The Thiol Groups of Fumarase*

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SUMMARY

Several reagents have been employed to determine the role of the thiol groups in fumarase. The nature of the reaction of these reagents indicates that the thiol groups are not associated with active site structures but are buried in a hydrophobic environment in the interior of the enzyme. Several observations are in accord with this view. The rate of reaction of the thiol groups is much slower in the native, active enzyme than in the unfolded, inactive molecule. The rate of reaction is also a function of the polarity of the thiol reagent. Uncharged aliphatic reagents react faster than more polar reagents. The rate of reaction of a series of alkyl mercury nitrates increases in proportion to the number of methylene groups in the alkyl side chain. In dilute aqueous solutions of aliphatic alcohols, the rate of reaction of p-chloromercuribenzoate with the thiol groups increases with alcohol concentration and the number of methylene groups in the alcohol. The rate of reaction of iodoacetate at pH 6.5 is minimal at 23.5°C but increases at lower or higher temperatures. An increase in reactivity at low temperatures appears to occur as a consequence of a decreased strength of hydrophobic interactions at low temperatures.

Earlier studies have shown that fumarase (fumarate hydratase, EC 4.2.1.2) from swine heart muscle has a molecular weight of 194,000 and is composed of four polypeptide chains, each with a molecular weight of 46,500 (1-4). The enzyme is devoid of disulfide bonds, but each of the four chains, which have an identical sequence, contains three thiol groups.

The studies reported here have been designed to define the role of the thiol groups in fumarase. In some enzymes, such as papain (5) or streptococcal proteinase (6), the thiol groups participate directly in the active site. In contrast, the thiol groups of fumarase do not participate directly in the active site, but are buried within the enzyme in such a manner that their modification leads to structural alterations sufficient to destroy enzyme activity. Furthermore, the "buried" thiols seem to exist in hydrophobic regions of the molecule as judged by the fact that the rate of reaction of the thiols with various reagents is affected not only by the chemical nature of the reagent itself but also by the solvent employed for the reaction.

EXPERIMENTAL PROCEDURE

Materials and Methods

Fumarase—Fumarase was prepared by the method of Kanarek and Hill (1) and stored at 5°C as a crystalline suspension in 55% ammonium sulfate, containing 0.05 M potassium phosphate buffer, pH 7.3. The protein was solubilized in the following manner. An appropriate aliquot of the crystalline suspension was centrifuged at 27,000 x g for 15 min at 5°C. The crystals were suspended at 25°C in 3 to 5 ml of a 16% solution of ammonium sulfate, pH 7.3, and centrifuged. The crystals are insoluble in 15% ammonium sulfate whereas amorphous protein is soluble. The crystals were then dissolved in 0.01 M potassium phosphate buffer, pH 7.3, and dialyzed for 1 hour at room temperature against 10³ to 10⁴ volumes of the same solvent. The clear solution was then centrifuged at 27,000 x g for 1 hour to remove the last traces of insoluble material. Fumarase prepared in this manner has a specific activity of 31,500 to 35,000 units per mg when assayed as described previously (1). When fumarase was used in other buffers, the solution obtained after the last centrifugation was redialyzed against the appropriate buffer.

Alkyl Mercury Nitrates—The insoluble alkyl mercury halides were converted to the corresponding soluble nitrates by the action of potassium cyanide.
method of Godchall and Veldstra (8). Approximately 1 mmole of the alkyl mercurial halide was treated with 1.7 mmoles (0.4 g) of silver oxide in a mixture of methanol and water (7:1). The reaction mixture (approximately 8 ml, final volume) was stirred in the dark overnight. Because of the extreme toxicity of these compounds, all reactions were carried out in a well ventilated hood. The suspension was then filtered, and the precipitate was washed once with 1 to 2 ml of absolute methanol.

The pH of the filtrate was adjusted with 0.1 N nitric acid to pH 6.97 to 7.00. A small aliquot was removed and was shown to be free of silver (9). The solution was then concentrated under vacuum to a volume of 1 to 2 ml. One milliliter of water was added to precipitate any residual alkyl halide. This solution was reconcentrated under vacuum to a final volume of 1 to 2 ml and stored at 5°C. Prior to use, the alkyl mercury nitrates were diluted with water to a volume of 5 to 7 ml and standardized by a modification of the method of Chinard and Hellerman (10).

Reagents — p-Chloromercuribenzoate (CMB) was a commercial preparation (Sigma). Solutions of this reagent were prepared by adjusting aqueous suspensions containing 0.01 M potassium phosphate to pH 10 to 11 to achieve solubilization and then readjusting to pH 7.5 to 8.0. Insoluble material, if present, was removed by filtration. The concentrations of the CMB² solutions were determined spectrophotometrically as reported earlier (11) or by titration with standard solutions of mercapto- succinic acid.

