Purification of a Salt-Requiring Enzyme from an Obligately Halophilic Bacterium

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ABSTRACT

HOLMES, P. K. (University of Illinois, Urbana), and H. O. HALVORSON. Purification of a salt-requiring enzyme from an obligately halophilic bacterium. J. Bacteriol. 90: 312-315. 1965.—The discovery that some halophilic enzymes may be reactivated from the salt-free and inactive state has facilitated the purification of nicotinamide adenine dinucleotide-linked malic acid dehydrogenase from crude extracts of Halo-bacterium salinarium. In the absence of large amounts of salt, the enzyme was totally inactive; yet, in this salt-free state, it could be purified by conventional techniques. The highly purified halophilic enzyme was shown to require a high concentration of salt for activity.

The enzymes which have been assayed in crude extracts of the extreme obligate halophile, Halo-bacterium salinarium, become inactivated if the salt concentration is reduced to low levels (Baxter and Gibbons, 1954, 1956, 1957; Larsen, 1962). The work of Baxter (1959) and Baxter and Gibbons (1954, 1956, 1957) has shown that such enzymes generally require very high concentrations of salt to exhibit maximal activity. The precise effect of salt on those enzymes is unknown, and we considered it desirable to obtain a purified halophilic protein for study. This report presents the results of some attempts towards this goal.

MATERIALS AND METHODS

The strain of H. salinarium used in this work was derived from Larsen's strain 1 (Dundas and Larsen, 1963), originally isolated from salted fish. The growth medium used consisted of 0.2% yeast extract, 0.25% Trypticase, 3% MgCl2·6H2O, and 25% NaCl (w/v).

The organism was routinely grown in 16-liter batches at 37 C. A 20-liter Pyrex carboy held 15 liters of nonsterile medium and approximately 1 liter of a 3-day culture as inoculum. As much air as possible was introduced under pressure into the medium from below the surface, and the whole was stirred with a propeller which extended into the culture approximately 20 cm. Under these conditions the tendency of the culture toward frothing was immense, and the addition of several milliliters of Dow Corning Antifoam A did not always suppress the foam for the 2.5- or 3-day growth period. Growth was usually halted when

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The optical density (OD) of the culture was approximately 220 Klett units (filter no. 66), and the cells were harvested in a Sharples Super Centrifuge. The yield averaged about 3 g (wet weight)/liter. Cells stored at 5 C in either pellets or in growth medium did not lose significant amounts of malic dehydrogenase (MDH) activity for at least 2 weeks. The enzyme activity was assayed as the oxaloacetate-dependent oxidation of reduced nicotinamide adenine dinucleotide (NADH2). Protein concentrations were routinely measured by the method of Lowry et al., 1951. The maximal specific activity of MDH was obtained in cell extracts prepared in an Eppenbach colloid mill (Gifford-Wood Co., Hudson, N.Y.). A pellet of whole cells, suspended in several volumes of 0.01 M phosphate buffer (pH 7) in 25% NaCl and homogenized in the mill (70 v, 10 C, 0.004-inch gap) for 30 to 60 sec, was totally ruptured. The brei from the mill, centrifuged at 30,000 X g for 10 min, treated with 1 μg of deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo.) per 20 mg of protein, and dialyzed against 25% NaCl, was used as "crude extract." The MDH activity in crude extract is about 70 units/mg of protein.

The specific activity in extracts prepared in a French pressure cell was about 40 units/mg of protein. The sonic treatment of whole-cell suspensions resulted in relatively inactive preparations.

MDH activity was stable in crude extracts at 5 C for at least 2 weeks. Crude extracts could be frozen and thawed without loss of MDH activity.

The organism WR-1 is an otherwise unidentified aerobic, motile, nonpigmented, rod-shaped, facultative halophile which grows well in Difco Nutrient Broth supplemented with 0 to 20% NaCl. This organism was isolated by H. Orin Halvorson,
who found it as a probable contaminant in cultures of \textit{H. salinarium} which had been diluted with salt-free medium to the lysis point. Crude extracts of this bacterium were prepared in the French pressure cell.

MDH from liver was obtained from Calbiochem, Los Angeles, Calif.

Cellulose (DEAE-70, Brown and Co., Berlin, N.H.), washed with acetone, acid, alkali, and water, was collected in a column to form a cylindrical matrix, 8 by 180 mm. The column was washed with 0.033 M sodium phosphate buffer (pH 8.0), and the sample, containing about 2 mg of protein suspended in the same buffer, was placed on the top. Elution was performed with a NaCl gradient, running from 0 to 5% NaCl, in the above phosphate buffer. Twenty samples of 5 ml each were collected at a flow rate of 10 ml/hr, at 5 C.

