The Reactive Sulfhydryl Groups of Microsomal Cytochrome Reductase*

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The results of previous work have indicated (2, 3) that one sulfhydryl group of microsomal cytochrome reductase is essential for the interaction of nucleotides with this enzyme. Thioether results, however, do not rule out the possibility that there are other reactive sulfhydryl groups on the native enzyme and that they are involved directly or indirectly in nucleotide-enzyme interactions. In the experiments described below, sulfhydryl group reagents have been used to characterize the reactive sulfhydryl groups of the native enzyme. It will be shown that each sulfhydryl group can be distinguished from the others by at least one property. On the basis of these properties, N-ethyl maleimide derivatives of microsomal cytochrome reductase, containing between one and two reactive sulfhydryl groups per molecule of protein, have been prepared. These derivatives catalyze all of the reactions of microsomal cytochrome reductase thus establishing that only one specific sulfhydryl group is involved in nucleotide interactions with the enzyme.

EXPERIMENTAL PROCEDURE

Aerobic and anaerobic procedures for measuring both enzymatic activities and spectral changes have been described previously (2, 3). Measurements were made with a Beckman model DU spectrophotometer with a photomultiplier attachment. Water at various temperatures from 0 to 25° was circulated from a constant temperature bath through thermostepers, obtained from Beckman Instruments, Inc., to obtain desired chamber temperatures during spectral observations. The standard assays of the catalytic activity of microsomal cytochrome reductase were carried out at 25°, aerobically, in micro cells containing 0.025 μ mole of DPNH, 0.05 μ mole of potassium ferricyanide (added at zero time), and enzyme in 0.20 ml of 0.1 M Tris acetate, pH 8.1. The final enzyme concentration in the assay system was usually 1.1 x 10^{-5} M. The oxidation of DPNH was followed by the decrease in absorbancy at 340 μm at 15-second intervals for 2 minutes. A correction for the small contribution of potassium ferricyanide reduction to the 340 μm absorbancy change was made.

A modification of the method of Boyer (4) for the determination of protein sulfhydryl groups with p-chloromercuribenzoate was used. For the enzyme titrations, 0.01 ml of 10^{-3} M mercuribenzoate was added to a reference cell containing 0.20 ml of 0.1 M Tris acetate, pH 8.1, and to a cell containing 0.20 ml of 5 x 10^{-6} M microsomal cytochrome reductase and 0.1 M Tris acetate, pH 8.1, at 0 to 5°. The increase in absorbancy at 250 μm after 5 minutes was determined and corrected for the volume change due to mercuribenzoate addition. The increase in absorbancy at 250 μm on adding the mercury compound to standard glutathione solutions under the same conditions is 7.8 for a 10^{-3} M glutathione solution with a spectrophotometer slit opening that gives a band width for the incident light of 0.3 to 0.4 μm at half the maximal light intensity. This value of the increase in absorbancy was used to calculate the sulfhydryl group content of the enzyme.

Microsomal cytochrome reductase was prepared as described previously (2) with a slight modification, namely that the original homogenate was made with 3 rather than 9 volumes of sucrose media. The ratio of the absorbancy at 275 μm to that at 460 μm was between 6.9 and 7.3 for all preparations used. Any preparation with a higher ratio was purified to fall within this range by a second gel treatment and ammonium sulfate fractionation as described for the original purification procedure (9). The enzyme was stored at -20° in 0.1 M Tris acetate, pH 8.1. Protein concentrations for these experiments were based on the flavoprotein absorption spectrum (2).

The DPNH, p-chloromercuribenzoate, N-ethyl maleimide, and glutathione were obtained from the Sigma Chemical Company. Duponol® was a product of E. I. DuPont de Nemours and Company, Inc.

