Mercury(II) Stimulation of Malate Dehydrogenase Activity

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SUMMARY

The reduction of NAD catalyzed by pig heart mitochondrial malate dehydrogenase can be stimulated by low concentrations, approximately 0.05 mM, of Hg++. This activation is dependent on the presence of the substrate malate at the time the enzyme is exposed to Hg++. The stimulation is both pH- and temperature-dependent, with maximum stimulation occurring near pH 8.8 and 30°C. The activation is not the result of an alteration in the Kᵢ values of the enzyme for either NAD or malate. The reverse reaction, the oxidation of NADH, is markedly inhibited by Hg++ in potassium phosphate buffer, pH 7.4, but is unaffected in Tris-HCl buffer at the same pH. These observations suggest that the effects of Hg++ on enzymatic activity are the results of various structural changes produced following mercaptide formation with different sulfhydryl groups of the enzyme. Hg++ stimulation of NAD reduction could also be demonstrated with malate dehydrogenase from other animal sources, as well as with the enzymes from Escherichia coli, Aerobacter aerogenes, and Salmonella typhimurium. In contrast, the enzymes from Bacillus subtilis and Bacillus polymyxa could not be stimulated, while those from Bacillus licheniformis and plant sources were inhibited by Hg++.

Sulfhydryl reagents (Hg++, p-chloromercuribenzoate, N-ethylmaleimide, iodoacetate, etc.) have been utilized as classical enzyme inhibitors in the characterization of many enzymes. The observation that the activities of most enzymes are markedly reduced in the presence of sulfhydryl reagents indicates the essential nature of free sulfhydryl groups for enzyme catalysis. However, in some instances it has been observed that the addition of a sulfhydryl reagent will stimulate the activity of certain enzymes (1).

Pig heart mitochondrial malate dehydrogenase has been well characterized previously as far as amino acid composition, molecular weight, and other physical and chemical parameters are concerned (2-4). The essential nature of sulfhydryl groups for enzymatic activity was indicated by the observation that incubation of the enzyme with increasing levels of sulfhydryl reagents led to progressive inhibition (3, 4). In the course of examining some of the possible regulatory properties of pig heart mitochondrial malate dehydrogenase, it was observed that under suitable conditions the reduction of NAD could be stimulated by the addition of sulfhydryl reagents. Hg++ appeared to be the most effective reagent tested in this study. The properties of Hg++ activation and its relationship to enzyme conformation are discussed in the present communication. Malate dehydrogenase from other mammalian sources, together with the enzymes from bacteria and plants, were also examined for Hg++ sensitivity.

EXPERIMENTAL PROCEDURE

Materials—Purified pig heart mitochondrial malate dehydrogenase (720 to 1000 units per mg) was obtained from Boehringer Mannheim Corporation and used without further purification. One unit of activity is defined as the amount of enzyme catalyzing the turnover of 1 μmole of substrate per min at 30°C. The enzyme purchased from either Nutritional Biochemicals or Serva Laboratories exhibited similar behavior in the presence of Hg++. Pig heart supernatant malate dehydrogenase was obtained from Serva Laboratories, and the enzyme from beef thymus was extracted from lyophilized beef thymus powder purchased from Nutritional Biochemicals. The partially purified enzyme from pea flour, treated with streptomycin and ammonium sulfate and chromatographed over DEAE-Sephadex resin, was kindly supplied by Miss Valerie Williamson, and the extract of wheat germ by Dr. V. Tuli. Cooking potatoes, Solanum tuberosum, were used to prepare extracts for measurement of dehydrogenase activity. B. subtilis, B. polymyxa, and B. licheniformis were grown in nutrient broth (Difco) plus 0.10% glucose; A. aerogenes in a tryptone, yeast extract, phosphate, and glucose medium, and E. coli in minimal medium minus CaCl₂ (5) supplemented with glucose. Extracts of S. typhimurium were kindly supplied by Mr. Naoto Sakamoto.

All reagents were obtained from regular commercial sources.

