Studies on Adrenal Steroid Hydroxylases

THE REACTIVITY OF TRIPHENYL PHOSPHINE WITH LABILE SULFUR IN ADRENAL IRON-SULFUR PROTEIN (ADRENODOXIN)*

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SUMMARY

The labile sulfur of adrenodoxin was attacked by a nucleophile, triphenyl phosphine. The product was triphenyl phosphine sulfide regardless of the presence of O₂. The stoichiometry between labile sulfur which disappeared and triphenyl phosphine sulfide formed was found to be 1:1. The initial rate of the reaction follows second order kinetics with a rate constant of 2 to 3 × 10⁴ M⁻¹ min⁻¹ at 38°C (pH 8.8) under aerobic and anaerobic conditions.

The product of the minor reaction was found to be pyruvate after acid hydrolysis of the protein. The amount of pyruvate formed (about 1 mole per mole of protein) is roughly equal to those of cysteine residues lost and H₂S evolved. There was no significant formation of alanine and serine by this reaction. The triphenyl phosphine-treated protein has no iron, no labile sulfur, one disulfide bond, 1 dehydroalanine, and 2 cysteine residues per molecule of the protein.

On the basis of these observations, it is concluded that, upon reaction of triphenyl phosphine with adrenodoxin, the labile sulfur is removed as the phosphine sulfide, and that one of the sulfur atoms of the cysteine residues is liberated as H₂S and results in the dehydroalanine residue in the polypeptide chain, which is quantitatively converted into pyruvate upon acid hydrolysis. This phenomenon is in contrast with the acid treatment which removes the labile sulfur as H₂S but does not produce dehydroalanine.

From the results of the triphenyl phosphine reactivity of model metal-sulfur compounds, Ni(C₃H₇CS₂)(C₆H₅CS₂) and Fe(C₃H₇CS₂)(C₆H₅CS₂), it would be likely that the iron-sulfur grouping of adrenodoxin as its oxidation-reduction center consists of Fe-S-S- linkage, because the above model compounds contain the metal-S-S- linkage, as judged by x-ray crystallography (D. COUCOUVANIS and S. J. LIPPARD, J. Amer. Chem. Soc., 90, 3281 (1968)), and they react with triphenyl phosphine to yield its sulfide.

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Optical absorption spectroscopy was carried out by the use of Hitachi spectrophotometer (EPS-3 and 124), and radioactivity was measured by a liquid scintillation counter (Packard Tri-Carb 314). For calculations, the molecular weight of adrenodoxin was assumed to be 13,000.

Ni(C\textsubscript{3}H\textsubscript{5}CS\textsubscript{2})\textsubscript{2} was synthesized as follows. C\textsubscript{6}H\textsubscript{5}SSH was prepared by the reaction of C\textsubscript{6}H\textsubscript{5}MgBr and CS\textsubscript{2}. To 0.1 M aqueous solution of dithio-benzoic acid potassium salt, calculated amounts of solid NiSO\textsubscript{4}·7H\textsubscript{2}O were added at equimolar ratio. The precipitate formed was washed with methanol and benzene and the compound was then extracted with methanol by the use of a Soxhlet apparatus. After evaporation of methanol, blue crystals were obtained. From elementary analyses of this sample, this compound was identified as Ni(C\textsubscript{3}H\textsubscript{5}CS\textsubscript{2})\textsubscript{2}.

C\textsubscript{6}H\textsubscript{5}SSH

Calculated: C 46.0, H 2.8, S 33.12

Found: C 45.4, H 2.5, S 34.72

The melting point was found to be 220° decomposed. The literature value is 219° decomposed (5). To the benzene solution of (C\textsubscript{3}H\textsubscript{5}CS\textsubscript{2})\textsubscript{2}Ni, an excess amount of the aqueous solution of Na\textsubscript{2}S\textsubscript{2} was added and shaken in a separatory funnel. The benzene layer became reddish violet. Alternatively, piperidine sulfur complex or P\textsubscript{2}S\textsubscript{5} reacts in a similar manner as Na\textsubscript{2}S\textsubscript{2} to produce the reddish violet material. After evaporation of benzene, the solid material was obtained, and then extracted with methanol by the Soxhlet method. Blue violet crystals were obtained.