RESULTS

Inhibition of Enzyme Activity and Flavin Reduction by Mercurybenzoate—In agreement with the observations with p-chloromercuribenzenesulfonate (2), the stoichiometry of mercuribenzoate inhibition of enzyme activity shows that one sulfhydryl group of microsomal cytochrome reductase is essential for enzyme activity (Fig. 1, Curve 1). Experimentally, complete inhibition requires more than one equivalent of either inhibitor, suggesting that another protein sulfhydryl group or groups are beginning to react with the mercurial reagent as saturation of the essential sulfhydryl group is approached. With either reagent, however, an extrapolation of the data between 0 and 0.7 equivalent of inhibitor yields a value of 1 equivalent at 100% inhibition. If DPNH is added to tubes containing microsomal cytochrome reductase and varying amounts of mercuribenzoate before dilution, a decrease in absorbancy at 250 μm was measured. A preliminary report of this work has appeared (1). This investigation was supported by research grants H-7768 from the National Heart Institute, United States Public Health Service, and NSF-G 4552 from the National Science Foundation.
tion for enzymatic activity assays, the inhibition of DPNH oxidation by the mercurial is partially reversed (Fig. 1, Curve 2). The DPNH concentration in these incubation mixtures is the same as that used in the assay systems. This apparent reactivation of mercuribenzoate-inhibited enzyme at relatively high enzyme concentrations in the presence of DPNH is also observed when the extent of flavin reduction by DPNH is measured (Fig. 1, Curve 3). Two equivalents of p-chloromercuribenzoate prevent both the reactivation of the enzyme activity and flavin reduction by DPNH. These data suggest that at the higher protein concentrations a slow migration of the mercuribenzoate from the essential protein sulfhydryl group to another protein sulfhydryl group with only a slightly lower affinity for mercu-
benzoate may occur in the presence of DPNH. The increased concentration of protein sulfhydryl groups as well as the presence of DPNH, which protects the essential sulfhydryl group (3), would favor such a reaction.

Titratable Sulfhydryl Groups of Microsomal Cytochrome Reductase

Titrations of several preparations of microsomal cytochrome reductase with mercuribenzoate at pH 8.1, by the modification of the method of Boyer (4) described earlier in this paper, show that the active enzyme contains three reactive sulfhydryl groups per flavin (Table I). Under these conditions all of the mercuribenzoate can be removed by dialysis against glutathione to regenerate the active enzyme. The rather acid conditions of Boyer (4) were not used in these titrations since microsomal cytochrome reductase is irreversibly denatured at pH 4.8 at 0 to 5° in 5 to 10 minutes. When the enzyme is denatured an additional sulfhydryl group is exposed (Table I) and the flavin is released from the enzyme as indicated by the loss of the typical flavoprotein spectrum (2). Duponol denaturation at pH 8.1 yields reproducible values for the sulfhydryl group content, but acid denaturation at pH 4.8 gave rather erratic and lower values. Since some turbidity was usually observed under acid conditions of denaturation, this variation is not surprising. The experiments described below will be concerned only with the characterization and reactions of the three reactive sulfhydryl groups of the native enzyme.

Table I

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Conditions of analysis*</th>
<th>Flavin content†</th>
<th>Sulfhydryl groups</th>
<th>Sulfhydryl groups per flavin</th>
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<tbody>
<tr>
<td>3</td>
<td>pH 8.1</td>
<td>0.00130</td>
<td>0.00380</td>
<td>2.92</td>
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<tr>
<td>7</td>
<td>pH 8.1</td>
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<td>0.00321</td>
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<tr>
<td>8</td>
<td>pH 8.1</td>
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<td>0.00380</td>
<td>3.02</td>
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<tr>
<td>7</td>
<td>pH 8.1, 0.45%</td>
<td>0.00105</td>
<td>0.00433</td>
<td>4.13</td>
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<tr>
<td>7</td>
<td>pH 8.1, 0.45%</td>
<td>0.00105</td>
<td>0.00425</td>
<td>4.05</td>
</tr>
<tr>
<td>7</td>
<td>pH 4.8</td>
<td>0.00100</td>
<td>0.00394</td>
<td>3.75</td>
</tr>
<tr>
<td>7</td>
<td>pH 4.8</td>
<td>0.00105</td>
<td>0.00372</td>
<td>3.54</td>
</tr>
</tbody>
</table>

* See "Experimental Procedure" for the analysis. In the last two trials a 0.1 M acetate buffer, pH 4.8, was used in place of 0.1 M Tris acetate, pH 8.1.
† Determined by flavoprotein spectrum (2).
‡ This concentration of Duponol inactivates the enzyme and releases the flavin as indicated by the change in absorption spectrum from that of the flavoprotein to that of free flavin adenine dinucleotide (2).