2,2'-Bis(dithioethanol) and 2,2'-bisdith&aethr;ethylamine were prepared by air oxidation of 2-mercaptoethanol and 2-mercaptop- ethylamine, respectively. Solutions (1 ml) of the mercaptans were adjusted to pH 9 with dilute NaOH and a sufficient amount of copper sulfate was added to give a final concentration of 0.001 M. Air was bubbled through the solution at 25°C until the characteristic color of the metal mercaptide had just disappeared. The resulting solution gave a negative nitroprusside test for free thiol groups (12). Metals were removed by passing the solution through a column, 0.9 X 3 cm, of Chelex resin (Bio-Rad).

Iodoacetate was recrystallized from petroleum ether (b.p. 66-75°C). Solutions of urea were deionized just prior to use on columns (0.9 X 10 cm) of Amberlite MB-3.

Analytical Methods — Sedimentation velocity analyses were performed with the Spinco model E ultracentrifuge equipped with schlieren optics. All runs were performed at 59,780 rpm at 25°C with 12-mm cells in 0.01 M NaCl. The protein concentration varied from 0.1 to 0.5%. Sedimentation coefficients were corrected to water at 20°C.

Optical rotatory dispersion measurements were made with the Carey model 60 recording spectropolarimeter with the use of 1-cm cells. All measurements were performed at 25°C ± 1°C.

Amino acid analyses were performed with the model 120 B Spinco automatic amino acid analyzer. Acid hydrolysates were prepared as described by Moore and Stein (13).

RESULTS

Reaction of Fumarase with p-Chloromercuribenzoate

Effects of CMB Concentration and Substrate — When fumarase is titrated with CMB in 8 M urea according to the methods of Boyer (11), the 12 thiol groups in the molecule react rapidly and completely with 12 molecules of CMB (3). This result is not unexpected since it has been established that in 8 M urea the enzyme is completely inactive and its four polypeptide chains are fully dissociated as well as extensively unfolded (3). In order to assess the nature of the reaction with native enzyme, fumarase was allowed to react with CMB at different concentrations of mercurial to enzyme. Fig. 1 (Curve A) shows the course of the reaction at pH 7.3 in phosphate buffer under conditions where the molar ratio of CMB to fumarase is 18. This ratio corresponds to 1.5 molecules of CMB per thiol group in the enzyme. The reaction is quite slow, and the thiols have been modified completely only after 40 hours. During the course of the reaction, the extent of modification of the thiols is directly proportional to the extent of inactivation. At a molar ratio of CMB to fumarase of 0.3 (3.6 molecules of CMB per 12 thiol groups), the reaction proceeds as shown in Curves B in Fig. 1. As expected under these conditions, an average of only 3.6 thiol groups per molecule are modified. On the other hand, the extent of inactivation is not directly proportional to the number of thiol groups modified except after 20 to 24 hours. Between 6 and 14 hours, a larger fraction of the enzyme is inactivated than would be expected on the basis of the fraction of thiol groups modified.

A number of conclusions may be drawn from the results shown in Fig. 1. The rate of reaction of the thiol groups in native fumarase with CMB is much slower than that found on reaction of CMB with free thiols, or with enzymes which contain a thiol group in their active site. Such sluggish reactivity not only suggests that the thiol groups are not present in the active site of fumarase, but also indicates that the 12 thiol groups may be buried within the enzyme and are unavailable for reaction until exposed to solvent. However, the loss of activity is directly proportional to the extent of thiol groups modified when an excess of CMB is permitted to react with enzyme. Although this observation could indicate that all 12 thiols of fumarase must have

Fig. 1. The reaction of fumarase with p-chloromercuribenzoate (PCMB). Fumarase (0.93 mg per ml; 4.8 X 10⁻⁴ µ) was incubated in 0.01 M phosphate buffer, pH 7.3, at 25°C with 8.7 X 10⁻⁴ M CMB. The change in absorbance of the mixture at 250 µw was determined at the time intervals indicated and provided a measure of the number of moles of CMB reacted per mole of enzyme (8). Aliquots of the mixture were removed and assayed for fumarase activity with 0.05 M malate at pH 7.3 (1) in the usual manner. Curve A shows the moles of CMB bound per mole of enzyme (O) and the percentage inactivation (DA). Curves B show the same relationship under the identical conditions except that 1.6 X 10⁻⁷ M CMB was used.

* The abbreviation used is: CMB, p-chloromercuribenzoate.
reacted before complete loss of enzymatic activity, the experiment with less than stoichiometric amounts of CMB (Curves B, Fig. 1) suggests that the reaction proceeds with all-or-none type of kinetics (14). The inactive fraction of enzyme in this experiment binds all of the added CMB whereas the active fraction possesses its full complement of unmodified thiol groups. This type reaction would also explain why loss in activity is initially greater than the amount of CMB bound. Many molecules of fumarase appear to react with CMB and are rendered inactive. However, in time, some partially reacted molecules lose CMB to other partially reacted molecules, so that at equilibrium, 30% of all of the fumarase molecules are fully reacted and the remainder of the enzyme is unmodified. This mechanism has not been established by isolation and analysis of the active and inactive forms, but it seems most plausible in view of the studies with other thiol reagents, which will be described below.

