Biochimica et Biophysica Acta, 321 (1973) 84–89

BBA 66979

THE EFFECT OF CATIONS ON THE HEAT STABILITY OF A HALOPHILIC NITRATE REDUCTASE

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(Received March 1st, 1973)

SUMMARY

1. Nitrate reductase was partially purified from extracts of an extremely halophilic bacterium. The temperature optimum of nitrate reductase activity was a function of both the concentration and the specific cation present in the assay mixture. Maximal activity was found at 85 °C in the presence of 4.27 M NaCl, 73 °C in the presence of 2 M KCl and 56 °C in the presence of 0.5 mM $MgCl_2$.

2. At suboptimal NaCl concentrations (0.17 M), 0.5 mM $MgCl_2$ enhanced nitrate reductase activity whereas at 4.27 M NaCl, 0.5 mM $MgCl_2$ was inhibitory.

INTRODUCTION

Enzymes of extreme halophiles are adapted to function at high salt concentrations. The response of cytochrome oxidase, cysteine desulfhydrase, and several dehydrogenases in crude extracts of *Halobacterium salinarium* to various concentrations of NaCl were tested[1–3]. More recently, the activity of many other enzymes from other extreme halophiles has been examined as a function of salt concentration[4–11]. In general, these enzymes were most active at high salt concentrations and were inactive in the absence of salt.

The internal salt concentrations of these bacteria have been found to be comparable to those found in the media in which they grow although the ionic composition may be different. It thus appears that besides Na^+ and Cl^-, K^+ is the major component of the internal salt concentration[12].

It is the purpose of this investigation to study the effects of various salt concentrations and temperatures on the activity of the extremely halophilic enzyme nitrate reductase.

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MATERIALS AND METHODS

Culture and growth conditions

The organism was an extreme halophile isolated from the Great Salt Lake, Utah and identified as *Halobacterium* sp.[13]. The growth medium consisted of 7.5 g casamino acids (Difco), 10 g yeast extract (General Biochemicals), and 3.0 g sodium citrate, 2.0 g KCl, 20 g KNO_3, 20 g $MgSO_4 \cdot 7H_2O$, 23 mg $FeCl_2$, and 250 g NaCl per liter of water. The pH was adjusted to 7.5 and the medium was autoclaved. The culture was shaken aerobically at 50 °C until it had entered the logarithmic growth phase. The flasks were then sealed and incubated anaerobically for an additional three days to induce nitrate reductase activity. The cells were harvested by centrifugation and washed with 4.3 M NaCl.

Partial purification of nitrate reductase

The washed cells were resuspended in a salt solution (4.27 M NaCl, 0.05 M Tris–HCl, pH 7.6, and 0.01 mM $MgCl_2$), sonically disrupted and separated into particulate and supernatant fractions as previously described[15]. The supernatant fraction (670 mg protein) was fractionated with acetone in successive 10% (volume acetone/initial volume supernatant) steps. The acetone and supernatant were kept in a salt water ice bath at −5 °C throughout the addition of the acetone which was added slowly with continuous stirring. At each step the precipitate was removed by centrifugation and resuspended in 5.0 ml of 4.3 M NaCl in 0.05 M Tris–HCl, pH 7.6. The protein suspensions were then dialysed against two changes of 4.3 M NaCl to remove the acetone and restore the nitrate reductase activity.

The enzyme was further purified on DEAE-cellulose (Cellex-D, Bio-Rad Labs) previously washed with acetone, 0.1 M NaOH, 0.1 M HCl, water, and finally suspended in 0.05 M Tris–HCl, pH 7.6, containing 0.17 M NaCl. The enzyme preparation from the acetone-precipitated fraction (80 mg protein) was dialyzed against the 0.17 M NaCl buffer solution, placed on the column (3 cm × 20 cm internal diameter), and eluted with a NaCl gradient ranging from 0.17 to 1.7 M NaCl. The samples eluted from the DEAE were then dialyzed against 4.3 M NaCl before assaying for activity.

Nitrate reductase assay

Nitrate reductase was measured using a modification of the procedure described by Showe and DeMoss[14]. The assay mixtures consisted of the enzyme preparation, 0.1 M Tris–HCl buffer, pH 7.5, 0.1 M KNO_3, 10^{-4} M methyl viologen, and 4.27 M NaCl.

Protein determinations

Protein determinations were carried out using either the turbidometric method of Stadtman *et al.*[16] or by the method of Lowry et al.[17] using crystalline bovine serum albumin as a standard.