Methods—The reduction of NAD was measured in the following standard reaction mixture: 0.10 M Tris-HCl buffer, pH
8.8, 1.0 mM NAD, 10.0 mM malate, and enzyme, in a total volume of 3.0 ml. Measurements at room temperature were made at 340 μM in a Beckman model DU spectrophotometer coupled to a Gilford model 2000 multisample recorder. For assays at elevated temperatures, thermoregulated water from a water bath (Forma Scientific Inc., Marietta, Ohio) was circulated through the Gilford thermospacers of the spectrophotometer.

The oxidation of NADH was followed in a reaction mixture containing 0.10 M Tris-HCl or potassium phosphate buffer, pH 7.4, 0.047 mM NADH, 0.13 mM oxalacetate, and enzyme, in a total volume of 3.0 ml. Oxalacetate was usually added to initiate the reactions. The decrease in absorbance at 340 μM was followed at room temperature. Hg²⁺ was included in the reaction mixtures, where indicated, in the form of either mercuric nitrate or mercuric acetate. Malate dehydrogenase activity responded identically to either salt.

Sucrose gradient sedimentation was carried out following the procedure of Martin and Ames (6). Sulfhydryl titrations of pig heart mitochondrial malate dehydrogenase were performed according to the method of Sere (7).

The enzymes obtained from commercial sources were used without further purification. The extract from beef thymus was prepared by extracting the lyophilized powder with 7 volumes of 0.02 M Tris-HCl buffer, pH 7.4, for 30 min at 4°C. The supernatant following centrifugation at 30,000 × g for 15 min served as the source of the enzyme. The potato extract was prepared by homogenizing sliced potatoes in a Waring Blender with a small amount of 0.02 M Tris-HCl buffer, pH 7.4. Following low speed centrifugation to remove large debris, the extract was centrifuged at 30,000 × g for 15 min and the supernatant was utilized without further purification. All of the bacterial extracts were prepared by sonically treating cell suspensions in a Raytheon 10-kc sonic oscillator in 0.02 M Tris-HCl buffer, pH 7.4, for 5 min. The supernatants following centrifugation at 30,000 × g were used to assay bacterial malate dehydrogenase activity.

All enzyme dilutions were made in 0.10 M ammonium sulfate-0.02 M Tris-HCl buffer, pH 7.4, to insure maximal activity (8).

**RESULTS**

In agreement with earlier observations (3, 4), recent experiments in this laboratory indicated that purified pig heart mitochondrial malate dehydrogenase was strongly inhibited by the addition of Hg⁺⁺ (9). These assays of NAD reduction were carried out under conditions in which the reactions were initiated by the addition of malate. However, the results of the present study indicate that the inhibition of NAD reduction by Hg⁺⁺ occurs only when the sulfhydryl reagent is added to the enzyme prior to the addition of malate (Table I). When the enzyme is first incubated with malate or when the enzyme is exposed to Hg⁺⁺ and malate simultaneously, stimulation of enzymatic activity is observed. Within the limits of the standard enzyme assay, immediate stimulation occurred upon addition of the enzyme, with no suggestion of a time-dependent activation process. The addition of Hg⁺⁺ to the enzyme preincubated with the other substrate NAD resulted in inhibition of dehydrogenase activity. Since the stimulatory effect of Hg⁺⁺ could only be demonstrated when the enzyme was exposed to the sulfhydryl reagent in the presence of malate, all assays were initiated by the addition of enzyme to the reaction mixtures.