Fig. 1. The effect of p-chloromercuribenzoate (CMB) on enzyme activity and flavin reduction. For all trials, 0.20 ml of 0.1 M Tris acetate, pH 8.1, containing $4.5 \times 10^{-5}$ M microsomal cytochrome reductase, and from 0 to $2.5 \times 10^{-4}$ M CMB were incubated 10 minutes at 0 to 5°. Curve 1, aliquots of the incubation mixture were diluted with 0.1 M Tris acetate, pH 8.1, to a final concentration of $1.1 \times 10^{-4}$ M enzyme for the standard assay for enzymatic activity (“Experimental Procedure”). Curve 2, 0.01 ml of $2.5 \times 10^{-4}$ M DPNH was added to each incubation mixture and after 10 minutes aliquots were diluted to a final concentration of $1.1 \times 10^{-4}$ M enzyme with 0.1 M Tris acetate, pH 8.1, for the standard assay for enzymatic activity. Curve 3, 0.01 ml of $2.5 \times 10^{-4}$ M DPNH was added to each incubation mixture and the extent of flavin reduction measured by the decrease in absorbance at 460 mμ (2).

Fig. 2. The reaction of N-ethyl maleimide with microsomal cytochrome reductase. Curve 1, for each trial 0.025 amole of DPNH was added to 0.20 ml of 0.1 M Tris acetate, pH 8.1, containing $5 \times 10^{-4}$ M N-ethyl maleimide and $5 \times 10^{-4}$ M microsomal cytochrome reductase which had been incubated at 0 to 5° for the time indicated. The percent of flavin reduction at 400 mμ was measured (2). Curve 2, after incubation of 0.20-ml samples of 0.1 M Tris acetate, pH 8.1, containing $5 \times 10^{-4}$ M N-ethyl maleimide and $5 \times 10^{-5}$ M microsomal cytochrome reductase at 0 to 5° for the specified time, the sulfhydryl group content of the enzyme was determined (“Experimental Procedure”).
Characteristics of N-Ethyl Maleimide Reaction with Microsomal Cytochrome Reductase—The rate of reaction of N-ethyl maleimide with the sulfhydryl groups of microsomal cytochrome reductase and the rate of inhibition of DPNH reduction of the flavin by this reagent are shown in Fig. 2. When two sulfhydryl groups have reacted, the enzyme is completely inhibited. The third sulfhydryl group is clearly distinguished by its slow reaction with N-ethyl maleimide. Since the inhibition of enzymatic activity continues throughout the entire period during which the first two groups react, this experiment does not indicate whether one or both of these groups are involved in nucleotide-enzyme interactions.

When microsomal cytochrome reductase is incubated with N-ethyl maleimide in the presence of excess DPNH only one sulfhydryl group reacts rapidly with the sulfhydryl reagent and only two such groups react even on prolonged incubation (Fig. 3). One of the sulfhydryl groups which react rapidly with N-ethyl maleimide is therefore protected from the sulfhydryl reagent by the reduced pyridine nucleotide.

Properties of N-Ethyl Maleimide Derivatives of Microsomal Cytochrome Reductase—The properties of a typical N-ethyl maleimide derivative of microsomal cytochrome reductase, obtained by incubating the enzyme with the sulfhydryl reagent in the presence of excess DPNH, are summarized in Table II. This preparation, which contained only 1.15 reactive sulfhydryl groups per flavin, catalyzes the nucleotide reactions which have been observed with untreated microsomal cytochrome reductase (2, 3). Both the extent of flavin reduction by DPNH and the rate of reduced nucleotide oxidation catalyzed by the N-ethyl maleimide derivative indicate that there was no gross alteration in nucleotide-enzyme interaction by the addition of N-ethyl maleimide to the enzyme. Data similar to those shown in Table II have been obtained with several derivatives containing from 1.05 to 1.40 sulfhydryl groups per molecule by p-chloromercuribenzoate (CMB). The preparation of the enzyme derivative is described in Table II. For all experiments, 0.20 ml of 0.1 M Tris acetate, pH 8.1, containing 5 X 10^-4 M enzyme, and from 0 to 2.0 X 10^-5 M CMB were incubated 10 minutes at 0 to 5°. Curve 1, aliquots of the incubations were diluted with 0.1 M Tris acetate, pH 8.1, to 1.1 X 10^-5 M enzyme for the standard assay for enzymatic activity (“Experimental Procedure”). Curve 2, 0.01 ml of 2 X 10^-3 M DPNH was added to each incubation mixture and the extent of flavin reduction measured by the decrease in absorbancy at 460 mp (2).

