Biochimica et Biophysica Acta, 403 (1975) 58—66

BBA 67597

STUDIES OF A HALOPHILIC NADH DEHYDROGENASE

II. KINETIC PROPERTIES OF THE ENZYME IN RELATION TO SALT ACTIVATION

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(Received April 1st, 1975)

Summary

1. An NADH dehydrogenase, obtained from an extremely halophilic bacterium, was activated by various salts when enzyme activity was measured as the observed velocity, whereas the maximum velocity was unaffected by either the salt concentration or the nature of the salt.

2. Two ion effects were observed; a quantitative cation effect, reflected in changes in the apparent Michaelis constant for 2,6-dichlorophenolindophenol, and a qualitative anion effect, reflected in the apparent Michaelis and dissociation constants for NADH.

3. The data suggest that cations act by neutralizing electrostatic charges surrounding the 2,6-dichlorophenolindophenol-binding site, whereas the anions affect the conformation of the enzyme by altering the accessibility of the NADH-binding site to the bulk solvent.

4. Thus, the apparent activation of this enzyme, obtained from an extremely halophilic bacterium, is a reflection of measuring enzyme activity at non-saturating substrate concentrations.

Introduction

The preceding paper in this series [1] described the purification and some properties of an NADH dehydrogenase (reduced NAD:2,6-dichlorophenolindophenol oxidoreductase, EC 1.6.99.3) from an extremely halophilic bacterium

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCIPH, reduced 2,6-dichlorophenolindophenol.

(strain AR-1). Like certain other enzymes obtained from extremely halophilic bacteria [2], the NADH dehydrogenase activity from strain AR-1 was enhanced by the presence of NaCl. However, considerable activity was observed in the absence of added salt (15 mM NaCl). While other enzymes obtained from halophilic bacteria have been reported to be active either at low salt concentrations or in their absence [3—6], the NADH dehydrogenases from *Halobacterium salinarium*, strain 1 [7], and *Halobacterium cutirubrum* [8] were inactive in the absence of added salt. In order to clarify the discrepancy between these NADH dehydrogenases, the ionic activation of the solubilized enzyme from strain AR-1 was studied in more detail. Particular attention was devoted to the effect of salts on the kinetic parameters of the enzyme since a previous study indicated that failure to take this into account could lead to anomalous data [9].

Materials and Methods

The enzyme employed in this study was the QAE fraction prepared as previously described [1]. It had a specific activity of 376 μmol DCIP reduced / min per mg protein when assayed in a standard reaction mixture.

The standard reaction mixture contained the following additions in a total volume of 1 ml: imidazole hydrochloride, pH 7.0, (50 μmol); KCN (1 μmol); DCIP (70 nmol); NADH (100 nmol); bovine serum albumin (40 μg); NaCl (2.5 mmol). The assays were carried out at 30°C by the addition of 0.036 unit of enzyme, where a unit of enzyme activity is that amount of enzyme catalyzing the reduction of 1 μmol of DCIP per min. Kinetic assays were carried out in standard reaction mixtures over a concentration range of from 25 to 71 μM DCIP and from 30 to 150 μM NADH. When Na^+ was replaced by other cations, all solutions were made up with the cation supplied as the chloride salt. When Cl^- was replaced by other anions, they were supplied as the sodium salts, and the buffer was neutralized with the appropriate acid with the exception of thiocyanate, where HCl was employed. The amount of NaCl added with the enzyme was 30 μmol. The data were corrected for any non-enzymic reduction of DCIP by NADH; no NADH-independent reduction of DCIP was observed.

The results from the kinetic experiments were first plotted in double reciprocal form. If linear, the results were analyzed using a computer program for sequential kinetics made available by Dr V. Schramm (Department of Biochemistry, Temple University School of Medicine).

NADH, NAD and DCIP were obtained from the Sigma Chemical Co. DCIPH was prepared in situ since it was rapidly auto-oxidized regardless of the reductant employed. The desired amount of DCIPH was obtained by adding sufficient DCIP to a standard reaction mixture so that reduction with an appropriate amount of ascorbic acid resulted in the desired amounts of both DCIP and DCIPH. The concentration of DCIP was determined from the absorbance at 600 nm prior to the addition of the enzyme (using 20 for the millimolar absorbance). The concentration of DCIPH was determined from the difference in absorbance at 600 nm prior to the addition of ascorbic acid and the absorbance at 600 nm before initiating the reaction by the addition of enzyme. In separate experiments, it was determined that dehydroascorbic acid

did not inhibit NADH dehydrogenase activity up to a concentration of at least 140 μM. This was the maximum amount ever present in the experiments reported in this paper. All other chemicals were obtained from the usual commercial sources.