As a supporting medium for electrophoresis, polystyrene particles (Geon-474, B. F. Goodrich Co., Cleveland, Ohio) provided more resolution of MDH than did either starch gel or cellulose. The washed particles of polymer, suspended in 0.022 M sodium phosphate buffer, were allowed to settle in a Plexiglass trough to form a bed, 28 by 2.8 by 0.9 cm. A transverse section was removed from the center of the bed, and the cavity was filled with a mixture containing Geon-474, buffer, and about 1 mg of protein containing 1.4 X 10^4 to 1.8 X 10^4 units of MDH. A potential of 20 per cm was applied with a current of 3 ma. After sufficient time for resolution had elapsed, the Geon bed was sectioned; the sections were individually eluted with water, reactivated by dialysis against 25% NaCl, and assayed for content of MDH and protein. Electrophoretic separations were conducted at 4 C.

All references to pH in this paper pertain to values determined for the buffer with the glass electrode before the addition of salt.

One unit of MDH produces a change in OD at 340 m\textmu of 0.010 in the presence of 0.0003 M oxaloacetic acid and 0.0001 M NADH_2 (pH 7.2).

\textbf{RESULTS}

Figure 1 illustrates the effect of NaCl on MDH activity in crude extracts at pH 7.2. Also included are curves for liver MDH and for MDH from WR-1 grown in a medium containing 20% NaCl. The MDH from \textit{H. salinarium} was most active at 5% NaCl, and showed no activity at 1% NaCl. The enzyme from the facultative halophile WR-1 showed a salt optimum not much higher than that for nonhalophilic enzymes, yet it was more active at the higher salt concentrations than was the enzyme from liver.

Crued extracts from \textit{H. salinarium} were maintained in 25% NaCl until immediately prior to the assay for MDH, when they were diluted to the salt concentrations shown.

Reactivation of MDH from the salt-free state.

If crude extracts were exhaustively dialyzed against 0.02 M phosphate buffer or water, all MDH activity was lost. If this salt-free preparation was then dialyzed against 25% NaCl, an average of 55% of the activity was restored. The purification of this enzyme by conventional methods could thus be performed in the absence of salt.

\textbf{Fractionation of crude extracts with acetone.}

MDH could be selectively precipitated with acetone from crude extracts containing salt. Although the separation was not clean, and considerable activity was lost, the ease with which some fractions of good purity could be obtained rendered the method useful.

Crude extracts containing 10 mg of protein/ml in 0.03 M phosphate buffer (pH 7.2) plus 25% NaCl were chilled in an ice-salt bath to -15 C. Acetone at the same temperature was added, at the rate of 10 ml/min, with vigorous stirring. After a volume (ca. 250 ml) of acetone corresponding to one-half the original volume of extract had been added, the mixture was stirred at -20 C for 15 min, and centrifuged at 10,000 X g for 5 min; the pellet was discarded. The supernatant fluid was returned to the cold bath, a volume of acetone equal to the first addition was introduced in the same manner, and the whole was stirred and centrifuged as before. The resulting pellet, suspended in and dialyzed against 25% NaCl, contained less than 2% of the original protein and more than 10% of the original MDH activity. This fraction was colorless; only the first pellet contained any of the red carotenoid pigment present in crude extracts.

Of the protein originally present in solution,
30% could be precipitated with acetone. From the precipitated protein, 25% of the original MDH activity could be recovered. Another 10% was found in the final supernatant liquid, leaving some 65% unaccounted for and presumably denatured. This nonrecoverable portion of activity could be reduced considerably by increasing the protein concentration in the original extract (e.g., increasing the protein concentration to 15 mg/ml resulted in a total loss in activity of only 30%), but this resulted in a concomitant loss of resolution in all precipitable fractions.

The sixfold or sevenfold purified enzyme, called the "acetone preparation," was completely free from NADH$_2$ oxidase activity. This preparation was further purified by a salting out in the absence of NaCl.

Fractionation of the acetone preparation by salting out. Since it is almost impossible to salt out any halophile protein from solutions already containing 25% NaCl, the ammonium sulfate fractionation was performed on aqueous solutions. Over 96% of the protein in crude extracts can be precipitated with ammonium sulfate once the NaCl has been removed.

The acetone preparation was freed from NaCl by dialysis against 0.01 M phosphate, pH 7.0 (two changes of 1,000 volumes each). The first proteins to precipitate from this preparation in the presence of ammonium sulfate contained most of the recoverable MDH. The method of addition of ammonium sulfate was critical, and the pattern of precipitation was not absolutely predictable even under the conditions finally chosen.