C\textsubscript{6}H\textsubscript{5}SSH

Calculated: C 42.3, H 2.5, S 39.35

Found: C 41.8, H 2.6, S 39.11

The melting point was observed as 200° decomposed. The literature value is 195° decomposed (5). To the benzene solution of (C\textsubscript{3}H\textsubscript{5}CS\textsubscript{2})(C\textsubscript{6}H\textsubscript{5}CS\textsubscript{2})Ni, an excess amount of the aqueous solution of Na\textsubscript{2}S\textsubscript{2} was added and shaken in a separatory funnel. The benzene layer became reddish violet. Alternatively, piperidine sulfur complex or P\textsubscript{2}S\textsubscript{5} reacts in a similar manner as Na\textsubscript{2}S\textsubscript{2} to produce the reddish violet material. After evaporation of benzene, the solid material was obtained, and then extracted with methanol by the Soxhlet method. Blue violet crystals were obtained.

C\textsubscript{6}H\textsubscript{5}SSHNi

Calculated: C 46.0, H 2.8, S 33.12

Found: C 45.4, H 2.5, S 34.72

The identification of the reaction product with triphenyl phosphine sulfide was carried out by measuring the melting point, of which literature value is 159-161° (8), and also by the use of infrared absorption spectroscopy. The authentic sample displayed maxima at 14.45, 13.93, 13.21, and 9.05 µm. The band at 9.05 µm was assigned to phosphorus-oxygen double bonds.
same order kinetics, indicating to be a complex reaction. Under anaerobic conditions, a similar rate constant was obtained (2.27 X 10^-3 M^-1 min^-1). The fitting of the experimental values to a second order reaction is illustrated in the inset of Fig. 2.

Effect of pH—The effects of pH on the rates of the triphenyl phosphine reaction with adrenodoxin were examined in a solution of 33% ethanol over the region between pH 6.1 and 9.4. The pH profile indicated a broad maximum at pH 8.4 with decreases in the rates at alkaline pH values, whereas the rates below pH 7.8 were essentially unchanged. These results are consistent with the interpretation of the reaction as a nucleophilic attack of triphenyl phosphine.

Stoichiometry between Triphenyl Phosphine and Adrenodoxin—Adrenodoxin was treated with various amounts of triphenyl phosphine. After the reaction ceased, the decrease in absorbance at 414 nm was determined. The amount of adrenodoxin decomposed was calculated by the use of 9.8 X 10^5 M^-1 cm^-1 as molar extinction coefficient at 414 nm. As shown in Table II, the molar ratio of reacted adrenodoxin to added triphenyl phosphine is approximately 1:2 under all of the conditions used. When the absorption spectrum in the visible region of the reaction mixture containing adrenodoxin and triphenyl phosphine in a ratio of 0.125 was examined, the spectral shape was found to be quantitatively identical with that of native adrenodoxin with no new peak and shoulder. Similarly, it may be recalled that no such new peak was observed during the course of complete bleaching of adrenodoxin by triphenyl phosphine (Fig. 1). These results suggest that there is no accumulation of a modified adrenodoxin containing only 1 mole of labile sulfur per mole of protein, as one could expect such molecule to give a quite different absorption spectrum from the original one. It is, therefore, concluded that when the first atom of labile sulfur reacts with triphenyl phosphine, the second one reacts immediately with the tertiary phosphine.
The experimental conditions for Experiment I are described in the text, and those for Experiment II are as follows. The reaction mixture contained equimolar amounts of the labile sulfur (795 nmoles) in adrenodoxin (5.08 mg of protein) and triphenyl phosphine in 1:2 (v/v) mixture of ethanol and 0.01 M phosphate buffer (pH 7.4). The reaction was carried out at 37°C for 22 hours under aerobic conditions.

The amounts of carrier triphenyl phosphine sulfide added were 99.18 and 61.07 mg, respectively, in Experiment I, A and B. The specific activity of tritiated triphenyl phosphine used was 3.013 x 10^10 cpm per mole. The final constant specific activity of recrystallized triphenyl phosphine sulfide was 6.53 x 10^10 and 1.06 x 10^9 cpm per mole for Experiment I, A and B, respectively.

For iron and labile sulfur determination of the treated samples, the phosphine sulfide was removed by passing through a Sephadex G-25 column. For amino acid analyses, the samples were passed through a Sephadex G-25 column, and precipitated by 5% trichloroacetic acid. After washing with alcohol and ether, the precipitates were dissolved in water and dialyzed against distilled water extensively. The dialysate was lyophilized to dryness. After measuring the weight, the sample was oxidized by performic acid for 4 hours at 0°C (10), and after removal of performic acid by lyophilization the dried material was subjected to acid hydrolysis.

p-Hydroxymercuribenzoate titrations were performed spectrophotometrically by measuring the absorbance increase at 250 nm after allowing the samples to stand for 20 hours at 23°C (4).