Results

Substrate kinetics

When initial velocity studies were carried out in the presence of 2.5 M NaCl, with DCIP as the variable substrate and NADH as the changing fixed one, a family of intersecting lines were observed in which both the ordinate intercept and the slopes changed (Fig. 1a). A similar pattern was observed when NADH was the variable substrate and DCIP was the changing fixed one (Fig. 1b). In all cases, replots of the slopes and intercepts were linear and are shown in the insets. These results were consistent with the operation of a sequential mechanism in which both substrates add to the enzyme prior to the release of products. The kinetic constants obtained from these experiments are summarized in Table I and are those suggested by Cleland [10], where K_a and K_b are the Michaelis constants equivalent to the concentrations of the appropriate substrates which give half of the maximum velocity, while K_{ia} is the dissociation constant of the appropriate binary complex.

Product inhibition studies

When NAD was used as the product inhibitor, competitive inhibition was observed with NADH as the variable substrate (Fig. 2a). Non-competitive inhibition was observed when DCIP was used as the variable substrate in the presence of NAD (Fig. 2b). When DCIPH was used as the product inhibitor, non-competitive inhibitions were observed when either NADH (Fig. 2c) or DCIP (Fig. 2d) was present as the variable substrate. The results of the product

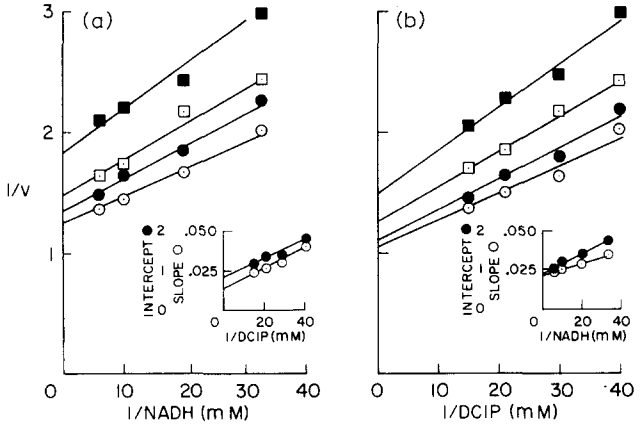

Fig. 1. Initial velocity patterns for NADH dehydrogenase. Reciprocal velocities are plotted as a funtion of NADH (a) or DCIP (b) as the varied substrates at various fixed concentrations of DCIP (a) or NADH (b). The insets represent secondary plots of intercepts and slopes from the related primary plots. Velocity units are changes in absorbance at 600 nm/min.

TABLE I

THE APPARENT KINETIC CONSTANTS FOR NADH DEHYDROGENASE IN 2.5 M NaCl

The kinetic constants were obtained from the initial velocity data in Fig. 1 and were analyzed using a computer program for sequential kinetics. The units for V are μmol DCIP reduced/min per mg protein. Values are means ± S.E.

Parameter	Constants
V	633 ± 15.0
K_a (μM)	25.4 ± 1.8
K_b (μM)	30.0 ± 1.4
K_{ia} (μM)	9.8 ± 2.6

inhibition experiments suggested that an ordered BiBi mechanism most adequately described the behavior of the NADH dehydrogenase from strain Ar-1 since it is the only one which predicts one competitive and three non-competitive inhibition patterns [11]. Assuming that the kinetic mechanism was ordered BiBi, it was possible to associate the various kinetic constants with their substrates, since NADH must add first ($K_a = K_{NADH}$, $K_{ia} = K_{iNADH}$) and DCIP second ($K_b = K_{DCIP}$).

The effect of cations

The effect of NaCl on the kinetic parameters was investigated at several salt concentrations. The results, summarized in Table II, indicated that the maximum velocity (V) was not significantly affected by the concentration of NaCl. Of the kinetic constants measured, only K_{DCIP} was affected by the salt