RESULTS

Purification of nitrate reductase

Partial purification of nitrate reductase was carried out on the supernatant

TABLE I

A SUMMARY OF AVERAGE YIELDS AND SPECIFIC ACTIVITIES OF NITRATE REDUCTASE AFTER THE VARIOUS PURIFICATION STEPS

Preparation	*Protein recovery (mg)*	*Percent recovery protein (total)*	*Specific activity (nmoles NO_2^-/ min per mg protein)*	*Total activity**
Supernatant	670	100	3.2	2144
Acetone	80	12	31.0	2480
DEAE-cellulose	29	4.3	76	2204

* The apparent high total recovery can be explained by experimental error in determining protein concentrations in high concentrations of NaCl after each purification step.

fraction[15] since it contained nitrate reductase activity presumably liberated from the particulate fraction by sonication. Approx. 12% (80 mg protein) of the total protein was recovered as soluble protein in the 40–50% (v/initial volume) acetone precipitate, resulting in a purification of approx. 10-fold (Table I). The enzyme activity was eluted from DEAE at approximately 1.1 M NaCl. Approx. 4.3% of the total protein was recovered with a 24-fold increase in the specific activity (Table I).

The effects of temperature on nitrate reductase activity at various NaCl concentrations

Since maximal activity of various halophilic enzymes was found in the presence of 25% NaCl[4], the effect of NaCl concentration at various temperatures were investigated. In the absence of NaCl no activity was observed at any temperature (Table II). In the presence of 0.17 M NaCl maximum activity was observed at 65 °C with no activity at 85 °C. At 0.85 M NaCl the maximum activity was seen at 75 °C and some activity beginning to appear at 85 °C. At 2.56 M NaCl the maximum activity was at 85 °C and some activity seen at 93 °C while at 4.27 M NaCl the maximal activity still was observed at 85 °C but the amount at this temperature and at 93 °C had increased. Finally at 5.31 M NaCl the maximal activity had shifted to 93 °C although lower than that at 85 °C.

No NO_2^- formation was observed in the absence of enzyme or of dithionite.

TABLE II

THE EFFECT OF TEMPERATURE ON NITRATE REDUCTASE ACTIVITY AT VARIOUS NaCl CONCENTRATIONS

Identical aliquots of enzyme were dialysed against distilled water, 0.17, 0.85, 2.56, 4.27, and 5.31 M NaCl. The aliquots were assayed as described in Materials and Methods in the presence of the respective concentrations of NaCl.

NaCl concn (M)	*nmoles NO_2^-/min per mg protein*						
Temp:	*45*	*55*	*65*	*75*	*85*	*93*	*97* °C
0	0	0	0	0	0	0	0
0.17	15	25	28	2	0	0	0
0.85	19	30	38	43	2	0	0
2.56	12	22	28	42	46	26	2
4.27	9	18	30	51	56	45	7
5.31	5	16	24	34	38	42	33

The effect of temperature on nitrate reductase activity at various KCl concentrations

Since the K^+ concentrations inside extremely halophilic bacteria are very high (about 4.6 M)[12], the effects of KCl concentration on nitrate reductase activity was studied. The results observed were similar to those observed with NaCl except that the greatest activity was seen in the presence of 2.01 M KCl and appeared at 75 °C.

The effects of $MgCl_2$ concentration and temperature on nitrate reductase activity

Since increasing concentration of NaCl and KCl resulted in an increase in the maximal activity of nitrate reductase at higher temperatures, the effect of a divalent ion, Mg^{2+}, was studied. The greatest activity was seen with $5 \cdot 10^{-4}$ M $MgCl_2$, a much lower concentration than that needed for NaCl or KCl. The maximal activity, however, was seen at a much lower temperature, 55 °C. As the ionic strength was increased to 0.05 M $MgCl_2$ the maximal activity decreased but now occurred at 65 °C. Further increases in ionic strength to 0.99 and 1.48 M $MgCl_2$ further decreased the maximal levels of activity and were seen at a higher temperature, 65 °C. The maximal values were, however, seen at comparatively lower temperatures than those seen with NaCl.

The effects of a combination of monovalent and divalent ions on nitrate reductase activity at various temperatures

Since the maximal activity with NaCl was seen in 4.27 M and 85 °C (Table II) while the maximal activity with $MgCl_2$ was seen at 0.5 mM and 55 °C experiments were carried out to determine the effects of combining Na^+ and Mg^{2+}. The maximal activity seen with 0.17 M NaCl at 65 °C was increased by approximately 17% in the presence of 2.5 mM $MgCl_2$. The maximal activity in the presence of 4.27 M NaCl was seen at 85 °C but when $MgCl_2$ was added to a final concentration of 2.5 mM, the level of maximal activity was decreased by approximately 24%. Also, the presence of Mg^{2+} appeared to enhance the resistance to heat denaturation seen with 0.17 M NaCl. In 4.27 M NaCl however, less activity was seen at 90 °C in the presence of Mg^{2+}.

