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## Factors affecting the cation requirement of a halophilic NADH dehydrogenase

It has been suggested that enzymes prepared from extremely halophilic bacteria differ from other enzymes in that they require high concentrations of monovalent cations to neutralize intramolecular electrostatic charges for the maintenance of enzymatically active conformations[1]. Recently, the oxidation of NADH by extracts prepared from various extreme halophiles was shown to require high concentrations of monovalent cations[2]. This requirement could be satisfied by relatively low concentrations of $MgCl_2$. These data could be interpreted as reflecting the low molar efficiency with which monovalent cations replaced a normally low divalent cation requirement[3]. During studies on an NADH dehydrogenase derived from the NADH oxidase, we observed that the cation concentration required for stability and maximum activity could be varied over a wide range depending on the cation and the buffer in which the enzyme was maintained. This communication summarizes these data.

Crude extracts were prepared from the extreme halophile AR-1 as previously described and used as the source of NADH dehydrogenase[3]. Enzyme activity was determined at 30° in reaction mixtures containing the following additions in a total volume of 1 ml: imidazole hydrochloride, pH 7.0 (50 $\mu$moles); KCN, pH 7.0 (1 $\mu$mole); sodium 2,6-dichlorophenolindophenol (0.071 $\mu$mole); NADH (0.1 $\mu$mole); and the desired cation, usually NaCl (2.5 mmoles). The reaction was started by the addition of enzyme, and the initial rate of dye reduction (determined at 600 m$\mu$ and corrected for nonenzymic activity) was taken as a measure of enzyme activity. Stability of the NADH dehydrogenase was determined by diluting crude extracts 1:34 in appropriate buffers and incubating at 22°. Aliquots were removed at various times, added to reaction mixtures which were 2.5 M with respect to NaCl, and the initial rate of dye reduction was assumed to reflect the residual NADH dehydrogenase activity. No evidence of salt reactivation[4] was observed during the course of the reaction.

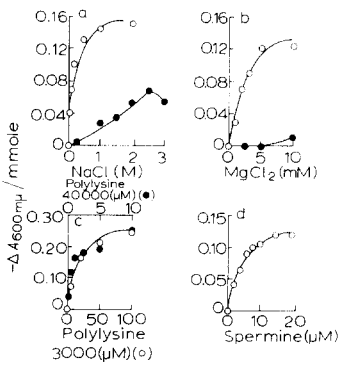


Fig. 1. The effect of cations on NADH dehydrogenase activity. In Figs. 1a and 1b, the buffer was either 50 mM Tris hydrochloride, pH 7.4 (●), or 50 mM imidazole hydrochloride, pH 7.0 (○). Otherwise standard reaction conditions were used. The cations were varied as indicated in each figure.

As shown in Fig. 1a, NADH dehydrogenase was activated in a manner characteristic of numerous halophilic enzymes[4–6]. No activity was observed in the absence of added NaCl, and enzymic activity progressively increased with increasing concentrations of NaCl. The concentration of NaCl required for maximum activity depended on the assay buffer. In the presence of Tris, maximum oxidation occurred at approx. 2.5 M NaCl. With imidazole, maximum oxidation was observed at 1 M NaCl. Two additional buffer effects were present: significantly greater activity was observed when the assay was carried out in imidazole rather than Tris buffer; and the rate of NADH oxidation as a function of NaCl concentration increased in a hyperbolic manner when the assay was carried out in imidazole, while in Tris, the response appeared to be sigmoidal. As shown in Fig. 1b, the ability of $MgCl_2$ to satisfy the cation requirement also was affected by the buffer. In the presence of imidazole, maximum oxidation took place at 5 mM $MgCl_2$. In the presence of Tris, no activity was observed at $MgCl_2$ concentrations less than 10 mM, and the level of activity was extremely low.