Fig. 4. The inhibition of the N-ethyl maleimide derivative of microsomal cytochrome reductase containing 1.15 sulfhydryl groups per molecule by p-chloromercuribenzoate (CMB). The preparation of the enzyme derivative is described in Table II. For all experiments, 0.20 ml of 0.1 M Tris acetate, pH 8.1, containing 5 X 10^-4 M enzyme, and from 0 to 2.0 X 10^-3 M CMB were incubated 10 minutes at 0 to 5°. Curve 1, aliquots of the incubations were diluted with 0.1 M Tris acetate, pH 8.1, to 1.1 X 10^-5 M enzyme for the standard assay for enzymatic activity (“Experimental Procedure”). Curve 2, 0.01 ml of 2 X 10^-3 M DPNH was added to each incubation mixture and the extent of flavin reduction measured by the decrease in absorbancy at 460 mp (2).

Table II

<table>
<thead>
<tr>
<th>Reaction tested</th>
<th>Activity of derivative (% of untreated enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation of DPNH</td>
<td>78</td>
</tr>
<tr>
<td>Extent of flavin reduction by DPNH</td>
<td>97</td>
</tr>
<tr>
<td>Oxidation of deamino analogue of DPNH</td>
<td>85†</td>
</tr>
<tr>
<td>Oxidation of 3-acetylpyridine analogue of DPNH</td>
<td>80†</td>
</tr>
</tbody>
</table>

† These activities were determined in the standard assay system described in “Experimental Procedure” with the particular nucleotide analogue substituted for DPNH.
of the native enzyme in which all of the sulfhydryl groups are intact. By allowing two of the sulfhydryl groups of microsomal cytochrome reductase to react with N-ethyl maleimide only one reactive site for mercuribenzoate remains, and this site has a much higher affinity for the mercurial than DPNH. It is protected by DPNH during the incubation with N-ethyl maleimide because the reduced nucleotide first reacts with the enzyme to yield a complex in which the DPN is bound tightly to reduced enzyme (3, 5).

**DISCUSSION**

Microsomal cytochrome reductase is an example of an enzyme of fairly low molecular weight in which several cysteine residues can be distinguished from one another by the differences in the reactivities of their sulfhydryl groups. As the present study shows, the number of reactive sulfhydryl groups is fortunately quite small. Only three such groups react with p-chloromercuribenzoate when the enzyme is in an active, undenatured form. Earlier experiments (2, 3) had already indicated that one sulfhydryl group of the protein is essential for enzymatic activity and is protected from p-chloromercuribenzenesulfonate by DPNH. This group has now been identified as the sulfhydryl group which reacts rapidly with N-ethyl maleimide but is protected from the sulfhydryl reagent by DPNH. The second sulfhydryl group reacts rapidly with N-ethyl maleimide when DPNH is present or absent. Finally, the third group is clearly distinguished by its very slow reaction with N-ethyl maleimide.

Two types of evidence establish that only the one specific sulfhydryl group protected by DPNH is involved in nucleotide reactions with microsomal cytochrome reductase. First, the N-ethyl maleimide derivative, which contained only 1.15 reactive sulfhydryl groups per flavin, catalyzed all of the nucleotide reactions observed with the untreated enzyme. Second, both the extent of flavin reduction by DPNH and the catalytic activity of the N-ethyl maleimide derivative were completely inhibited by exactly 1 equivalent of mercuribenzoate.

The three reactive sulfhydryl groups identified in these experiments are not the only sulfhydryl groups of microsomal cytochrome reductase. It has been shown here that denaturation of the enzyme exposes a fourth such group. It is also possible that other sulfhydryl groups do not become reactive or are destroyed by this treatment. The observation that denaturation with Duponol does result in the exposure of one new protein sulfhydryl group as well as in the release of the flavin, raises the question whether these two effects are merely coincidental or whether this sulfhydryl group is involved in flavin binding. Experiments on the effect of mercuribenzoate on the reactivation of the apoenzyme should help to decide between the two alternatives.

**SUMMARY**

1. Titrations of microsomal cytochrome reductase with p-chloromercuribenzoate showed that the active enzyme contains three reactive sulfhydryl groups.
2. Denaturation of microsomal cytochrome reductase releases a fourth sulfhydryl group.
3. The three reactive sulfhydryl groups of the native enzyme have been distinguished from one another by differences in their reactivities to various sulfhydryl group reagents.
4. The properties of N-ethyl maleimide derivatives of the enzyme establish that only one specific sulfhydryl group is involved in nucleotide interactions with microsomal cytochrome reductase.

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**REFERENCES**

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