂-Uptake of DTT-Iron Complexes**

Fig. 2 shows the result of \( O₂ \)-uptake by the DTT-Fe complexes.

It was distinctly observed that the rates of the oxygen consumption of the DTT-Fe-S and DTT-Fe-Se complexes were much faster than that of the original DTT-Fe complex. The rate of \( O₂ \)-uptake was accelerated according to the order, DTT-Fe-Se > DTT-Fe-S > DTT-Fe complexes. Of special note is the promotive effect of inorganic sulfur or selenium. The effect of concentration of each complexes upon the rate of \( O₂ \)-uptake is shown in Fig. 3.

![Fig. 2. \( O₂ \)-Uptake of DTT-Fe Complexes](image)

![Fig. 3. Dependence of Rate of \( O₂ \)-Uptake on Concentration of DTT-Fe Complexes](image)

Fig. 2. \( O₂ \)-Uptake of DTT-Fe Complexes

Reaction mixtures contained DTT\( (4.0 \, \text{mM}) \) and FeCl₃\( (0.4 \, \text{mM}) \), or DTT\( (4.0 \, \text{mM}) \), Na₂S or H₂SeO₃\( (0.4 \, \text{mM}) \) and FeCl₃\( (0.4 \, \text{mM}) \). The reaction was carried out at 29° and pH 9.2. Initial amount of \( O₂ \) was approximately 280 \( \mu \text{L} \).

The rate of \( O₂ \)-consumption increased gradually with increasing concentration of the DTT-Fe complex. In contrast, the system containing the DTT-Fe-S or DTT-Fe-Se complex displayed a saturation behavior and revealed an effective uptake of oxygen with smaller concentra-

tion of the complex. As shown in Fig. 4, the maximal O₂-uptake of the DTT-Fe, DTT-Fe-S and DTT-Fe-Se complexes occurred at pH regions from 9.2 to 10.7, from 9.0 to 10.5 and from 8.5 to 11.0, respectively.

On the other hand, the optimum pH region for the complex formation of these iron complexes was approximately between 8.5 and 10.5. Both pH effects were substantially parallel. This fact indicates strongly that the complex formation plays an important role for the O₂-uptake of these model complexes.

Generally, it is well-known that metal ions, such as iron and copper, catalyze the oxidation of thiol compounds\textsuperscript{11).} If the consumption of O₂ occurs only through the following reaction mechanism, therefore, an estimate of the rate of O₂ uptake by the DTT-Fe(III)-S complex can be made from the first-order decay rate of the DTT-Fe(III)-S complex. Spectrophotometric and iodometric experiments showed that DTT-Fe-S complex had decayed with half-life of about 90 min. Accordingly, it can be roughly calculated that 0.4 mM of the DTT-Fe-S complex should be able to consume about 2–3 μM of O₂ per min. under the reaction conditions. However, the determined rate of O₂-uptake by the model compounds is about 230 μM per min. Although no direct evidence for O₂-activation mechanism is obtained in the present study, these model complexes, especially the DTT-Fe-S and DTT-Fe-Se complexes, may be able to activate O₂ very efficiently by bonding O₂ directly to the iron-sulfur environment. Of interest, in any event, is the effect of the inorganic sulfur and selenium for the O₂-uptake and stabilization of the DTT-Fe complex.

**Aniline Hydroxylation by DTT-Iron Complexes and Adrenodoxins**

Fig. 5 shows the typical result of thin-layer chromatography of the reaction solution obtained by the incubation of [\textsuperscript{14}C]-aniline with the model complexes.

![Figure 4](image)

**Fig. 4.** pH-Dependence on O₂-Uptake of DTT-Fe Complexes

Conditions were the same as in Fig. 2, except for pH of reaction.

![Figure 5](image)

**Fig. 5.** Thin-Layer Chromatogram of Reaction Solution Obtained from Incubation of [\textsuperscript{14}C]-Aniline and Model Complexes

The A–E represent the chromatogram of the following systems: A, aniline and DTT-Fe-Se complex; B, aniline and DTT-Fe-S complex; C, aniline and DTT-Se complex; D, aniline and Fe; E, aniline and DTT. Reference compounds were: 1, β-aminophenol; 2, α-aminophenol; 3, β-aminophenol; 4, DTT; 5, phenylhydroxylamine; 6, aniline; 7, mixture of compounds 1–6. Mobile phase, benzene: methanol:acetic acid = 45:8:4. The chromatogram was run for 2 hours.

Aniline is hydroxylated by the DTT-Fe-S and DTT-Fe-Se complexes and subsequently forms $\beta$-aminophenol as the main product, together with a very little amount of $o$-aminophenol and phenylhydroxylamine. The $para$: $ortho$-ratio is about 10:1 and no $m$-aminophenol was obtained. In general, the $ortho$-position always is subject to appreciable steric effect. This favored attack at the $para$-position clearly points to an electrophilic and considerable selective hydroxylation reaction. In contrast, the stannous/phosphate/oxygen system attacks unselectively the aromatic nucleus and reveals a hydroxylation pattern which would be expected for a random substitution. The effect of complex concentration on aniline hydroxylation by the DTT-Fe complexes is shown in Fig. 6.