The lactate dehydrogenase assay for pyruvate was carried out in neutralized aliquots of the acid hydrolysate without performic acid oxidation by measuring the decrease of absorbance at 340 nm.

The determination of H2S produced during the reaction was performed as follows. In a Thunberg-type tube, the reaction mixture contained 0.75 µmole of the labile sulfur in adrenodoxin and 0.75 µmole of triphenyl phosphine in 1:2 (v/v) mixture of ethanol and 0.1 M Tris buffer (pH 8.4). The reaction was carried out for 5 hours at 33°C. A completely bleached sample was subjected to the H2S determination.

The number of cysteic acid and alanine residues per mole of the native protein were calculated as 5 and 7, respectively. These values and the total number of -SH groups obtained here are somewhat higher than those of our previous report (11), but agree well with the recent data of the total amino acid sequence determination of adrenodoxin done by Tanaka, Hanju, and Yasunobu (12). Therefore, the theoretical value of the total -SH per mole is 2 x 2 + 5 = 9 (0.692 µmole per mg of protein), since 1 mole of H2S or labile sulfur consumes 2 moles of p-hydroxymercuribenzoate. Also, the low value for the serine residues reported in this table is the result of decomposition of this amino acid by performic acid oxidation.

All values in this table are micromoles or microatoms per mg of protein.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Adrenodoxin</th>
<th>Iron</th>
<th>Labile sulfur</th>
<th>SH</th>
<th>Cysteic acid</th>
<th>Alanine</th>
<th>Serine</th>
<th>H2S produced</th>
<th>Pyruvate formed</th>
<th>Triphenyl phosphine sulfide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated sample</td>
<td>0.154</td>
<td>0.140</td>
<td>0.062</td>
<td>0.36</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated sample</td>
<td>0</td>
<td>0</td>
<td>0.056</td>
<td>0.83</td>
<td>0.43</td>
<td>0.434</td>
<td>0.04</td>
<td>0.006</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Treated sample</td>
<td>0</td>
<td>0</td>
<td>0.056</td>
<td>0.83</td>
<td>0.43</td>
<td>0.434</td>
<td>0.04</td>
<td>0.006</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Difference</td>
<td>-0.155</td>
<td>-0.140</td>
<td>-0.051</td>
<td>-0.078</td>
<td>+0.005</td>
<td>+0.012</td>
<td>+0.054</td>
<td>+0.070</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- The amount of protein is expressed by the dry weight.
- The value for the native protein without any treatment.
- The small increases are regarded as within experimental errors.

**Reaction Products**—A reaction mixture containing 0.80 ml of adrenodoxin (670 natriums of iron, 610 nmoles of labile sulfur, and 4.35 mg of protein) and 0.80 ml of an alcohol solution of tritiated triphenyl phosphine (3.43 µmoles) was kept at room temperature for 16 hours. The color of adrenodoxin disappeared within 2 hours. Unlabeled triphenyl phosphine sulfide dissolved in benzene was then added to the reaction mixture. Triphenyl phosphine sulfide was extracted three times with benzene. The benzene solution was washed three times with water. The resulting benzene solution was then passed through an alumina column (1.5 x 2 cm). Eluted with benzene, the collected fractions containing the sulfide was evaporated to dryness. The white crystals were recrystallized from the alcohol solution until the specific activity became constant.

From the values of added carrier and final specific activity, the amounts of triphenyl phosphine sulfide formed in the reaction between triphenyl phosphine and adrenodoxin were calculated, and shown in the last column of Table III. It is clear that approximately equimolar amounts (732 and 734 nmoles in Experiment I, A and B, respectively) of triphenyl phosphine sulfide relative to the labile sulfur content (610 nmoles) of adrenodoxin were recovered from the reaction mixture.

The other product was the labile sulfur-free protein which was recovered by passing the reaction mixture through a Sephadex G-25 column to remove the phosphine sulfide and other small molecules. The colorless protein collected was then subjected to iron and labile sulfur analyses, p-hydroxymercuribenzoate titrations, pyruvate determinations, and amino acid analyses after performic acid oxidation and acid hydrolysis. As also seen from the Table III, the modified protein lost all of the iron and labile sulfur originally present in adrenodoxin. From the amino acid analysis, a portion of cysteine residues in the protein was found to be decomposed by this treatment. The product was subsequently found to be pyruvate but not alanine.