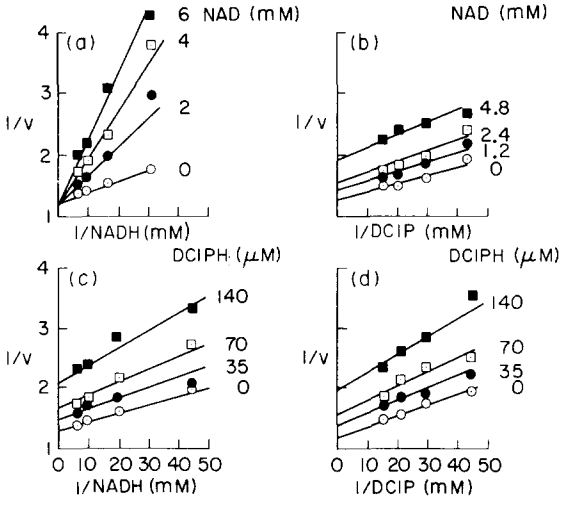

Fig. 2. Inhibition patterns for NADH dehydrogenase. (a) Product inhibition by NAD with NADH as the varied substrate; DCIP concentration was 70 μM. (b) Product inhibition by NAD with DCIP as the varied substrate; NADH concentration was 150 μM. (c) Product inhibition by DCIPH with NADH as the varied substrate; DCIP was 70 μM. (d) Product inhibition by DCIPH with DCIP as the varied substrate; NADH was 150 μM. Velocity units are changes in absorbance at 600 nm/min.

TABLE II

THE APPARENT KINETIC CONSTANTS FOR NADH DEHYDROGENASE AT VARIOUS CONCENTRATIONS OF NaCl

The kinetic constants were obtained from initial velocity data and were analyzed using a computer program for sequential kinetics. The units for V are μmol DCIP reduced/min per mg protein. Values are means ± S.E.

Parameter	NaCl concentration			
	100 mM	250 mM	500 mM	1.0 M
V	723 ± 37.0	635 ± 51.0	616 ± 34.0	579 ± 51.0
K_{DCIP} (μM)	79.5 ± 6.5	76.8 ± 10.0	34.6 ± 8.2	41.6 ± 8.4
K_{NADH} (μM)	27.8 ± 4.6	19.1 ± 5.9	18.1 ± 5.0	21.5 ± 8.1
K_{iNADH} (μM)	12.8 ± 2.2	10.0 ± 3.3	13.4 ± 4.4	11.4 ± 7.9

concentration. Thus, in the presence of 2.5 M NaCl, the value observed was 28 μM, whereas in the presence of 100 mM NaCl the value for K_{DCIP} was 79 μM. Neither the apparent Michaelis constant nor the dissociation constant for NADH was affected by the NaCl concentration.

The nature of the cation had no significant effect on any of these parameters when the ions were present at a concentration of 2.5 M (Table III). When the monovalent cations were assayed at low concentrations (100 mM), the only parameter affected was K_{DCIP}. In all cases the values of K_{DCIP} were indistinguishable from those observed in the presence of 100 mM NaCl.

The effect of anions

Four anions, added as the sodium salts, were examined for their effects on NADH dehydrogenase activity. As shown in Fig. 3, sulfate, chloride and nitrate stimulated enzyme activity; the extent of stimulation was greatest with sulfate and least with nitrate. The results with thiocyanate were ambiguous since it inhibited NADH oxidation at all concentrations tested. If, like other NADH dehydrogenases [12—14], the NADH dehydrogenase from strain AR-1 contains iron, then it is not clear whether the inhibition produced by thiocyanate is a consequence of ion chelation or an extreme extension of the anion effect.

TABLE III

THE APPARENT KINETIC CONSTANTS FOR NADH DEHYDROGENASE IN THE PRESENCE OF VARIOUS CATIONS

The kinetic constants were obtained from initial velocity data and were analyzed using a computer program for sequential kinetics. The units for V are μmol DCIP reduced/min per mg protein. Values are means ± S.E.

Cation	Concentration	V	K_{NADH} (μM)	K_{DCIP} (μM)	K_{iNADH} (μM)
Li^+	2.5 M	561 ± 61.0	19.6 ± 9.2	27.2 ± 7.8	18.2 ± 6.1
K^+	2.5 M	606 ± 17.0	17.9 ± 1.9	16.5 ± 1.4	12.0 ± 1.8
Cs^+	2.5 M	656 ± 33.0	16.5 ± 1.4	20.4 ± 3.1	10.3 ± 2.6
Li^+	100 mM	733 ± 117	35.2 ± 14.8	77.9 ± 19.9	8.9 ± 2.9
K^+	100 mM	617 ± 50.0	25.5 ± 6.6	75.5 ± 16.4	11.8 ± 3.4

