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# Reduction of Activity of Reduced Nicotinamide Adenine Dinucleotide Oxidase by Divalent Cations in Cell-Free Extracts of *Bacillus cereus* T

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A rapid and effective method was devised for the reduction of activity of reduced nicotinamide adenine dinucleotide (NADH) oxidase in crude extracts of *Bacillus cereus* T. The addition of 25 μmoles of $MnCl_2$ per mg of extract protein in tris(hydroxymethyl)aminomethane-hydrochloride buffer reduced NADH oxidase activity by 90% within 1 min, and this reduction was independent of *p*H between *p*H 7.0 and 8.5. Other divalent cations such as $Mg^{2+}$, $Ba^{2+}$, $Ca^{2+}$, and $Co^{2+}$ also reduced NADH oxidase activity, but monovalent cations such as $Na^+$ and $K^+$ were ineffective. The reduction of NADH oxidase activity by divalent cations was presumably due to the removal of an essential flavine cofactor, since the addition of riboflavine and flavine mononucleotide to treated extracts was shown to completely restore NADH oxidase activity. The specificity, convenience, and efficiency of the procedure were shown to be applicable to crude extracts of *B. megaterium* and *B. subtilis* and should facilitate spectrophotometric measurements of nicotinamide adenine dinucleotide-dependent dehydrogenases in these and other microorganisms.

Soluble reduced nicotinamide adenine dinucleotide (NADH) oxidases in vegetative cells and spores of *Bacillus cereus* T have been reported, partially characterized, and purified (1, 2, 4). These enzymes, also detected in sporulating cells at relatively high levels, have created much difficulty in spectrophotometric measurements of nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenases in crude extracts prepared from spores or cells at various stages of growth and sporulation. Methods to reduce or eliminate NADH oxidase activity in crude extracts have been previously described, but these were largely nonspecific, time-consuming, or so rigorous that enzymes desired were often inactivated. Basically, these methods included the use of chelating and sulfhydryl reagents (5), ultracentrifugation (13), and heat treatments (12).

In the course of investigating β-hydroxybutyrate dehydrogenase in sporulating cells of *B. cereus* T, it was noted that relatively high concentrations of manganese reduced NADH oxidase activity in cell-free preparations from this organism. This observation was extended to include other divalent and monovalent cations to determine what effect these had on NADH oxidase activity. From this study, a procedure was developed for the rapid and efficient elimination of NADH oxidase activity in cell-free extracts of *B. cereus* T which did not significantly alter the activities of several NAD-dependent dehydrogenases examined.

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## MATERIALS AND METHODS

**Organisms and cultural conditions.** Cells of *B. cereus* T were grown in modified G medium by using the active culture technique (9). Immediately after commitment to sporulation, as evidenced by the characteristic and sudden rise in *p*H, the cells were harvested and transferred to the chemically defined sporulation medium buffered at *p*H 6.4 (10).

Cells of *B. megaterium* and *B. subtilis* were inoculated directly into modified G medium and incubated for 10 hr. Commitment of these organisms to sporulation was estimated microscopically by observing morphological changes characteristic of sporulating bacilli.

All cultures were incubated at 30 C on a rotary shaker set at 300 rev/min.

**Preparation of cell-free extracts.** Crude extracts of *B. cereus* T, unless otherwise specified, were prepared at 4 C from cells 2 hr after replacement into the sporulation medium. The cells were harvested by centrifugation, washed once in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 8.5, and concentrated 40-fold in the same buffer. The concentrated cell suspension was then passed through a French pressure cell at 20,000 psi. Unbroken cells and large cell fragments were removed by centrifugation at 27,000 × *g* for 10 min.

Ultracentrifuged extracts were prepared by centrifuging crude extracts for 1 hr at 240,000 × *g* in an International Equipment Co. preparative ultracentrifuge (model B-60) at 4 C.

Crude and ultracentrifuged extracts of *B. megaterium* and *B. subtilis* were prepared in the same manner as described for *B. cereus* T.