**Model Experiments with Metal Sulfur Compounds**—In order to define the reactivity of the labile sulfur toward triphenyl phosphine, some model experiments were carried out. Ni-
adrenodoxin. However, there is much supporting evidence to indicate that the oxidation-reduction center in all proteins of the ferredoxin family are closely similar to each other. One difference might be that the labile sulfur in the Chromatium protein is liberated only by drastic acid hydrolysis (17), whereas that of adrenodoxin and plant and bacterial ferredoxins is very liable to acid.

It has been established by us that benzyl and benzhydryl hydrosulfide, C₆H₅CH₂S⁻SHII, (C₆H₅)₂C⁺⁺S⁻SHII, the sulfenyl sulfur atom of which is labeled with radioactivity, is attacked by triphenyl phosphate both on sulfenyl and sulphydryl sulfur atoms by triphenyl phosphate as shown below (18, 19).

\[
\text{R}⁺⁺\text{SH} + \text{PR}⁺⁺ \rightarrow \left[ \text{R}⁺⁺\text{SH} \right]⁺⁺ \rightarrow \text{R}⁺⁺\text{S}⁺⁺ \rightarrow \text{R}⁺⁺\text{S}⁺⁺
\]

The experiments with the hydrosulfides (18, 19) indicate that Sequence A occurs to a great extent and Sequence B only occurs to a minor extent. It is also known that triphenyl phosphate does not react with RSH, RSSR, and H₂S under the conditions similar to those used in this investigation.

From the data presented here, it can be concluded that triphenyl phosphate reacts with the labile sulfur in adrenodoxin regardless of the presence of O₂. The major product was triphenyl phosphate sulfide. After acid hydrolysis of the treated protein, the minor product was found to be pyruvate but not alanine which is expected under Sequence B. Thus, rather than a reductive desulfhydration of cysteine, a β elimination of H₂S occurs.

In contrast with the fact that the acid treatment of adrenodoxin does not produce dehydroalanine (pyruvate is quantitatively produced from dehydroalanine by acid hydrolysis) from the cysteine residues, the triphenyl phosphate treatment does produce pyruvate after hydrolysis. The amount of pyruvate (0.91 mole per mole of protein) and H₂S (0.70 mole per mole of protein) produced is roughly equal to that of the cysteine residues lost (1.01 moles per mole of protein). It shall be noted here that the excess amount of triphenyl phosphate sulfide formed (2.19 - 2.00 = 0.19 mole per mole of protein) is considerably smaller than the amount of pyruvate formed from adrenodoxin.

About one-half of the untreated cysteine residues in the treated protein is oxidized to cystine residue under these conditions: 0.562 - (2 × 0.154 + 0.075) = 0.174 mole per mg of protein = 2.28 moles per mole of protein. Thus, the stoichiometry among cysteine lost, pyruvate formed, H₂S evolved, triphenyl phosphate sulfide formed, half-cystine (disulfide) formed, and remaining cysteine is 1.01:0.91:0.70:2.19:2.28:1.70 = 1:1:1:2:2:2.2. The sum of cysteine lost and half-cystine formed and remaining cysteine (1.01 + 2.28 + 1.70 = 4.99 moles per mole of protein) is exactly identical with the number of the total residues in the native protein (5 moles per mole of protein).

It has been postulated for the iron-sulfur grouping that the

\[
\text{Fe} + \text{S}⁻⁻ \rightarrow \text{FeS}⁻⁻ \rightarrow \text{FeS}⁺⁺ \rightarrow \text{Fe}⁺⁺\text{S}⁺⁺ \rightarrow \text{Fe}⁺⁺\text{S}⁺⁺
\]

The detailed chemical structure of labile sulfur in ferredoxins is not yet known. Although x-ray crystallography would be the best approach to this problem, some difficulties must be overcome to elucidate the structure by this method. A preliminary x-ray analysis of Chromatium high potential nonheme iron protein (19) indicated that the oxidation-reduction iron sulfur center is similar to that of (C₆H₅)₂FeS₂ (14, 15). A recent proton nuclear magnetic resonance study on the same protein supported this model (16). There is yet no such information available on the structure of the oxidation-reduction center of adrenodoxin. However, there is much supporting evidence to indicate that the oxidation-reduction center in all proteins of the ferredoxin family are closely similar to each other. One difference might be that the labile sulfur in the Chromatium protein is liberated only by drastic acid hydrolysis (17), whereas that of adrenodoxin and plant and bacterial ferredoxins is very liable to acid.