Phosphate and malate have marked effects on the reaction with CMB. When fumarase (0.485 mg per ml) was allowed to react with 0.001 M CMB at 31° in 0.005 M phosphate buffer, pH 7.3, 50% of its activity was lost in 4 min. In 0.05 M phosphate, the half-life of fumarase activity increased to 17 min. In 0.05 M phosphate and malate, half of the activity of fumarase was lost only after 75 min. Although protection by substrate is generally interpreted to indicate reaction with active site structures, this need not be the case here, because phosphate as well as malate markedly influences those interactions among the four polypeptide chains which are essential to maintain the active, four-chained structure (3). Thus, stabilization of the enzyme by substrate could be expected to alter the reactivity of buried thiol groups.

Reactivation of CMB-inhibited Fumarase—The inactivation of fumarase by CMB is reversed to a considerable degree by 2-mercaptoethanol. When fumarase was allowed to react with a large excess of CMB (mole ratio of CMB to fumarase = 400:1) at pH 7.3, inactivation was complete within 30 min. On addition of 2-mercaptoethanol reactivation proceeded as shown in Fig. 2. The rate of reaction appeared to be first order with respect to the concentration of fumarase, and after 3 min, 50% of the enzymatic activity was restored. In the experiment shown in Fig. 2, 88% of the original activity was restored at 2 hours. In other experiments the CMB-treated enzyme was seldom reactivated to greater than 95% of its original activity.

Inactivation of Fumarase by CMB in Dilute Aqueous Solutions of Alcohols—The above studies with CMB suggested that the 12 thiol groups in fumarase were buried in the molecule; thus it was of interest to determine whether solvents which may be expected to weaken hydrophobic bonds in proteins would alter the rate of reaction between CMB and fumarase. For this purpose, the reaction with CMB was studied in dilute aqueous solutions of methanol, ethanol, n-propyl alcohol, and n-butyl alcohol. The effect of these alcohols is shown in Fig. 2. As expected, at equivalent concentrations, the alcohols increase the rate of the CMB reaction in the order methanol < ethanol < n-propyl alcohol < n-butyl alcohol < n-pentyl alcohol. Fig. 5 shows this effect more clearly and indicates that there is a linear relationship between the logarithm of the concentration required to give a reaction of 95% inhibition and the mass of the side chain of the alcohols.

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To clarify further the role of the alcohols, the effects of t-butyl alcohol on the reaction with CMB were compared to those obtained with n-butyl alcohol. Both isomers have the same molecular weight and about the same dielectric constant but differ markedly in their solubility in water. t-Butyl alcohol is completely miscible with water whereas n-butyl alcohol is soluble to the extent of 2.8 g/100 ml water at 25°. Thus, t-butyl alcohol may be expected to disrupt hydrophobic bonds to a lesser degree than n-butyl alcohol. As shown in Fig. 6, t-butyl alcohol proved to have much less effect than n-butyl alcohol on the rate of the reaction and gave a result which corresponded approximately to that given by methanol.
The experiments with aliphatic alcohols showed that the rate of reaction of CMB with fumarase was increased significantly by solvents which may be expected to weaken hydrophobic interactions in the enzyme. Thus, it was of interest to determine if the rate of reaction of the thiol was a function of the hydrophobic character of the mercurial itself. For these studies, methyl, ethyl, n-propyl, and n-butyl mercury nitrates were employed, reagents which can be expected to show an increasingly greater degree of hydrophobicity from the methyl through the n-butyl derivative. Fig. 7 shows the rate of inactivation of fumarase by this series of compounds at 19°C. In these studies it is assumed that the loss in activity is directly proportional to the amount of mercurial bound, as is the case with CMB. Inactivation follows apparent first order kinetics, and the rate increases with increasing size of the aliphatic side chain. Similar relationships were obtained at 15, 23, and 27°C. Arrhenius plots for the reaction with each alkyl derivative were constructed.

Control studies showed that, over 5 hours, the activity of fumarase was unaffected at all concentrations of each alcohol used in these experiments. In addition, gross structural alterations in fumarase are not induced by the alcohols as judged by optical rotatory dispersion studies. At all wave lengths between 225 and 345 mμ, the dispersion curves of aqueous solutions of fumarase did not differ markedly from curves in 2.5 to 5.0% solutions of methanol, ethanol, n-propyl alcohol, or n-butyl alcohol. The reduced mean residue rotation [m]′ of fumarase at 234 mμ in 0.095 M phosphate buffer, pH 7.3, was -6925°, whereas in the same buffer in 2.5% n-butyl alcohol, [m]′ was -7018°. Although these measurements indicate that gross structural alterations do not result from exposure to these alcohols, it is evident that compounds which weaken hydrophobic interactions in proteins markedly alter the reactivity of thiols.