**Enzyme assays.** All enzyme assays were performed at 30 C in 0.16 M Tris-hydrochloride buffer at *p*H 7.0. Specific activities are expressed in international units (micromoles of substrate converted per minute per milligram of extract protein).

NADH oxidase was assayed spectrophotometrically by measuring the oxidation of NADH at 340 nm with a Beckman DU spectrophotometer. The reaction mixture consisted of buffer, extract, and water to a total volume of 3.0 ml, and the reaction was started by the addition of NADH. Where indicated, 0.1 μmole of flavine mononucleotide or riboflavine was added to the reaction mixture.

The assay procedure for β-hydroxybutyrate dehydrogenase was modified from that previously described by Jurtshuk et al. (8). Magnesium chloride was added to a final concentration of $10^{-2}$ M, and the total volume in the cuvette was increased to 3.0 ml.

The assay procedure for 6-phosphogluconic acid dehydrogenase was previously described by Doi et al. (3), and Goldman and Blumenthal (6).

Malic dehydrogenase was assayed as described by Ochoa (11).

**Protein determination.** Protein was determined by the procedure described by Groves et al. (7). To prevent interference by the spontaneous oxidation and precipitation of manganese at alkaline *p*H values, all protein determinations were done in 0.1 M Tris-hydrochloride, *p*H 7.0.

**Reduction of NADH oxidase activity.** After the protein concentrations of crude extracts were determined, appropriate amounts of a 1.0 M solution of a specific cation were added. The enzyme-cation mixture was stirred gently for 5 min at 4 C. The resulting precipitate was then removed by centrifugation at 27,000 × *g* for 10 min. The chloride salt of the cations was used in all cases.

## RESULTS

To determine the concentration of $Mn^{2+}$ required for maximum reduction of NADH oxidase activity, various amounts of $MnCl_2$ were added to crude extracts containing 10 mg of protein per ml. Table 1 shows that NADH oxidase activity in crude extracts was reduced more than 85% when 25 or more μmoles of $Mn^{2+}$ was added per mg of extract protein. (This ratio was found to be effective for extracts varying in protein concentration from 5 to 25 mg/ml, and protein losses resulting from these $Mn^{2+}$ treatments were negligible.) Table 1 further shows that this reduction of NADH oxidase activity was not reversed when excess $Mn^{2+}$ was removed by passing the extract through a column (0.9 by 30 cm) containing a 10-cm bed of G-25 Sephadex.

To determine the effect of *p*H on the inactivation of NADH oxidase by $Mn^{2+}$, cell-free extracts were prepared in 0.1 M Tris-hydrochloride buffers at *p*H 7.0, 7.5, 8.0, and 8.5. The *p*H values for the resulting extracts were 7.5, 8.0, 8.4, and 8.7, respectively. Each extract was assayed for NADH oxidase activity, treated with 25 μmoles of $Mn^{2+}$ per mg of protein for 10 min at 4 C, and then assayed again for NADH oxidase activity after removing the resulting precipitate by centrifugation. All of the extracts showed comparable losses in NADH oxidase activity, suggesting that the inactivation of NADH oxidase by $Mn^{2+}$ was not *p*H-dependent. Since the initial NADH oxidase activity in the *p*H 8.7 extract was approximately twice that of the *p*H 7.5 extract, all subsequent extracts used in this investigation were prepared in 0.1 M Tris-hydrochloride buffer at *p*H 8.5.

TABLE 1. *Inactivation of soluble NADH oxidase in treated extracts of Bacillus cereus T*

| Treatment of extract | Specific activity | Per cent activity |
|---|---|---|
| None | 0.048 | 100 |
| Desalted | 0.048 | 100 |
| Ultracentrifuged | 0.0096 | 20 |
| $Mn^{2+}$-treated[a] | | |
| 5.0 | 0.03 | 62 |
| 11.0 | 0.017 | 35 |
| 18.0 | 0.013 | 27 |
| 25.0 | 0.007 | 14 |
| 33.0 | 0.007 | 14 |
| 43.0 | 0.007 | 14 |
| Desalted $Mn^{2+}$-treated[b] | 0.007 | 14 |

[a] Micromoles of $Mn^{2+}$ added per milligram of extract protein.