**Table I**

<table>
<thead>
<tr>
<th>Order of additions</th>
<th>ΔA40 per min</th>
<th>Activity</th>
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</thead>
<tbody>
<tr>
<td>NAD, malate, enzyme</td>
<td>0.026</td>
<td>100</td>
</tr>
<tr>
<td>Enzyme, Hg⁺⁺, NAD, malate</td>
<td>0.000</td>
<td>35</td>
</tr>
<tr>
<td>Enzyme, NAD, Hg⁺⁺, malate</td>
<td>0.013</td>
<td>50</td>
</tr>
<tr>
<td>Enzyme, malate, Hg⁺⁺, NAD</td>
<td>0.043</td>
<td>105</td>
</tr>
<tr>
<td>NAD, malate, Hg⁺⁺, enzyme</td>
<td>0.044</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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</tr>
<tr>
<td>Mercuric acetate</td>
<td>1.9</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>1.1</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>1.2</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effect of Hg⁺⁺ concentration on NAD reduction catalyzed by pig heart mitochondrial malate dehydrogenase. The incubation components were added in the following order: 0.10 M Tris-HCl buffer, pH 8.8; 1.0 mM NAD; 10.0 mM malate; Hg⁺⁺ at the indicated concentrations; and enzyme to initiate the reaction. The initial rate in the absence of Hg⁺⁺ was designated 100% activity.

Other sulfhydryl reagents can also stimulate NAD reduction, but Hg⁺⁺ appears to be the most effective reagent tested (Table II). Increasing the concentration of the other reagents resulted in increased stimulation, but not to the extent produced by Hg⁺⁺. The formation of an Hg⁺⁺-mercaptide complex with
MTCtialation of Nalate Dehydrogenase

1-01. 2x3, so. 5

PH

effect of pH on Ig++ stimulation of XA11 reduction catalyzed by pig heart mitochondrial malate dehydrogenase. The assays were carried out as described under Fig. 1. l, relative activity in the absence of Ig++; o, relative activity in the presence of 0.033 mM Ig++. The activity at pH 8.0 in the absence of Ig++ was arbitrarily assigned the relative value of 1.0.

TEMPERATURE (°C)

FIG. 3. Effect of temperature on Ig++ stimulation of XA11 reduction catalyzed by pig heart mitochondrial malate dehydrogenase. Assays were performed under the standard assay conditions described under Fig. 1, except that each reaction mixture was first incubated at the designated temperatures for 5 min before the addition of enzyme. The indicated temperatures were maintained during the measurements of activity. l, enzymatic activity in the absence of Ig++; o, enzymatic activity in the presence of 0.033 mM Ig++.

malate dehydrogenase is suggested not only by the results of Table II but also by the observation that the addition of 2-mercaptoethanol to the reaction mixture, either before or after the initiation of the reaction, abolishes the stimulatory effect of Ig+++. This effect of 2-mercaptoethanol may result either from a direct chelation of Ig+++ or by cleavage of the Ig+++ mercaptide bonds of the enzyme. The addition of a chelating agent such as EDTA also reverses the stimulatory effect of Ig+++. The degree of stimulation was shown to be dependent on the concentration of the sulphydryl reagent utilized (Fig. 1). Maximum stimulation was observed near 0.05 mM Ig+++. Increasing the concentration of Ig++ above 0.10 mM resulted in progressive inhibition of enzymatic activity.

Since the pH of the reaction mixture can influence both the ionization of the individual sulphydryl groups of the enzyme and also the availability of these groups for titration, the effects of pH on Ig+++ activation were investigated. Maximum stimulation occurred between pH 8.0 and pH 9.0 (Fig. 2). At pH 10.0, the pH optimum of the reaction, no Ig+++ stimulation could be detected. The substitution of phosphate for Tris-HCl buffer below pH 8.0 resulted in Ig++ inhibition of enzymatic activity.

The rate at which sulphydryl groups of an enzyme react with Ig+++ may also be dependent on temperature. It was, therefore, of interest to determine the effects of this parameter on the Ig+++ effect. Stimulation of NAD reduction by Ig+++ could be demonstrated at temperatures below 40° (Fig. 3). Above this temperature the presence of Ig+++ produced inhibition, even though enzymatic activity in the absence of the sulphydryl reagent was increased. Above 50°, negligible enzymatic activity could be detected in the presence of Ig+++. The results of Fig. 3 have not been corrected for the decrease in pH of Tris buffer which results from an increase in temperature (10). Although such a correction would quantitatively alter the observed activities, it would not affect the principal observation that Ig+++ produces a marked decrease in enzymatic activity at elevated temperatures.