**Reaction of Fumarase with Alkyl Mercury Nitrates**

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The rate constants were calculated from the half-time for inactivation by each derivative. The activation energy for the reaction of the four mercurials was calculated from the slope of each curve and is presented in Table I along with the values calculated for $\Delta H^\ddagger$, $\Delta F^\ddagger$ and $\Delta S^\ddagger$. The activation energies for the reactions decrease progressively from the methyl to the n-butyl derivative, whereas the $\Delta S^\ddagger$ values for the same series become more positive. The last column lists the change in $\Delta F^\ddagger$ between successive members of the series. If these values are considered to correspond to the free energy of transfer of a methylene carbon atom from an aqueous to a nonaqueous environment, then they are of the same order of magnitude as the free energy of transfer of a methylene carbon from water to alcohol, as judged from solubility studies (15).

Fig. 8, which shows the relationship between the logarithm of the first order rate constant at 25° and the mass of the aliphatic side chain on the mercurial, shows quite clearly that the mercurials react with the thiols of fumarase in the order n-butyl > n-propyl > ethyl > methyl.

The values for $\Delta S^\ddagger$, given in Table I suggest that structures in the enzyme may be rate limiting in the reaction of the thiol groups with alkyl mercurials. It was important, however, to determine whether the different mercurials reacted at the same rate with unhindered thiols. For this purpose attempts were made to estimate the rate of reaction of each mercurial with a small molecular weight mercaptan. With 10−4 M 3-carboxy-4-nitrothiophenol (Ellman’s reagent, reduced form) in 0.1 M potassium phosphate buffer, pH 7, each of the alkyl mercury nitrates (0.01 M) reacted completely within 2 sec as judged by the changes in absorbance at 412 μ. These results indicate that the differences in the rate of reaction of the alkyl mercurials with fumarase do not result from inherent differences in the rate of reaction with free thiols.

### Table I

<table>
<thead>
<tr>
<th>Mercury nitrate derivative</th>
<th>$k$ (sec⁻¹ $\times 10^4$)</th>
<th>$E_a$ (cal/mole)</th>
<th>$\Delta H^\ddagger$ (cal/mole)</th>
<th>$\Delta S^\ddagger$ (cal/degree·mole)</th>
<th>$\Delta F^\ddagger$ (cal/mole)</th>
<th>$\Delta F^\ddagger$ (cal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl.</td>
<td>6.42</td>
<td>17,752</td>
<td>17,110</td>
<td>21.67</td>
<td>21,700</td>
<td>178</td>
</tr>
<tr>
<td>Ethyl.</td>
<td>8.65</td>
<td>16,016</td>
<td>15,424</td>
<td>−21.08</td>
<td>21,621</td>
<td>428</td>
</tr>
<tr>
<td>n-Propyl.</td>
<td>17.83</td>
<td>15,883</td>
<td>15,291</td>
<td>−19.64</td>
<td>21,193</td>
<td>389</td>
</tr>
<tr>
<td>n-Butyl.</td>
<td>34.00</td>
<td>15,897</td>
<td>15,305</td>
<td>−18.36</td>
<td>20,811</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 8.** The relationship between the rate of inactivation of fumarase by alkyl mercurials and the mass of the alkyl group.
Marized in Fig. 9, it was found that the extent of carboxymethyl-
zyme (1) (12 residues per molecule). In other studies, sum-
cule, a value in good agreement with the thiol content of the
Amino acid analysis of a 24-hour acid hydrolysate of this deriva-
tive revealed 11.9 residues of S-carboxymethylcysteine per mole-
gives a single peak in the ultracentrifuge with an $s_{20,w}$ of 5.27.
8
with an $s_{20,w}$ of 5.36. When fumarase is carboxymethylated in
carboxymethylation. About 22% of the thiols are modified
called dimer) which is formed as a function of the extent of
component has not been determined, its $s_{20,w}$ value is close to
of the total carboxymethylated enzyme. The other component
which represented 20% of the enzyme had an apparent $s_{20,w}$
of 5.36. Although an exact molecular weight of the 5.36 S
component has not been determined, its $s_{20,w}$ value is close to
for a fumarase molecule which contains only two
of four polypeptide chains (3). Thus, native fumarase,
which is tetrameric (four subunit polypeptide chains per mole-
cule) seems to dissociate into a two-chained enzyme (hereafter
called dimer) which is formed as a function of the extent of
carboxymethylation. About 22% of the thiols are modified
(Table II), and 20% of the same preparation sediments as a dimer
with an $s_{20,w}$ of 5.36. When fumarase is carboxymethylated in
8 M urea at pH 8.6 (0.02 M Tris buffer, 0.10 M iodoacetate, 15
min, 26°), the product, after removal of urea and iodoacetate,
gives a single peak in the ultracentrifuge with an $s_{20,w}$ of 5.27.
Amino acid analysis of a 24-hour acid hydrolysate of this deriv-
revealed 11.9 residues of S-carboxymethylcysteine per mole-
cule, a value in good agreement with the thiol content of the
enzyme (1) (12 residues per molecule). In other studies, sum-
marized in Fig. 9, it was found that the extent of carboxymethylation
of the thiols in fumarase is directly proportional to the
amount of dimer observed in the ultracentrifuge.