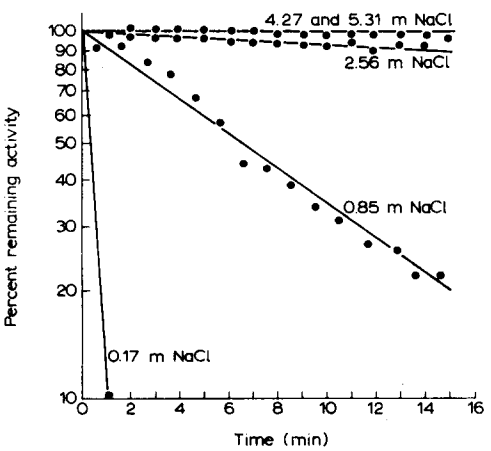

Fig. 1. Heat inactivation of enzyme at various NaCl concentrations. Identical aliquots (0.5 ml total volume) of enzyme in various concentrations of NaCl were heated to 80 °C for various time periods, immediately cooled, and assayed for activity at 50 °C.

Heat inactivation of the enzyme

Identical aliquots of the enzyme in 0.17, 0.85, 2.56, 4.27, and 5.31 M NaCl, respectively, were heated at 80 °C for various times and assayed at 50 °C for activity remaining. As can be seen in Fig. 1, more than 90% of the original activity was lost after 1 min of heating at 80 °C in the presence of 0.17 M NaCl. In the presence of 0.85 M NaCl, 50% of the original activity remained after about 7 min. This protection against heat inactivation was greatest at 4.27 and 5.31 M NaCl where virtually no inactivation was seen at 80 °C in 15 min.

Lack of heat activation of nitrate reductase

Several enzymes are heat activated[18]. Identical aliquots of the enzyme preparation in 4.27 M NaCl were assayed at 50 °C. One aliquot was first heated to 85 °C for 5 min and then cooled to 50 °C. Both aliquots produced identical amounts of nitrite indicating that the enzyme is not irreversibly heat activated.

DISCUSSION

Several hypotheses have been presented concerning the stability of halophilic enzymes in salt solutions. Baxter[19] suggested that this stability could be ascribed to electrostatic interactions, and other investigators[5,20,21] concluded that the stability of the enzymes was due to shielding of the negative electrical charges found in extremely halophilic proteins. The latter hypothesis was supported by the findings that halophilic proteins contain an excess of amino acids with a net negative charge[22,23] which could be shielded by Na^+ and other cations. Lanyi and co-workers[10,24], however, suggest that the high salt requirement of halophilic enzymes is due not only to charge shielding but also to hydrophobic forces. The native conformation of the enzyme is achieved through the interaction of hydrophobic groups due to their decreased solubility in the solvent[24]. They further suggest that at low salt concentrations and especially in the presence of polyvalent ions such as Mg^{2+} the enzyme is thought to be in a partially unfolded conformation and a partial stability is achieved by counterion shielding.

The results presented in this study seem to substantiate the hypothesis of Lanyi and co-workers[10,24] since salts have a profound effect on the activity and stability of nitrate reductase. At 0.17 M NaCl the enzyme may be in a partially folded configuration which is stabilized by electrostatic shielding. The increase in activity with increased temperature can be explained by regular enzyme kinetics. As the temperature is further increased beyond that at which conditions are optimal the enzyme's configuration is changed further until the protein is denatured. As shown in Fig. 1, 0.17 M NaCl did not protect against heat denaturation at higher temperatures. However, as the salt concentration was increased the enzyme was subjected to hydrophobic interaction forces so that the enzyme was in a tighter, more stable configuration. The presence of high salt concentrations in the assay medium allowed the enzyme to catalyze NO_3^- reduction at high temperatures and protected against inactivation of the enzyme at 80 °C (Fig. 1). The activity of the enzyme at lower temperatures was, however, inhibited by the higher salt concentrations possibly because the reactive center was not as accessible to the substrate. As the temperature was increased the enzyme became more "soluble", the configuration

more relaxed, permitting increased accessibility to the reactive site by the substrate. This effect, combined with the increased activation of the substrate by temperature, resulted in an increased total activity. This hypothesis is plausible since it is clear that increased stability of the enzyme at the higher salt concentrations does not account entirely for the increased activity at the higher temperatures. For example, although 2.56 M NaCl nearly fully protects the enzyme from inactivation at 80 °C, the activity of the enzyme in the presence of this concentration of salt at 75 °C and 85 °C is considerably less than at 85 °C with 4.28 M NaCl.

The combined effects of divalent and monovalent ions seemed to indicate that Na^+ and Mg^{2+} were competing for charged areas of the enzyme and that at higher salt concentrations the conformation of the enzyme is better suited for optimal activity in the presence of monovalent ions as compared to divalent ions such as Mg^{2+}.

ACKNOWLEDGMENTS

This work was supported in part by United States Public Health Services Predoctoral and Postdoctoral Fellowships, Grant Nos 1-FO1-GM-40, 359-01A1 and 1 F2 AM 51, 482-01 (E. D. M.); also by grants from the National Institutes of Health, U.S. Public Health Services (AI 05637) and the National Science Foundation (GB6257X). The authors wish to thank Dr A. A. Benson, Scripps Institution of Oceanography, in whose laboratory part of this work was carried out and Dr R. E. McCarty for his advice in preparation of this manuscript.

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