It was also of interest to determine whether the activation produced by Ig+++ involved any alteration of the kinetic constants of the enzyme. Fig. 4 shows that the presence of Ig+++ did not appear to significantly alter the $K_v$ values for either NAD or malate, but did increase the $V_{\text{max}}$ of the reaction.

It was of further interest to determine the effects of Ig+++ on the reverse reaction catalyzed by malate dehydrogenase, the oxidation of NADH in the presence of oxalacetate. In the presence of 0.10 M potassium phosphate buffer, pH 7.4, Ig+++ caused a marked inhibition of enzymatic activity (Fig. 5). The apparent progressive inhibition of NADH oxidation in phosphate buffer may be a consequence of the progressive titration of essential sulphydryl groups on the dehydrogenase. In contrast, the presence of Ig+++ produced a slight stimulation of activity in the presence of 0.10 M Tris-HCl buffer, pH 7.4. Incubation of the enzyme with oxalacetate prior to Ig+++ exposure protected the enzyme from inactivation. However, simultaneous exposure of the enzyme to both oxalacetate and Ig+++ provided no significant protection. Prior incubation of the enzyme with NADH also resulted in Ig+++ inactivation.

It was also of interest to determine the effects of Ig+++ on the two buffer systems cannot be explained solely on the basis of a difference in ionic strength.
This is suggested by the observations that (a) increasing the ionic strength of the reaction mixture containing Tris-HCl buffer, by either the addition of salts or more buffer, did not result in Hg²⁺ inhibition of NADH oxidation; and (b) reducing the concentration of phosphate buffer did not significantly alter the inhibition by Hg²⁺. Thus, the buffer systems appear to have specific effects on malate dehydrogenase. A difference in the structure of the enzyme incubated in phosphate buffer compared to that in Tris-HCl buffer is suggested by a comparison of the rates of titration of the sulfhydryl groups of the enzyme with 5,5'-dithiobis(2-nitrobenzoic acid). The sulfhydryl groups of the enzyme in the presence of Tris-HCl buffer are titrated at about twice the initial rate of those in phosphate buffer. Apparently, this increased rate of titration does not involve a sulfhydryl group necessary for enzymatic activity. Furthermore, the enzyme incubated in phosphate buffer is much more resistant to heat inactivation than the enzyme in Tris-HCl buffer.

Since malate dehydrogenase activity has been demonstrated in a variety of different organisms, the effects of Hg²⁺ on the enzyme from other sources were examined. Under the standard assay conditions, Hg²⁺ stimulation of NAD reduction, catalyzed by malate dehydrogenase from other animal sources, could be readily demonstrated (Table III). This stimulatory effect of Hg²⁺ was shown to be independent of the ionic strength of the enzyme preparation. In contrast to the properties of the enzymes from animal sources, malate dehydrogenase activity from a variety of plants could not be stimulated by Hg²⁺, but instead was inhibited. Changing the assay conditions did not result in any detectable stimulation by Hg²⁺.

When bacterial malate dehydrogenases were examined for Hg²⁺ sensitivity, different effects were observed, depending on the organism examined. The enzymes from the family Enterobacteriaceae (A. aerogenes, E. coli, and S. typhimurium) were all stimulated in the presence of Hg²⁺. However, the enzymes examined from the genus Bacillus could not be stimulated by Hg²⁺. The enzyme from B. subtilis was inhibited, and those from B. subtilis and B. polymyxa were unaffected by Hg²⁺ under the standard assay conditions. Thus, the enzymes from bacterial sources showed all three possible responses to Hg²⁺.

**Fig. 5.** Effects of Tris-HCl and potassium phosphate buffers on Hg²⁺ inhibition of NADH oxidation catalyzed by pig heart mitochondrial malate dehydrogenase. The reaction components were added in the following order: 0.10 M Tris-HCl or potassium phosphate buffer, pH 7.4; enzyme; 0.047 mM NADH; and 0.13 mM oxalacetate to initiate the reactions. 0, enzymatic activity in the absence of Hg²⁺; residual enzymatic activity in the presence of 0.033 mM Hg²⁺.