[b] Extract was treated with 25 μmoles of $Mn^{2+}$ per mg of protein, and the resulting precipitate was removed by centrifugation before desalting.

Although extracts treated with 25 μmoles of $Mn^{2+}$ per mg of protein were initially stirred for 10 min at 4 C, it was observed that a 90% reduction of NADH oxidase activity occurred within 1 min after cation addition. No significant increase in per cent reduction was noted when samples were examined after stirring in the presence of $Mn^{2+}$ for up to 30 min, and thus an incubation period of 5 min was routinely employed thereafter.

Since $Mn^{2+}$ was effective in reducing NADH oxidase activity in crude extracts, it was of interest to examine the specificity of this phenomenon. Table 2 shows that other divalent cations such as $Mg^{2+}$, $Ba^{2+}$, $Ca^{2+}$, and $Co^{2+}$ were as effective as $Mn^{2+}$, whereas the monovalent cations $Na^+$ and $K^+$ were ineffective in reducing NADH oxidase activity in crude extracts.

To determine the mechanism of NADH oxi-

TABLE 2. *Effect of various cations on soluble NADH oxidase in crude extracts of Bacillus cereus T*

| Treatment of extract[a] | Specific activity | Per cent activity |
|---|---|---|
| None | 0.046 | 100 |
| $Mn^{2+}$ | 0.002 | 5 |
| $Mg^{2+}$ | 0.004 | 9 |
| $Ca^{2+}$ | 0.003 | 7 |
| $Ba^{2+}$ | 0.002 | 5 |
| $Co^{2+}$ | 0.004 | 9 |
| $Na^{+}$ | 0.041 | 89 |
| $K^{+}$ | 0.040 | 90 |

[a] Cations were added to a final concentration of 25 μmoles per mg of protein.

dase inactivation by divalent cations, various attempts were made to restore enzyme activity in $Mn^{2+}$-treated extracts. It was shown earlier that the reduction of NADH oxidase activity by $Mn^{2+}$ treatment was not due to reversible enzyme inhibition, since extracts did not regain activity of this enzyme when excess $Mn^{2+}$ was removed by G-25 Sephadex column chromatography. It also seemed unlikely that this enzyme was selectively removed with the nucleic acids in the precipitate formed upon the addition of $Mn^{2+}$, because the oxidase was already inactive in extracts which were assayed before the precipitate was removed by centrifugation. Table 3 shows that NADH oxidase activity could be completely restored by the addition of either riboflavine or flavine mononucleotide to desalted extracts, suggesting that $Mn^{2+}$ and other divalent cations effectively removed a flavine cofactor essential for NADH oxidase activity.

The final objective of this study was to determine the effect of the $Mn^{2+}$ treatment on NAD-dependent dehydrogenases in crude extracts.

TABLE 3. *Effect of flavine mononucleotide (FMN) and riboflavine on soluble NADH oxidase in treated extracts of Bacillus cereus T*

| Treatment of extract | Specific activity | Per cent activity |
|---|---|---|
| None | 0.026 | 100 |
| FMN | 0.048 | 185 |
| Riboflavine | 0.054 | 208 |
| $Mn^{2+}$-treated[a] | 0.002 | 4 |
| $Mn^{2+}$-desalted[b] | 0.004 | 8 |
| Desalted plus FMN[b] | 0.045 | 173 |
| Desalted plus riboflavine[b] | 0.055 | 210 |

[a] Crude extract was treated with 25 μmoles of $Mn^{2+}$ per mg of protein, and the resulting precipitate was removed by centrifugation before assay.

[b] Extract was treated with 25 μmoles of $Mn^{2+}$ per mg of protein, and the resulting precipitate was removed by centrifugation before desalting.

