THE REACTION OF METHYL MERCURY NITRATE WITH THE SULFHYDRYL GROUPS OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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The importance of two of the thiol groups of yeast triosephosphate dehydrogenase for its activity has been shown by the abolition of activity and diphosphopyridine nucleotide binding by the addition of 2 equivalents of p-chloromercuribenzoate (PCMB) (1, 2). The reaction of methyl mercury nitrate with this enzyme has been found to be anomalous in the sense that this mercury compound, unlike PCMB, does not react first with the enzyme sulfhydryl groups which are required for activity. These results and some determinations of the total numbers of sulfhydryl groups available in the enzyme after various treatments are presented here.

EXPERIMENTAL

The enzyme was prepared from Fleischmann’s yeast by the procedure of Krebs et al. (3). It has not been found possible to obtain preparations fully active in the absence of cysteine, even if ethylenediaminetetraacetic acid (EDTA) is present during all stages of the preparation. It has also been found that the enzyme loses activity on storage (whether activity is tested with or without cysteine) even in the presence of EDTA. In general, the enzyme has 20 to 30 per cent less activity with EDTA than after cysteine activation. Enzyme concentrations were determined by absorption at 280 m\(\mu\) and corrected for any nucleic acid by the absorption at 260 m\(\mu\) (3, 4). The enzyme was dialyzed against a phosphate buffer (pH 6.7 or 7.9) before use.

The diphosphopyridine nucleotide (DPN) used was a Pabst Laboratories preparation and assayed 90 per cent pure by reduction with excess substrate.

dL-Glyceraldehyde-1-bromide-3-phosphoric acid dimer was kindly furnished as the dioxane addition compound by Dr. C. A. Dekker and assayed 95 to 100 per cent pure.

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The methyl mercury bromide used was generously supplied by Dr. P. E. Wilcox. The PCMB used was a Sigma preparation.

Enzyme activity was determined in the following system. 2.6 ml. pyrophosphate buffer (0.03 M) at pH 8.3 containing 0.0024 M EDTA, 0.1 ml. of enzyme solution (usually containing 0.001 mg. of protein), and 0.1 ml. of 0.195 M sodium arsenate were mixed. The reaction was started by the addition of 0.2 ml. of a solution containing 0.15 mg. of the triose phosphate addition compound and 1.6 mg. of DPN. The optical density at 340 nm was read at 0.5 minute intervals against a blank containing buffer and the triose phosphate-DPN mixture. The final pH was 8.1 to 8.3. Activities were calculated as a first order rate constant from the 3.0 minute reading. With fresh cysteine-activated preparations $k_1$ is usually 180 to 200 ml. per minute per mg. of protein at this level of triose phosphate. The reaction follows no simple kinetics exactly, but approximates a first order reaction, particularly if the 0.5 minute reading, which is generally high, is neglected. In some cases the reaction was started by the addition of triose phosphate alone, the DPN having been put in the reaction mixture previously. In this case the activity is 10 to 20 per cent higher than that observed with the other method of starting the reaction. All reactions were performed in duplicate.

PCMB concentrations were determined by direct titration with dithizone, standardized against mercuric nitrate, as for the determination of methyl mercury (5). Sulfhydryl groups in the native protein were determined by dithizone titration of the unchanged methyl mercury extracted from the aqueous protein solution into a toluene layer. Equilibration of the two phases was carried out in a rocking dialyzer at 0-2°. Sulfhydryl groups in the denatured protein were determined by direct titration with methyl mercury (5). All treatments of the protein with mercurials were carried out in phosphate buffers, pH 6.7 or 7.9.

**Results**

Table I contains the results of the determinations of sulfhydryl groups in the native protein and in the guanidine hydrobromide-denatured (5) protein. The number of sulfhydryl groups found on the native molecule, namely four, is similar to the number of sulfhydryl groups which react with iodoacetic acid in the case of triosephosphate dehydrogenase from muscle (6). The total number of sulfhydryls (six and a half after denaturation) is, however, considerably less than in the latter protein (7). The figures for the sulfhydryl groups of the native and denatured protein are likely to be lower than the true values; i.e., the values for a protein preparation which is fully reduced. If it is considered that 20 per cent of the protein

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1 Unpublished method of Dr. W. L. Hughes, Jr., transmitted by Dr. P. E. Wilcox.
sulfhydryl groups are oxidized (judging from a 20 per cent activation by
cysteine which was observed with the preparations used for these deter-
minations), the figures become 5.0 and 8.1 for sulfhydryl groups of the
native and denatured protein.