### DISCUSSION

The earlier observations of Wolfe and Nielsens (4) and Thorne and Kaplan (3) clearly indicated that sulphydryl groups for maintaining the activity of pig heart mitochondrial malate dehydrogenase. In the later study, the enzyme was first incubated with the sulphydryl reagent HMB for 1 hour in the absence of substrates. Under these conditions no marked stimulation of NAD reduction could be demonstrated, but, instead, progressive inactivation was observed as the concentration of sulphydryl reagent was increased. In the present study the presence of malate together with the relatively shorter exposure period of the enzyme to Hg²⁺ might allow different sulphydryl groups to be titrated than those of the earlier studies. This would suggest that the contrasting effects of sulphydryl reagents on enzymatic activity under a variety of conditions are reflections of differential mercaptide bond formation. In the present study the inhibition resulting from concentrations of Hg²⁺ above 0.10 mM may be the result of the further titration of additional sulphydryl groups, including those required for the maintenance of enzymatic activity.

The present data also suggest that the presence of malate induces a structural change of the enzyme, which not only protects sulphydryl groups necessary for enzymatic activity but also exposes groups the titration of which leads to an increase in the rate of NAD reduction. That mercaptide formation results in an altered enzyme structure is also suggested by the observation that Hg²⁺-treated malate dehydrogenase is more sensitive to heat inactivation than the untreated enzyme. It seems very unlikely that the role of malate in the stimulatory effect of Hg²⁺ is simply to protect sulphydryl groups involved in the binding of this substrate to the enzyme. This role for malate would...
explain the protective effect of the substrate against Hg^{2+}
inactivation, but not the stimulatory effects of the sulfhydryl
reagent. The other substrate, NAD, does not appear to induce
similar structural changes, since prior incubation of the enzyme
with NAD before the addition of Hg^{2+} results in enzyme inactiva-
tion.

Therefore, alterations of enzyme conformation by environ-
mental changes might also determine whether the presence of
Hg^{2+} results in either stimulation or inhibition of NAD reduc-
tion. This suggestion is supported by the observation that
heating the enzyme alone for 5 min at 50°C or incubation of the
enzyme in high salt concentrations markedly decreases the ex-
tent of Hg^{2+} stimulation. Both treatments apparently alter
the structure of the enzyme, since activity in the absence of
Hg^{2+} is reduced in each case. The effects of both pH and tem-
perature on Hg^{2+} stimulation may also be interpreted in this
regard. The increase of pH from neutrality to more alkaline
regions in Tris-HCl buffer results in increased Hg^{2+} stimulation
(Fig. 2). This change to a more alkaline pH favors the ioniza-
tion of sulfhydryl groups and, hence, mercaptide formation.

That this ionization effect is not solely responsible for the pH
effects is indicated by the observation that at pH 10.0, at which
point sulfhydryl ionization would be maximized, negligible
stimulation could be demonstrated. Therefore, it appears that
an effect of pH on enzyme conformation may play a role in Hg^{2+}
stimulation.

At temperatures below 40°C the sulfhydryl groups available
for titration may not be essential for maintaining enzymatic
activity, and mercaptide formation could then result in a stimu-
lation of enzymatic activity. However, increasing the tempera-
ture above this point may result in the titration of sulfhydryl
groups essential for NAD-reducing activity. Thus, the confor-
mation of the enzyme at elevated temperatures in the presence
of Hg^{2+} may not allow the expression of any detectable activity.
An alternate explanation for these results might be that the
titration of the enzyme with Hg^{2+} results in an enzyme structure
which is extremely sensitive to the elevation of temperature.
However, it appears that increasing the temperature in the pres-
ence of the substrates alters the conformation of the enzyme
directly, since activity is increased in the absence of Hg^{2+}.
Furthermore, Hg^{2+}-treated enzyme preparations show reduced
but demonstrable activity following incubation at 50°C for periods
up to 10 min.