These results suggest that the thiol groups of fumarase are
buried in a hydrophobic environment, in accord with the studies
with CMB and the aliphatic mercurials. Although iodoacetate
inactivates fumarase more rapidly than N-ethylmaleimide at
pH 7.3, only 4 thiol groups are modified. Iodoacetate would
not be expected to penetrate hydrophobic regions as well as
N-ethylmaleimide because it carries a full negative charge. On
the other hand, once carboxymethylation begins, complete
carboxymethylation of all thiols in a molecule seems to occur, and
the molecule tends to dissociate into a dimeric form. Dissocia-
tion may not be unexpected when a charged group which resides
on the substituted thiol is placed in the interior of the molecule.
Treatment with N-ethylmaleimide or iodoacetamide does not
produce derivatives of the thiol groups which possess a charge,
thus these derivatives retain their tetrameric structure.

Effect of Temperature on Fumarase-Iodoacetate Reaction—From
the preceding studies with the organic mercurials and other
thiol reagents, it seemed that the thiol groups of fumarase are in
hydrophobic regions of the molecule and are not readily accessi-
ble for reaction. Because hydrophobic bonds in proteins are
weakened at lower temperatures (17), it was of interest to deter-
mine the effects of temperature on the S-carboxymethylation
reaction of fumarase. Fig. 10 shows the change in enzyme
activity with time on reaction with iodoacetate at temperatures
between 15 and 30°. These experiments were performed at pH
6.5 and pH 8.0. As anticipated, the rate of inactivation at pH
6.5 and 8.0 increased with temperature and was greater at pH
8.0 than pH 6.5. However, the extent of S-carboxymethylation
of the thiol groups appeared to differ at the two pH values. At
pH 8.0, the extent of carboxymethylation increased in propor-
tion to the temperature, although at pH 6.5, the extent of carboxy-
methylation was at a minimum at 23.5° but increased at both
higher and lower temperatures. Fig. 11 shows the number of
thiol groups which remain unmodified after 10 hours of reaction
with iodoacetate at both pH values and at several temperatures.
These results suggest that at the two pH values, the thiol groups
are in somewhat different environments. They react more
readily at pH 8.0 than at pH 6.5 in accord with the expected
change in degree of ionization of the thiols with pH. However,
at pH 6.5 the thiols become increasingly reactive as the tempera-

3 Iodoacetate, at pH 6.5, has been found to carboxymethylate
methionyl as well as histidyl residues in fumarase. Modification
of these residues appears specific since only 1 of the 14 histidyl
residues and 2 of the 15 methionyl residues appear to react. R. A.
Bradshaw and R. L. Hill, unpublished observations.
ture decreases from 23.5°. This result may be explained if it is assumed that, at pH 6.5, the thiols are buried within hydrophobic regions. The hydrophobic bonds which protect the thiol groups increase in strength from 15 to 23.5° and consequently render reaction of the thiols more difficult. The increase in reactivity above 23.5° may be the result of an increasing instability of the hydrophobic regions imposed by structural changes other than those involving hydrophobic bonds.

The marked effects of temperature on the reactivity of thiols are also shown by the data in Table III which show the rate constants for carboxymethylation at pH 6.5 and pH 8.0 in the absence and presence of urea. In these experiments, fumarase was treated with iodoacetate under the conditions indicated in the table, and the amount of S-carboxymethylcysteine formed was determined on samples of the reaction mixture recovered at appropriate intervals. The S-carboxymethylcysteine content was determined by chromatographic analysis of acid hydrolysates of the samples or by titration of the free thiol groups with 5,5'-bisdithio-2-nitrobenzoate. From these data an apparent first order rate constant for S-carboxymethylcysteine formation was calculated. In urea, the rate of reaction is 200 to 300 times that in phosphate buffer at the same pH. The ratio of the rate constant at pH 8.0 \( (k_{a,8}) \) to the rate constant at pH 6.5 \( (k_{a,5}) \) is always about 2 except at 15° in phosphate buffer. The observed ratio of approximately 2 at 25° in phosphate buffer and at 15 and 25° in urea probably reflects differences in the degree of ionization of the thiol groups. The ratio of 0.45 at 15° in phosphate buffer may reflect the influence of structural differences either on the ionization of the thiol groups or on the availability of these groups for reaction with iodoacetate.