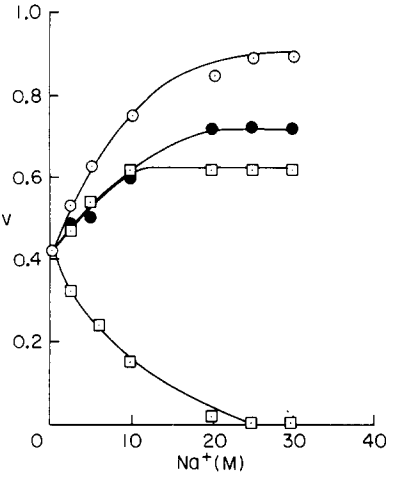

Fig. 3. The effect of anions on NADH dehydrogenase activity. Enzyme activity was determined in standard reaction mixtures in which the salt concentration was varied as indicated. The anions were added as the sodium salts. In the absence of added NaCl, the NaCl concentration was 30 mM, due to carryover with the enzyme. The velocity units are changes in absorbance at 600 nm/min. ⊙, Na_2SO_4; ●, NaCl; □, $NaNO_3$; ■, NaCNS.

In order to ascertain whether the anion effect was a consequence of enzyme activation, or due to an effect on some kinetic parameter, a series of experiments were carried out at various anion concentrations in which the concentration of both substrates was varied. The results of such experiments are summarized in Table IV. When the assays were carried out in the presence of 2.5 M Na^+, the only parameters affected were K_{NADH} and K_{iNADH}, with the values being least in the presence of sulfate, and greatest in the presence of nitrate. No significant differences were observed in the values for V or K_{DCIP}. When the assays were carried out in the presence of 100 mM Na^+, there was no effect on V. K_{NADH} exhibited a less pronounced response, although the value for sulfate was still significantly lower than those for chloride and nitrate. Since the assays were carried out in the presence of low cation concentrations, the increased values in K_{DCIP} were not unexpected.

TABLE IV

THE APPARENT KINETIC CONSTANTS FOR NADH DEHYDROGENASE IN THE PRESENCE OF VARIOUS ANIONS

The kinetic constants were obtained from initial velocity data and were analyzed using a computer program for sequential kinetics. The units for V are μmol DCIP reduced/min per mg protein. Values are means ± S.E.

Parameter	$[Na^+]$ = 100 mM		$[Na^+]$ = 2.5 M	
	SO_4^{2-}	NO_3^-	SO_4^{2-}	NO_3^-
V	570 ± 30.0	633 ± 52.0	706 ± 13.0	612 ± 26.0
K_{NADH} (μM)	12.3 ± 3.5	32.5 ± 5.9	10.2 ± 1.1	50.1 ± 5.4
K_{DCIP} (μM)	72.3 ± 5.2	61.2 ± 5.9	22.8 ± 1.2	31.3 ± 2.9
K_{iNADH} (μM)	3.5 ± 3.3	14.2 ± 5.3	2.7 ± 1.8	24.5 ± 4.3

Discussion

There have been few kinetic studies with enzymes obtained from extremely halophilic bacteria. McParland [6] described the effect of salt concentration on Mn^{2+}-activated alkaline phosphatase obtained from *H. salinarium*, strain 1. V was observed to increase with increasing concentrations of NaCl, with maximum activity observed in 1 M NaCl. At higher concentrations of NaCl, V decreased so that in the presence of 4 M NaCl, the value was about 60% that in 1 M NaCl. The apparent Michaelis constant for Mn^{2+} was also shown to increase with increasing salt concentration, while the apparent Michaelis constant for the substrate, *p*-nitrophenyl phosphate, decreased. These results were interpreted to suggest that salt interacted with the enzyme in two ways: by shielding electrostatic interactions which distorted the active site, and by affecting the electrostatic interactions between Mn^{2+} and its binding site. In the case of the isocitric dehydrogenase obtained from a colorless strain of *H. salinarium* [4], V was affected by the salt concentration, passing through a maximum value at about 1 M salt. Both apparent Michaelis constants were also affected by the salt concentration; however, each increased with increasing salt concentration. In addition, there appeared to be a qualitative ion effect since the enzyme exhibited greater activity in the presence of NaCl than in KCl, while the apparent Michaelis constants were significantly less in the presence of KCl rather in NaCl. It was proposed that the isocitric dehydrogenase existed in two forms. In the presence of high concentrations of NaCl (but not KCl) the predominant form of the enzyme was that for which the binding of substrates was inhibited by NaCl.