Preliminary experiments had indicated that 0.25–1.0 M lysine activated the NADH dehydrogenase with the activity at the latter concentration equivalent to the rate observed with 1.0 M NaCl. Attempts to study lower concentrations of lysine were complicated by a very rapid loss of activity. The enzyme appeared to be more

TABLE I

THE EFFECT OF SPERMINE ON THE STABILITY OF NADH DEHYDROGENASE

The enzyme was incubated in 0.1 M Tris hydrochloride at the indicated pH values and cation concentrations. The $-\Delta A_{600\ m\mu}$ was converted to $\mu$moles NADH, using 19.1 as the millimolar extinction coefficient for dichlorophenolindophenol[7]. Specific activity was defined as $\mu$moles NADH · $min^{-1}$ · mg $protein^{-1}$.

| *Incubation conditions* | *Specific activity* | | *Half-life* |
|---|---|---|---|
| *Incubation times:* | *0 min* | *120 min* | *(min)* |
| *Expt. 1: pH 7.4* | | | |
| 3.4 M NaCl | 0.71 | 0.68 | — |
| 0.1 M NaCl | 0.61 | 0 | 18 |
| 0.1 M NaCl + 3.3 mM spermine | 0.71 | 0.45 | 150 |
| 0.1 M NaCl + 33 mM spermine | 0.71 | 0.52 | 220 |
| *Expt. 2: pH 8.5* | | | |
| 3.4 M NaCl | 0.66 | 0.68 | — |
| 0.1 M NaCl | 0.63 | 0.03 | 30 |
| 0.1 M NaCl + 3.3 mM spermine | 0.68 | 0.49 | 200 |
| 0.1 M NaCl + 33 mM spermine | 0.66 | 0.63 | 1500 |

stable in the presence of polylysine, permitting determinations to be made at low concentrations. As shown in Fig. 1c, polylysine of an average molecular weight of 3000 satisfied the cation requirement at 100 $\mu$M; a preparation of average molecular weight 40 000 was effective at 10 $\mu$M. Assuming 14 and 192 lysine residues per mole, respectively, both polyamino acids appeared about equally effective in relation to their lysine content. In terms of ionic strength, polylysine 3000 ($I = 0.011$) was about 15 times more effective than polylysine 40 000 ($I = 0.17$) at these concentrations,

and the former was essentially as effective as 5 mM $MgCl_2$ ($I = 0.015$). Further indications that the efficacy of a cation was an intrinsic property of the ion rather than its concentration or the ionic strength were obtained with the polyamine spermine. As shown in Fig. 1d, 15 $\mu$M spermine satisfied the cation requirement (where the cation concentration was 60 $\mu$M and the $I = 1.5 \cdot 10^{-4}$). Double reciprocal plots of velocity *versus* cation concentration indicated that while the apparent $K_m$ values differed with each cation, the maximum velocities were virtually identical.

Studies on the ability of spermine to stabilize the enzyme revealed that it was effective in maintaining NADH dehydrogenase activity (Table I). When incubated in 0.1 M Tris buffer (pH 7.4) containing 0.1 M NaCl, the enzyme decayed exponentially with a half-life of 18 min. In the presence of 3.3 mM spermine, the half-life increased to 150 min, while in 33 mM spermine half of the initial activity was lost after 220 min. When the experiments were carried out at a more alkaline pH, the rate of decay was significantly less. At pH 8.5, the half-life in 0.1 M NaCl increased to 30 min, while in the presence of 3.3 mM spermine the half-life was 200 min. After 120 min, the dehydrogenase had lost little activity when incubated in the presence of 33 mM spermine, and after 25 h, retained approx. 50% of the initial activity.

These data point to the complexity of the cation requirement for one putatively halophilic enzyme in that whether an enzyme is "halophilic" or not depends on factors other than the enzyme *per se*. Preliminary experiments with a glycerol dehydrogenase prepared from *Halobacterium cutirubrum* indicated that this enzyme also functioned in the absence of added cations other than low concentrations of spermine. It is tempting to speculate that enzymes obtained from halophilic bacteria may not require concentrations of cations much different from enzymes obtained from non-halophilic cells. Rather, they may be stabilized and activated at low concentrations of specific cations. In the absence of these cations, they are maintained in a functional and stable state by extraordinarily high concentrations of monovalent cations.

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