**Mixed Disulfides of Fumarase**

The properties of the mixed disulfides of fumarase and cysteine, 2,2'-bisdithioethanol, and 2,2'-bisdithioethylamine also shed light on the nature of the thiol groups of fumarase. Fumarase and low molecular weight disulfides would be expected to react in general as follows:

\[
\text{Fumarase-(SH)\_12 + 12 R-S-S-R \rightarrow fumarase-(S-S-R)\_12 + 12 R-SH}
\]

At low concentrations of fumarase and high concentrations of disulfide, the reaction would proceed to give high yields of the fumarase mixed disulfide.

The mixed disulfide with cysteine was prepared as follows. A 30-mg portion of fumarase in 5 ml of 0.01 M phosphate buffer, pH 7.3, was dialyzed with stirring at 5° against 100 ml of 0.1 M phosphate buffer, pH 9.7, saturated with cystine (about 0.1 M). After 65 hours, the mixture was allowed to dialyze for an additional 10 hours at 25°. The reaction mixture was then dialyzed at 5° against 0.02 M phosphate, pH 7.3. The small amount of insoluble cystine which appeared initially on dialysis against the pH 7.3 buffer was removed by centrifugation, and the mixture was then dialyzed exhaustively against several changes of buffer. Although this method was used in earlier experiments, it was found later that the enzyme was fully modified at 30° in 24 hours under the same conditions. These conditions were used in some studies. The mixed disulfides of 2,2'-bisdithioethanolamine and 2,2'-bisdithioetheanol were prepared in the same way except that fumarase was dialyzed against 0.05 to 0.1 M solutions of these reagents.

On reaction of fumarase with each of the disulfides, the enzymatic activity diminishes during the first 4 hours of reaction, as shown in Fig. 12A. Some of the properties of the mixed disulfides are given in Table IV. Each derivative was devoid of enzymatic activity and did not contain free thiol groups as judged by titration with CMB in 6 M urea. The absence of free thiol groups is in accord with the observation that the mixed disulfide with cysteine contained close to the expected cysteic acid content of 24 residues per molecule after oxidation with performic acid (23). Similarly, the 2,2'-bisdithioethanolamine derivative contained 12 residues of cysteic acid and 10.8 residues of taurine per molecule after oxidation. Of particular interest is the observation that the two mixed disulfides in which a charged side chain is present on the thiol group, sediment in the ultracentrifuge with ~20,~ values of 5.1 and 5.5. In contrast, the dithioethanol derivative, which does not possess a formal charge, sedimented at a rate which suggests it retains its tetra-

*Fig. 11. The effect of temperature on the thiol content of fumarase after reaction with iodoacetate. Fumarase was allowed to react at various temperatures with iodoacetate as described for Fig. 0. The carboxymethylcysteine content was determined after 10 hours of reaction by amino acid analysis of acid hydrolysates of the reaction mixtures.*

**Table III**

*Apparent first order rate constants for reaction of fumarase and iodoacetate*

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>( k ) at 25°</th>
<th>( k ) at 15°</th>
<th>Ratio of ( k ) at pH 8.0 to ( k ) at pH 6.5 at 25° and 15°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.5</td>
<td>pH 8.0</td>
<td>pH 6.5</td>
</tr>
<tr>
<td>Phosphate buffer, 0.01 M</td>
<td>( 1.3 \times 10^{-3} )</td>
<td>( 2.5 \times 10^{-3} )</td>
<td>( 2 \times 10^{-3} )</td>
</tr>
<tr>
<td>Phosphate buffer, 0.01 M, urea, 8 M</td>
<td>( 3.5 \times 10^{-3} )</td>
<td>( 7.0 \times 10^{-3} )</td>
<td>( 1.1 \times 10^{-3} )</td>
</tr>
</tbody>
</table>
and with cystine (Curve 5). The formation of the mixed disulfides was allowed to react at 25° with 0.1 mm fumarase from mixed disulfides. Each of the mixed disulfides was used at a concentration of 0.05 M. B, the regeneration of bisdithioethylamine were used whereas 2,2'-bisdithioethanol is described in the text. During reaction, 0.1 M cystine and 2,2'-bisdithioethanol were removed at intervals and assayed in the usual manner. Curve 1, fumarase-2,2'-bisdithioethanol mixed disulfide; Curve 2, fumarase-cystine mixed disulfide.

**FIG. 12.** Enzymatic activity of the mixed disulfides of fumarase. A, the loss in activity of fumarase during reaction with 2,2'-bisdithioethanol (Curve 1), with 2,2'-bisdithioethylamine (Curve 2), and with cystine (Curve 3). The formation of the mixed disulfides is described in the text. During reaction, 0.1 mM cystine and 2,2'-bisdithioethylamine were used whereas 2,2'-bisdithioethanol was used at a concentration of 0.05 M. B, the regeneration of bisdithioethylamine from mixed disulfides. Each of the mixed disulfides (1.85 X 10^-4 M), in 0.05 M potassium phosphate buffer, pH 7.3, was allowed to react at 25° with 0.1 mM cysteine. Aliquots were removed at intervals and assayed in the usual manner. Curve 1, fumarase-2,2'-bisdithioethanol mixed disulfide; Curve 2, fumarase-2,2'-bisdithioethylamine mixed disulfide; Curve 3, fumarase-cystine mixed disulfide.