![Graph showing effect of complex concentration on hydroxylation](image)

**Fig. 6. Effect of Complex Concentration on Hydroxylation of Aniline by DTT-Fe Complexes**

The model complexes used were as follows:
- DTT-Fe-Se($\exists \triangleright$)
- DTT-Fe-S($\exists \triangleright$)
- DTT-Fe($\Delta \triangleright$) complexes and Fe($\times \times$). The reaction conditions are indicated in the experimental section and the $\beta$-aminophenol formed was estimated.

| Table II. Hydroxylation of Aniline by Adrenodoxin and Se-Adrenodoxin |
|-----------------------------|------------------|
|                           | $\beta$-Aminophenol formed (%) |
| Adrenodoxin (oxidized)    | 4.55             |
| Adrenodoxin (reduced)     | 10.90            |
| Se-Adrenodoxin (oxidized) | 6.75             |
| Se-Adrenodoxin (reduced)  | 7.64             |

The reaction solutions contained $^{14}$C-aniline (2.0 mg) and adrenodoxin (0.043 mg) or Se-adrenodoxin (0.006 mg) at pH 8.5, and were incubated for 90 min at 20°C.

Under all concentrations of the model complexes investigated, the activity of aniline hydroxylation increased in the order, DTT-Fe-Se > DTT-Fe-S > DTT-Fe complexes. This order corresponds well to that of $O_2$-uptake by these iron complexes.

On the other hand, Table II shows the result of aniline hydroxylation by iron–sulfur proteins.

Of quite interest is the fact that adrenodoxin can effect hydroxylation of aniline in the absence of a hydroxylase enzyme. However, it is noteworthy that the activity of native S-adrenodoxin(reduced) is clearly larger than that of Se-adrenodoxin(reduced). From the results of the optical absorption characteristics, the g-values of ESR absorptions and spin-orbit coupling constants, it has been indicated that the paramagnetic center of Se-adrenodoxin is more rhombohedral than that of the native protein, and that the Fe-Se bond is more covalent than the Fe-S bond. The activity of Se-adrenodoxin toward steroid-11$\beta$-hydroxylation, however, was found to decrease approx. 60% as active as the native adrenodoxin. Considering the differences concerning the hydroxylation-effect of the inorganic sulfur and selenium between the model complex and native protein, we strongly feel that the amino acid sequence and resulting three dimensional structure of adrenodoxin require reasonably the inorganic sulfur to produce its optimum biological activity. The yield of $\beta$-aminophenol formed by these systems was about 10% per 90 min, and especially the effect of native adrenodoxin was almost same level at lower concentration (approx. 1/10) in comparison with that of the model DTT-Fe complexes.

Using the reconstituted liver microsomal hydroxylation system, on the other

hand, Lu, et al.\textsuperscript{13} have found that the hydroxylation of aniline by rat liver microsomes requires the three components—cytochrome p-450, reductase and lipid fraction—for maximal activity, and that the yield of \( p \)-aminophenol formed is about 1\% per 15 min.

The aniline hydroxylation by the DTT-Fe complexes proceeded optimally at pH region, 8.5—10.5, and the activity was negligibly small at acid pH (<5) and strong alkaline pH (>12) regions. This result implicates strongly that the complex formation participates significantly in the aniline hydroxylation in the DTT-Fe systems. In addition, it is also suggested that superoxide anion radical is not main intermediate in this aniline hydroxylation, because the radical would be converted to perhydroxyl radical (O\(_2\)H\(^{+}\)) at acid pH region\textsuperscript{14} and subsequently it could be predicted that the hydroxylation would be enhanced at acid pH region. Certainly, we found that the systems of the model DTT-Fe complexes produced no superoxide anion radical, as determined by the co-oxidation of epinephrine to adrenochrome\textsuperscript{15} and by the reduction of nitroblue tetrazolium.\textsuperscript{16} On the other hand, superoxide anion radical has been shown to be generated during the autoxidation of reduced adrenodoxin,\textsuperscript{16b} as well as ferredoxins\textsuperscript{17} and xanthine oxidase.\textsuperscript{18} Accordingly, the following reaction scheme (Fig. 7) might be postulated to explain the aniline hydroxylation by the model DTT-Fe complexes and native adrenodoxin, although the proposed dioxygen intermediate is not detected.

\begin{equation}
\text{(A) DTT–Fe complexes}

2\text{DTT} + \text{Fe}^{2+} \rightarrow (\text{DTT})_2\text{FeO}_2

\end{equation}

\begin{equation}
\text{(B) adrenodoxin}

\text{adrenodoxin (reduced)} + \text{O}_2 \rightarrow \text{N}_2\text{S}_2\text{O}_4 \rightarrow \text{adrenodoxin (oxidized)} + \text{O}_2^{2-}

\end{equation}

\begin{equation}
\text{O}_2^{2-} + \text{NH}_2\text{OH} \rightarrow \text{NH}_2\text{OH} + 1/2\text{O}_2

\end{equation}

Fig. 7. Probable Reaction Scheme

In summary, the inorganic sulfur and selenium coordinated to the DTT-Fe complex and induced the change of spin state from high spin (S=5/2) to low spin (S=1/2) ferric ions. The DTT-Fe-S and DTT-Fe-Se complexes enhanced the ability of \( O_2 \)-uptake and the activity of aniline hydroxylation in comparison with the original DTT-Fe complex. In addition, native adrenodoxin and its selenium derivative catalyzed also aniline hydroxylation to produce \( p \)-aminophenol.

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\textsuperscript{17} H.P. Misra and I. Fridovich, \textit{J. Biol. Chem.}, 246, 6886 (1971).