The most satisfactory procedure involved the addition of solid ammonium sulfate to a buffer surrounding a dialysis bag which held the protein sample. The addition of powdered ammonium sulfate directly to the extract resulted in very poor resolution. As a rule, a concentration of 10 g of ammonium sulfate per 100 ml of buffer effected the precipitation, at 5 C, of the fraction containing MDH. In some runs, however, this first fraction could be discarded in favor of the protein precipitating in the range of 10 to 12 g of ammonium sulfate/100 ml of buffer. The recoverable enzyme precipitated within a narrow range of ammonium sulfate concentrations; an increase in concentration of 2 g/100 ml would remove 90% of MDH activity from a solution in which it had just begun to precipitate.

Dialysis of the protein against ammonium sulfate was carried out at 5 C, and 5 hr were allowed for precipitation. This is admittedly a slow salting-out, but the resulting selectivity of the fractionation justified the leisurely procedure. The method consistently gave a 10-fold to 14-fold increase in specific activity over the acetone preparation, with a total recovery of 25%.

The maximal total activity could be returned to the ammonium sulfate preparation only if the ammonium sulfate was removed before the return of NaCl. The precipitated protein was soluble in either water or 25% NaCl, but reactivation was hampered by the presence of ammonium sulfate; the precipitates were therefore dialyzed first against water, then against the sodium chloride solution, before assaying.

Fractionation by chromatography on cellulose. The ammonium sulfate preparation of MDH, with a specific activity 60 to 100 times greater than that of crude extract, could be purified further by selective elution from diethylaminoethyl (DEAE) cellulose. In the absence of salt, the enzyme adhered to the cellulose, from which it could be removed with added NaCl.

The ammonium sulfate preparation contained approximately 0.8% nucleic acid, as determined by the ratio of its absorption of light at 260 and 260 mm; a concentration of 4.5% sodium chloride was necessary to free the nucleic acid from the cellulose.

MDH was contained only in those fractions eluting at salt concentrations between 2 and 3%; over 75% of the recoverable activity was found in the fraction containing approximately 2.4% NaCl. This one fraction represented, as an average, a 30% recovery of the enzyme placed on

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**Table 1. Average individual and total recoveries, and specific activities of MDH fractions, at various stages of purification**

<table>
<thead>
<tr>
<th>Per cent recovery</th>
<th>Specific activity increase</th>
<th>Specific activity increase, total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Acetone</td>
<td>11</td>
<td>6.5</td>
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<tr>
<td>Ammonium sulfate</td>
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<td>12</td>
</tr>
<tr>
<td>DEAE cellulose</td>
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<tr>
<td>Electrophoresis 1</td>
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<td>2.5</td>
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<tr>
<td>Electrophoresis 2</td>
<td>80</td>
<td>1.5</td>
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the column, and showed an average 2.6-fold increase in specific activity. The MDH from this fraction was dialyzed against 0.022 M sodium phosphate buffer (pH 6.7) in preparation for electrophoretic separation.

Fractionation by electrophoresis. The salt-free preparation of MDH from the cellulose column could be purified almost another 4-fold by electrophoretic fractionation. A two-stage separation lead to the final recovery of about 0.5% of the original MDH, with a specific activity of 6.7 × 10^3 units/mg of protein.

MDH moved towards the cathode and, at a potential of 20 v/cm, at pH 6.7, migrated at a rate of about 18 mm/hr. After 3 hr of migration, a 1-cm band about 6 cm from the origin contained all of the recoverable (65% of the initial) MDH activity and showed an increase of specific activity averaging 2.5-fold.

Enzyme from this fraction was introduced into another bed of Geon-474 buffered at pH 6.4, and was subjected to a potential of 16 v/cm. After 4 hr under these conditions, a 1-cm-wide fraction about 6 cm from the origin contained 80% of the original activity and displayed an increase in specific activity of 1.5-fold. This latter fractionation has produced preparations of MDH which at best were some 960 times more active per milligram of protein than were crude extracts.

A summary of the average yields and specific activities of the enzyme in the various purification steps is seen in Table 1.

**DISCUSSION**

The relatively low total yield of enzyme has precluded our determination of the purity of this protein preparation. It is probable, moreover, that a considerable fraction of any contaminating protein consists of inactivated MDH. However, whatever state of purification this procedure effects, it is clear that a several-hundred-fold increase in specific activity can be achieved by conventional techniques. In the past, we have spent much time in attempts to purify halophilic proteins in the presence of high salt concentrations, and such endeavors have met with very little success.

The reactivation of halophilic enzymes other than MDH has been examined by dialysis against solutions containing only NaCl (Holmes and Halvorson, 1963). Apparently, cells of this organism contain large amounts of potassium (Christian and Waltho, 1962); hence, KCl may prove to be a more useful salt for reactivation.

It is possible that, in the absence of salt, halophilic proteins undergo a reversible conformational change. It would be interesting to know whether such a reversible change attends the disruption of halophilic structural proteins. A study of halophilic cell walls or flagella might answer this question.

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**Literature Cited**