It appears from Fig. 1 that methyl mercury reacts first, to a large extent
at least, with the two sulfhydryl groups which are not primarily involved
(1, 2) in the activity of the enzyme. This is indicated by the 74 per cent
of activity remaining after the addition of 2 equivalents of methyl mercury
as against the 50 per cent to be expected from random reaction with four
sulfhydryl groups per mole of protein. The presence of some oxidized, or

| TABLE I |
| Sulphhydryl Groups of Yeast Glyceraldehyde-3-phosphate Dehydrogenase |

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Age of preparation</th>
<th>Activity, * k1, ml. per min. per mg. protein</th>
<th>Treatment before sulphhydryl determination</th>
<th>Sulphhydryl groups per 120,000 gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>K21-3</td>
<td>1</td>
<td>145</td>
<td>None</td>
<td>4.0</td>
</tr>
<tr>
<td>K21-3</td>
<td>1</td>
<td></td>
<td>Denatured with guanidine</td>
<td>6.5</td>
</tr>
<tr>
<td>K32-2</td>
<td>6</td>
<td>117</td>
<td>None</td>
<td>3.6</td>
</tr>
<tr>
<td>04-5†</td>
<td>1</td>
<td>113</td>
<td>4 equivalents PCMB, reprecipitated with (NH₄)₂SO₄</td>
<td>2.1</td>
</tr>
<tr>
<td>04-5†</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Without cysteine activation.
† Edelhoch et al. (5).
‡ This preparation was activated with cysteine, recrystallized two times, and stored with 0.005 M EDTA. The activity was initially k1 = 170.

otherwise inactive enzyme, as is known to occur with urease (8), does not
affect this conclusion. The per cent inhibition after reaction with a mer-
cural equivalent in amount to a given fraction of the total native sulphy-
dryl groups present is predictable on the basis of random reaction regardless
of the fraction of the total native sulfhydryl groups required for activity.
PCMB appears to react only with the sulfhdyryls required for activity
(Table I and Fig. 1). The two mercurials are different both in the size of
the organic part and in that one (PCMB) bears a charge other than that
on the mercury. Either or both of these differences may be relevant to
the observed difference in reactivity. It may be noted that, although there
are four sulfhydryl groups reactive to methyl mercury, 4 equivalents of this
reagent do not completely abolish the activity of the enzyme (Fig. 1). A
similar situation obtains with PCMB. A lack of good agreement has been
observed in duplicates of assays of activity when the inhibition is nearly
ME(I)YL MERCURY AND THIOL GROUPS

The experiments of Fig. 1 show that the four sulfhydryls of the enzyme molecule are of two kinds with respect to their reactivity with methyl mercury as well as with respect to their necessity for enzymatic activity. Since about 25 per cent of the enzymatic activity (instead of none) is lost by the addition of 2 equivalents of methyl mercury, the interpretation that only two sulfhydryls are necessary for activity and that methyl mer-

![Graph](http://www.jbc.org/)

**Fig. 1.** Per cent loss of activity with the addition of methyl mercury nitrate (O) or p-chloromercuribenzoate (●) to the enzyme in 0.1 M phosphate buffer, pH 6.7 or 7.9. The points for 1 and 2 equivalents of methyl mercury are averages of five determinations in duplicate; the other points are averages of two determinations in duplicate. The percentages are not corrected for the presence of inactive enzyme in the preparations used (see the text). The preparations contained four sulfhydryl groups per native molecule.

cury reacts solely with the two sulphydryls not necessary for activity is inadequate. We may suppose that either the reaction of methyl mercury is not completely specific between the two classes or the two unnecessary sulphydryls do have some effect on the activity of the molecule. An experiment designed to test whether the reaction of methyl mercury with the protein was completely selective for the two unessential sulphydryl groups was inconclusive. The protein was first treated with 2 equivalents of methyl mercury, then with 2 equivalents of PCMB, and, after reprecipitation with ammonium sulfate, the residual sulphydryl was found to be 0.52 equivalent per mole. This would indicate an incomplete specificity of the methyl mercury reagent, assuming that PCMB reacts only with the two essential sulphydryl groups (Table I). However, a similar experi-
ment in which the protein was treated with 4 equivalents of methyl mercury gave a residual sulfhydryl only of 0.39 equivalent per mole. These results are probably caused by denaturation of the protein, as seems likely from the rapid appearance of protein precipitate in both experiments during the equilibration with toluene. Considering the amount of preliminary handling and the high concentration of protein (which has been observed by us to enhance denaturation of this protein) necessary for these determinations, it seems not unlikely that sufficient denaturation to account for the results may have occurred. In these circumstances the closeness of the figures for residual sulfhydryl in the two experiments may indicate that the methyl mercury reagent reacts completely with the unessential sulfhydryl groups before reacting with those necessary for enzymatic activity. If this is so, it becomes necessary to suppose that these groups do have a small effect on the activity of the enzyme.

SUMMARY

The number of sulfhydryl groups in the yeast triosephosphate dehydrogenase molecule has been determined for the native and guanidine hydrobromide-denatured enzyme by the use of methyl mercury.

The reaction of methyl mercury nitrate with yeast triosephosphate dehydrogenase indicates that the most reactive of the four sulfhydryl groups of the native molecule are the two not required for enzymatic activity.

The author wishes to thank Dr. Hans Neurath, in whose laboratory this work was performed, Dr. P. E. Wilcox for furnishing information on the use of methyl mercury, and Dr. E. G. Krebs, who supplied some of the enzyme samples.

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