Table 4 compares the specific activities for malic, β-hydroxybutyric, and 6-phosphogluconic acid dehydrogenases which were determined spectrophotometrically in both ultracentrifuged and $Mn^{2+}$-treated extracts prepared from cells of *B. cereus* T harvested 5 hr after replacement into chemically defined sporulation medium. Although the specific activity of malic acid dehydrogenase in the $Mn^{2+}$-treated extract was approximately half that of the ultracentrifuged extract, full activity of this enzyme was regained upon removal of excess $Mn^{2+}$ on a G-25 Sephadex column. The specific activity of β-hydroxybutyrate dehydrogenase in the $Mn^{2+}$-treated extract was nearly twice that of the ultracentrifuged extract due to enzyme stimulation by this cation, whereas 6-phosphogluconic acid dehydrogenase was equally active in both extracts. Thus, the reduction of NADH oxidase activity in crude extracts by $Mn^{2+}$ did not appear to negatively affect these NAD-dependent dehydrogenases despite differences in their cation requirements.

Since NADH oxidase activity is present at relatively high levels in sporeforming bacteria in general, the effect of $Mn^{2+}$ was also determined for extracts prepared from *B. megaterium* and *B. subtilis*. Although not shown, a 90% reduction of NADH oxidase activity in extracts prepared from these organisms was also obtained when 25 μmoles of $MnCl_2$ was added per mg of protein. The addition of riboflavine or flavine mononucleotide restored completely NADH oxidase activity in $Mn^{2+}$-treated extracts of *B. megaterium* but to a lesser extent in extracts of *B. subtilis*.

## DISCUSSION

The presence of a highly active soluble NADH oxidase in crude extracts prepared from *B. cereus*

TABLE 4. *Comparison of dehydrogenase activities between ultracentrifuged and manganese-treated extracts*

| Enzyme | Specific activity of extracts | | |
|---|---|---|---|
| | Crude | Ultracentrifuged | $Mn^{2+}$-treated[a] |
| NADH oxidase | 0.06 | 0.012 | 0.006 |
| Malic dehydrogenase | | 0.55 | 0.23[b] |
| β-Hydroxybutyrate dehydrogenase | | 0.014 | 0.024 |
| 6-Phosphogluconate dehydrogenase[c] | | 0.015 | 0.015 |

[a] Crude extracts were treated with 25 μmoles of $Mn^{2+}$ per mg of protein, and the resulting precipitate was removed by centrifugation before assay.

[b] Activity equivalent to that in the ultracentrifuged extracts can be restored by removal of $Mn^{2+}$.

[c] Requires $Mg^{2+}$.

T during growth and sporulation has greatly hindered studies on NAD-dependent dehydrogenases in this organism. Results from this investigation have shown that the addition of relatively high concentrations (25 $\mu$moles/mg of protein) of divalent cations such as $Mn^{2+}$, $Mg^{2+}$, $Ba^{2+}$, $Ca^{2+}$, and $Co^{2+}$ caused a rapid and effective (more than 90%) reduction of NADH oxidase activity in crude extracts. This reduction was independent of *p*H between the range of *p*H 7.0 and 8.5, and was shown to be due to the removal of some flavine prosthetic group essential for NADH oxidase activity. Extracts treated with $Mn^{2+}$ were further shown to retain enzymatic activities of several NAD-dependent dehydrogenases which could then be assayed spectrophotometrically without interference from NADH oxidase.

The procedure described is believed to be superior to others previously reported in the literature. The use of chelating and sulfhydryl reagents, although 90% effective in inhibiting NADH oxidase activity in crude extracts of *Tetrahymena pyriformis* (5), is not effective in reducing the activity of this enzyme in extracts of *B. cereus* T (4). Ultracentrifugation, although an effective method for removing NADH oxidase activity from crude extracts of *B. cereus* T (13), is time consuming, whereas the use of heat treatment is applicable to studying dehydrogenases which are relatively stable to the elevated temperatures used.

For instances in which a particular NAD-dependent dehydrogenase is inhibited by the cation treatment, it was shown that the cations could be removed by G-25 Sephadex column chromatography without restoring oxidase activity.

The specificity, convenience, and efficiency of this procedure to reduce NADH oxidase activity in crude extracts of *B. cereus* T, *B. megaterium*, and *B. subtilis* will undoubtedly facilitate future investigations on NAD-dependent dehydrogenases of these and other microorganisms.

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