**TABLE IV**

Some properties of mixed disulfides of fumarase

<table>
<thead>
<tr>
<th>Mixed disulfide*</th>
<th>Cysteic acid content</th>
<th>Thiouic content</th>
<th>S&lt;sub&gt;2&lt;/sub&gt; or</th>
<th>Activity regenerated</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine ..........</td>
<td>23.3 residues/molecule</td>
<td>5.07</td>
<td>252</td>
<td></td>
<td>79</td>
</tr>
<tr>
<td>2,2'-Bisdithioethylamine</td>
<td>12</td>
<td>10.8</td>
<td>5.47</td>
<td>200</td>
<td>69</td>
</tr>
<tr>
<td>2,2'-Bisdithioethanol.</td>
<td>12</td>
<td>10.8</td>
<td>5.47</td>
<td>200</td>
<td>69</td>
</tr>
</tbody>
</table>

* No thiol groups were present in the compounds studied.

**Fig. 13.** Separation of fumarase and fumarase-cystine mixed disulfide on Sephadex G-200. Fumarase (19 mg) was allowed to react with cysteine at pH 9.5 as described in the text until 70% of the enzymatic activity was lost. Excess cysteine was removed on dialysis against 0.01 M phosphate buffer, pH 7.3. The reaction mixture was then applied to a column (2 X 175 cm) of Sephadex G-200 and eluted with the same buffer. The column was developed at room temperature at a flow rate of 5 to 8 ml per hour. The effluent fractions (2.5 ml) were monitored at 280 mm. The activity was measured in the usual manner. Void volume of the column was 200 ml.

**DISCUSSION**

Reaction of fumarase with a variety of thiol reagents leads to a loss of enzymatic activity. The loss in activity is directly proportional to the number of thiol groups modified except for iodoacetate which seems to cause other modifications in the enzyme. It also appears that the thiol groups in an individual molecule react in an all-or-none fashion. This is best shown by the reaction with cysteine to form the fumarase-cystine mixed disulfide. When 70% of the disulfide is formed, 70% of the enzymatic activity is lost. On gel filtration of the partially reacted enzyme two components were obtained, one which is active, unmodified enzyme in an amount equal to 37% of the dimeric structure. Thus, the sedimentation behavior of these derivatives is similar to that of the alkylated derivatives described above, and confirms the view that the presence of a charged group on the thiols leads to dissociation of the four-chained molecule. In a separate experiment, fumarase was allowed to react with cysteine as described above until 40% of its activity was lost. At this time the cystine was rapidly removed by dialysis at pH 7.3. The resulting derivative gave two components in the ultracentrifuge, one with an s<sub>20w</sub> of 5.1 and the other with an s<sub>20w</sub> of 8.95. The 5.1 S component represented 36% of the total protein, or about the same amount of inactive enzyme formed. In another experiment, cystine was allowed to react with fumarase until 70% of the activity was lost. After removal of the excess cystine, the reaction mixture was applied to a column of Sephadex G-200 which had been equilibrated with 0.01 M phosphate buffer, pH 7.3, at room temperature. The resulting chromatogram is shown in Fig. 13. Two peaks were observed, one, which represented 37% of the total protein applied (peak at 283 ml), was fully active and contained 12 residues of half-cystine per molecule. The second component (peak at 309 ml) which represented 63% of the total protein applied, was inactive and contained 20.4 residues of half-cystine per molecule. The first peak corresponds to native fumarase and the second peak to the mixed disulfide. Thus, the amount of dimer formed is proportional not only to the loss in activity, but also to the amount of mixed disulfide formed. These results also show that the reaction of fumarase with thiol reagents proceeds by an all-or-none mechanism (14).

Finally, each of the mixed disulfide derivatives may be regenerated to give active fumarase on incubation with cysteine or 2-mercaptoethanol. The extent and rate of regeneration vary among the three derivatives as shown in Fig. 12B. The dithioethanol derivative regains activity more rapidly than the other two derivatives. The cystine derivative shows a marked lag period of about 30 min during which time no activity is regenerated. Perhaps the slower rates of regeneration reflect the fact that the cystine and dithioethylamine derivatives are dimeric structures that must form the four-chained, native enzyme before activity is regained. This is not necessary for the dithioethanol derivative, which retains its tetrameric structure. These studies, and those reported earlier for the regeneration of lysozyme from the mixed disulfide of lysozyme and cystine (18), indicate that the product formed on treatment of a mixed disulfide of a protein is determined by the amino acid sequence of the protein. On treatment with cysteine, the mixed disulfide of lysozyme and cysteine forms active lysozyme which contains no free thiol groups, whereas the mixed disulfide of fumarase and cysteine gives active fumarase free of disulfide bonds.
total protein and the other, which is the inactive mixed disulfide in an amount equal to 63% of the total protein. This type of reaction, which was noted earlier with phosphorylase (19, 20) and D-amino acid oxidase (14), suggests that once one of the thiols in a molecule is modified, there is sufficient structural change so that the remaining thiol groups react more rapidly.