The NADH dehydrogenase from strain AR-1 exhibited considerable activity in the absence of added NaCl. Increasing the concentration of NaCl enhanced enzyme activity, with a maximum observed at about 2 M NaCl. A similar enhancement was observed when NaCl was replaced with LiCl, KCl, or CsCl (Hochstein, L.I., unpublished data). If the maximum rather than the observed velocities were determined, the dependency of enzyme activity on the NaCl concentration disappeared. The effect of NaCl concentration was to increase the apparent Michaelis constant for DCIP (K_{DCIP}). Replacing Na^+ with other monovalent cations did not affect any of the kinetic parameters of the enzyme when the assays were carried out in the presence of 2.5 M monovalent cation. At lower monovalent cation concentrations, again the only kinetic parameter affected was K_{DCIP}. Thus, it is possible to account for the dependency of V on salt concentration as probably a reflection of the dependency of enzyme activity on the dye concentration, since the amount of DCIP employed in the standard reaction mixture was only 2.7 K_{DCIP}.

Since DCIP is an anionic dye, a possible interpretation of the data is that the dye-binding site on the enzyme is cationic and surrounded by a region relatively rich in negative charges. This assumption is not too unreasonable in view of the observation that bulk proteins from extremely halophilic bacteria [15] and an alkaline phosphatase from *H. salinarium* [6] are characterized by the presence of a relative excess of acidic amino acids. This interpretation then suggests that cations act by neutralizing negative charges near the dye-binding site and allow the approach of the anionic dye. In solutions of low ionic

strength, such an approach would be hindered by the presence of identical charges which would repel the approach of the dye. This interpretation was also consistent with the observation that K_{DCIP} and V were independent of the nature of either the cation or anion.

An entirely different response was observed when NADH dehydrogenase activity was determined in the presence of different anions. The observed velocities were dependent on the nature of the anion, with the greatest activity observed in the presence of sulfate, intermediate activity observed in the presence of chloride, and least activity observed in the presence of nitrate.

When V was determined in the presence of these anions, the dependency of enzyme activity on the nature of the anion disappeared. Instead, the only parameter affected was K_{NADH} and K_{iNADH}. The magnitude of the constants was greatest in the presence of nitrate and least in the presence of sulfate. Since the magnitude of K_{NADH} and K_{iNADH} was independent of the concentration of the anion, and since earlier studies demonstrated that NADH was probably bound to the enzyme via the 6-amino nitrogen of the adenosine moiety of NADH [1], it seemed unlikely that the anions functioned by neutralizing locally high concentrations of positive charges so as to allow the approach of the positively charged NADH to its binding site. This hypothesis seems particularly unattractive in view of the acidic nature characteristic of halophilic proteins [14].

Recent data have pointed to the importance of hydrophobic forces for stabilizing halophilic enzymes and the role of certain anions in destabilizing such interactions [16]. Assuming that a similar situation exists in the case of the NADH dehydrogenase from AR-1, and that the various conformational states are catalytically active, then the NADH-binding site may be contained within a region of the enzyme whose accessibility to bulk solvent, and hence substrate, is a function of the conformational state of the enzyme. Those ions which favor the most compact form of the enzyme may produce a conformation in which the NADH-binding site has maximal access to the bulk solvent, while those ions which favor the least compact form of the enzyme also result in removing the binding site from ready access to the solvent. These effects are, in turn, reflected in the K_{NADH} and K_{iNADH} values and suggest that, as in the case of the cation effect on enzyme activity, the stimulation of activity due to anions reflects the inability to saturate the enzyme with substrate in the presence of certain anions.

At the present time it is not possible to explain the apparent discrepancy in the behavior of the NADH dehydrogenases from strain AR-1 *H. cutirubrum* [8], and *H. salinarium* [7]. In a further attempt to understand these differences, we have recently undertaken a study of the NADH dehydrogenases from a number of extremely halophilic bacteria. Preliminary data indicate that those enzymes which are solubilized from the membrane in 2 M NaCl and stable at this salt concentration behave in a manner similar to the enzyme obtained from AR-1, whereas those which are not apparently solubilized but remain membrane bound (the NADH dehydrogenases from *H. salinarium* and *H. cutirubrum*), do not. It is interesting that all of the strains that possess a membrane-bound NADH dehydrogenase capable of being solubilized in 2 M salt also utilize one or more carbohydrates (Hochstein, L.I. and Tomlinson, G., unpublished data).

Acknowledgement

The author wishes to thank Ms B.P. Dalton for her technical assistance.

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