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\]
labile sulfur atom bridges the 2 iron atoms (Fe-S-Fe). This structure which does not contain disulfide bond might be attacked by triphenyl phosphate. In order to evaluate this possibility, we have carried out some model experiments with metal-disulfide compounds. The results obtained in this study show that the Structure VI is not attacked by triphenyl phosphate. Thus, the reactions of triphenyl phosphate with these model compounds can be described as follows.

\[
\begin{align*}
\text{Ni}(C_2H_2-C\bar{S})_2(C_2H_2-C\bar{S}) + P(C_6H_3) & \rightarrow \\
\text{Ni}(C_2H_2-C\bar{S})_3 + S=P(C_6H_3) & \\
(C_2H_2-C\bar{S})_2Fe(C_2H_2-C\bar{S}) + P(C_6H_3) & \rightarrow \\
(C_2H_2-C\bar{S})_2Fe(C_2H_2-C\bar{S})_2 + S=P(C_6H_3) & \\
Fe(C_2H_2-C\bar{S})_3 + S=P(C_6H_3) &
\end{align*}
\]

Fackler and Coucouvanis (6) and Coucouvanis and Lippard (7) reported the reactivity of triphenyl phosphate toward the same metal compound and Fe(C_2H_2-C\bar{S})_2(C_2H_2-C\bar{S})_2. Furthermore, they determined the structure of Fe(C_2H_2-C\bar{S})_2(C_2H_2-C\bar{S})_2 by x-ray crystallography, indicating the presence of Fe-S-S linkage in this compound. According to their observations, triphenyl phosphate reacts with metal-S-S linkage, but not with -S-S linkage. Our present results on the model experiments were essentially able to confirm their finding.

In this context, it is likely to suggest that the iron-sulfur grouping in adrenodoxin consists of Fe-S-S linkage. However, since Structure VI which was used for the present model experiments, is not exactly identical with the structure with dimeric iron atoms, we could not completely exclude the possibility of the presence of Fe-S-Fe linkage in adrenodoxin through our present model experiments. Yet, our preliminary results indicated that a brown dimeric iron compound, (o-phenanthroline)_2Fe(OH)_2Fe(o-phenanthroline)_2 does not react with triphenyl phosphate under anaerobic conditions. This fact strongly suggests that the bridging oxygen atoms of this compound are not attacked by this reagent, in contrast with the anaerobic reactivity of triphenyl phosphate with adrenodoxin. It should be emphasized here that our procedures for synthesis of the model metal-sulfur compounds are extremely similar to that of reconstitution of native ferredoxins from their labile sulfur and iron-free apoprotein, iron salt, Na\textsubscript{2}S, and mercaptoethanol as reductant. Furthermore, S\textsuperscript{2-} never reacts with triphenyl phosphate to yield its sulfide. These facts are, perhaps, strong support for our present conclusion. In this connection, Fackler and Coucouvanis (6) and Coucouvanis and Lippard (7) could not correlate their model compounds to the iron-sulfur grouping of ferredoxins through their structural works, although they appear to be in favor of the idea of the presence of Fe-S-S bound in ferredoxins.

Under these circumstances, together with all available information on the structure of this protein, we would propose the following hypothetical structure for the oxidation-reduction center of adrenodoxin. The final conclusion must await the X-ray crystallographic analysis.

\[\text{Hypothetical Structure for Oxidized Oxidation-Reduction Center of Adrenodoxin}\]

Another point of interest is the fact that only 1, but not 2 or more, dehydroalanine residue in the polypeptide chain of adrenodoxin is produced by the triphenyl phosphate reaction. It is believed that 4 cysteine residues out of the total 5 cysteine residues in this protein are participating in the 2 iron-2 labile sulfur grouping of adrenodoxin. If the 1 cysteine residue attacked by this reagent participates in the oxidation-reduction center, this cysteine must be in a different microenvironment than the other 3 cysteines. Thus, it would be of interest to see whether or not the different environment of this particular cysteine is responsible for the intrinsic distortion and optical asymmetry of the iron-sulfur chromophore (3). The location of this particular cysteine residue in this protein is under investigation together with the mechanism of dehydroalanine formation in the triphenyl phosphate reaction.

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REFERENCES

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