The proposed structural changes produced by Hg^{2+} in the
presence of malate do not result in significant alterations in the
K_{m} values of the dehydrogenase for the two substrates. The
increase in V_{max} under these conditions suggests that a step
subsequent to the binding of the substrates is altered in the
presence of Hg^{2+} and malate. Earlier kinetic data (11) have
suggested that the rate-limiting step in NAD reduction is the
removal of the reaction product NADH from the active site.
However, the oxidation of NADH in 0.10 M Tris-HCl buffer,
pH 8.8, is unaffected by Hg^{2+}. Furthermore, the binding of
NADH to the dehydrogenase at pH 8.8, measured by fluores-
cence spectroscopy, is not significantly altered by Hg^{2+} in the
presence of malate. Therefore, under the standard assay condi-
tions the binding of NADH to the coenzyme site does not appear
to be affected by Hg^{2+}. This suggests that some other aspect
of the reaction is altered in the presence of Hg^{2+}.

Hg^{2+}, along with other sulfhydryl reagents, has been used to
abolish the sensitivity of regulatory proteins to their allosteric
effectors (12, 13). The desensitizing effects of these reagents
appear to be the result of alterations in the subunit interactions
of these enzymes (13). Since recent evidence suggests that pig
heart mitochondrial malate dehydrogenase is composed of two
identical subunits (14), alterations in subunit interaction could
be involved in the stimulatory effects of the mercurial. How-
ever, the addition of Hg^{2+} to mitochondrial malate dehydro-
genase in the presence of malate does not cause any appreciable
change in sedimentation properties measured following sucrose
gradient sedimentation. Thus, any major alteration of subunit
interaction, i.e. dimer to monomer transition, does not appear to
occur following treatment with Hg^{2+} under the conditions
necessary to demonstrate stimulation.

These results suggest that, under the conditions of the standard
assay for NAD reduction, the structure of the enzyme is such
that maximal activity is not expressed, i.e. the enzyme is par-
tially inhibited. When Hg^{2+} is added in the presence of malate,
the enzyme is altered to a state in which maximum activity can
now be measured.

The effect of Hg^{2+} on NADH oxidation was markedly de-
dendent on the nature of the buffer utilized (Fig. 5). The
results of this study suggest that the structure of the enzyme may
differ in phosphate and Tris-HCl buffers at the same pH. Pre-
vious work with other proteins indicated that different confor-
mational changes occur depending on the nature of the anions
present in solution (15). Thus, the contrasting effects of Hg^{2+}
in the two buffers may reflect a difference in malate dehydro-
genase structure produced in the presence of phosphate and
chloride (from Tris-HCl) anions. This structural difference
may result from the direct binding of the anions to the enzyme
or from conformational changes induced in the two buffer sys-
tems. Direct measurements of conformational changes with
highly purified dehydrogenase preparations should differentiate
between these two types of structural changes.

A comparison of the effects of Hg^{2+} on malate dehydrogenases
from a variety of sources (Table III) is of interest in light of the
known structural similarities of these enzymes. Murphy et al.
(16) have shown by gel filtration measurements that malate
dehydrogenases from a variety of organisms fall into two dis-
tinct classes, the dimer model (molecular weights of approxi-
mately 60,000) and the tetramer model (molecular weights of
approximately 117,000). All of the animal and plant enzymes
examined conformed to the dimer model. Most bacterial en-
zymes, including the Enterobacteriaceae, also appear to be
dimers, with the notable exception of the enzymes from the
genus Bacillus, which all behave as trimers. Although malate
dehydrogenases from a large number of sources were not sur-
vveyed in the present study, the representative results indicate
that Hg^{2+} stimulation of NAD reduction catalyzed by malate
dehydrogenases is common to mammalian and dimeric bacterial
enzymes. On the other hand, the tetrameric bacterial malate
dehydrogenases are not stimulated by Hg^{2+} and, in fact, can be
inhibited, depending on the organism tested. Finally, plant
malate dehydrogenases are inhibited by Hg^{2+} under these con-
ditions. These results probably reflect the structural similarities
and differences which exist among the enzymes. Thus, the
different effects of Hg^{2+} on malate dehydrogenases from various
sources may reflect the evolutionary change in structure of the
enzyme.

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REFERENCES