The conditions necessary for reaction of the thiol groups in fumarase suggest that the thiols are not associated with the active site of the enzyme but are buried in hydrophobic regions. Rather high concentrations of all thiol reagents are required to rapidly produce a significant amount of modification or marked changes in enzymatic activity. This may explain why CMB, iodoacetate, and iodoacetamide were reported earlier to have no effect on the activity of fumarase (16).

There is ample evidence that the thiol groups are buried in the molecule and are not readily accessible to solvent. The rate of reaction with iodoacetate in 8 M urea is 200 to 300 times that in phosphate buffer at pH 8 and pH 6.5. Similar relationships are found with CMB. Furthermore, reaction with thiol reagents which carry a positive charge (2,2'-bisdithioethylamine), a negative charge (ioadoacetate), or a positive and negative charge (cystine) leads to dissociation of the enzyme. The extent of dissociation is directly proportional to the number of thiol groups modified as well as to the loss in enzyme activity. This suggests that a charge cannot be buried in the vicinity of a thiol group in the native molecule. However when a charged substituent is so placed, the interactions among the four subunit polypeptides are weakened and dissociation into a two-chained inactive molecule ensues. When uncharged thiol reagents are allowed to react with fumarase dissociation does not occur, as shown by ultracentrifugal analysis of the derivatives obtained with N-ethylmaleimide, iodoacetamide, and 2,2'-bisdithioethylamine.

CMB and the alkyl mercurials are generally more effective than alkylating agents or disulfides in reacting with fumarase. This is not unexpected in view of the marked hydrophobic character of mercury. Indeed, mercuric chloride is more soluble in ethanol and other organic solvents than in aqueous solutions. It is, of course, impossible from the data given here to obtain a quantitative relationship for the reactivity of these reagents, since each has a somewhat different rate of reaction with unhindered thiols.

The studies with aliphatic mercurials perhaps represent one of the best examples of how the character of the reagent is a measure of the hydrophobic environment of the thiols in fumarase. Clearly, the rate of modification by these reagents is in the order, the methyl < ethyl < n-propyl < n-butyl. As the hydrophobic nature of the alkyl group is increased, there is an increasingly greater rate of reaction with the thiol groups. Furthermore, analysis of the reaction of these compounds as a function of temperature, reveals that the $d\Delta F^\ddagger$ for the reaction is of the order of magnitude for the transfer of a methylene carbon from water to alcohol solution (15). Similar findings were observed by Godschalk and Veldstra on reaction of alkyl mercurials with turnip yellow mosaic virus (8).

The hydrophobic character of the environment of the thiol groups is also indicated by the studies of the reaction with CMB in dilute solutions of aliphatic alcohols. At equivalent concentrations, the alcohols affect the rate of reaction with CMB in the order methanol < ethanol < n-propyl alcohol < n-butyl alcohol < n-pentyl alcohol. Thus, as the carbon chain is lengthened, the alcohols become more hydrophobic and consequently exert a greater effect on those interactions in the enzyme which normally hinder the reaction with thiols. Others have used a series of alcohols to disrupt hydrophobic regions of proteins. Cecil and Thomas (21) demonstrated that unreactive thiols in hemoglobin became increasingly susceptible to reaction with a mercurial reagent in the presence of increasing concentrations of alcohols. Von Hippel and Wong (22) and Schrier and Scheraga (23) showed that aliphatic alcohols lowered the thermal transition of ribonuclease.

Finally, the effect of temperature on the reaction between iodoacetate and fumarase also provides evidence that the thiol groups are in a hydrophobic environment. The hydrophobic bonds of proteins in aqueous solution are weakened as the temperature of the solution is decreased. Thus, below 25.3° the thiols in fumarase become increasingly available for reaction with iodoacetate, just the opposite of the expected effect of temperature on the rate of reaction.

Although the observations described here indicate that the thiols exist in hydrophobic regions in the interior of the enzyme, a specific function of these groups remains unclear. Presumably they act in some manner to maintain the appropriate three-dimensional structure of the enzyme, including aggregation of subunits to form the four-chained structure. It may be tempting to suggest that the thiol groups exist at the interface between subunits, since some types of modification lead to dissociation. On the other hand, dissociation could occur as the result of conformational changes within a single subunit. A more detailed knowledge of the structure of fumarase may be necessary before these questions can be answered.

REFERENCES


The Thiol Groups of Fumarase
George W. Robinson, Ralph A. Bradshaw, Louis Kanarek and Robert L. Hill


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