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PURIFICATION AND PROPERTIES OF ALANINE DEHYDROGENASE FROM *HALOBACTERIUM SALINARIUM*

DIAMANDIS KERADJOPOULOS and AUGUST W. HOLLDORF

Institut für Physiologische Chemie der Ruhr-Universität Bochum, D-4630 Bochum 1 (F.R.G.)

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Summary

1. L-Alanine dehydrogenase (L-alanine:NAD^+ oxidoreductase (deaminating), EC 1.4.1.1) was purified about 500-fold from *Halobacterium salinarium*.

2. The enzyme appears to be homogeneous in polyacrylamide gel electrophoresis. The apparent molecular weight is about 60 000.

3. Activity and stability of the enzyme are largely affected by different salts. Full activity of the NADH-dependent reductive amination of pyruvate occurs at 4.3 M NaCl. This activation can be achieved also by KCl and several other salts instead of NaCl.

4. The NAD^+-dependent oxidative deamination of L-alanine occurs only in the presence of high concentrations of KCl. This reaction is not stimulated by NaCl. The K_m values for the substrates NADH, pyruvate and NH_4^+ are also salt dependent.

5. The thermal stability of the enzyme is considerably higher in the presence of high concentrations of NaCl than in the presence of KCl.

6. The enzyme is completely inactivated by the removal of salt. Full reactivation is achieved by addition of salt in the presence of 2-mercaptoethanol. Inactivation proceeds about ten times faster than reactivation. The inactivation after the withdrawal of salt and the reactivation following the readdition of salt show a characteristic hysteresis loop.

Introduction

Most enzymes from extreme halophilic bacteria are active and stable only in the presence of high salt concentrations [1—3]. A comparative study on eight enzymes from three different species of the genus Halobacterium has indicated a remarkable degree of thermophilicity of these enzymes [4]. Chemical and physical studies and especially the comparison of the primary structures of the

same enzyme from mesophilic and from thermophilic organisms have indicated, that thermophilicity is determined by very limited regions of the polypeptide sequences, in which hydrophobic interactions are of special importance [5,6]. The parallelism between halophilicity and thermophilicity raises the question, whether both phenomena have a common molecular basis.

A prerequisite for comparative studies on the same enzyme from mesophilic organisms, on the one hand, and thermophilic or halophilic organisms, on the other hand, is the availability of pure enzymes from both sources. This prerequisite is fulfilled in the case of numerous thermophilic bacteria, but rarely in the case of halophilic bacteria. The purification of enzymes from these organisms is severely impaired by the high salt concentrations, which are required for activity and stability of most halophilic enzymes. So far only malate dehydrogenase (EC 1.1.1.37) from several halobacteria [7,8], isocitrate dehydrogenase (EC 1.1.1.42) from *Halobacterium cutirubrum* (9), a NADH dehydrogenase from an extremely halophilic bacterium (strain AR-1) [10], DNA-dependent RNA polymerase (EC 2.7.7.6) from *Halobacterium halobium* [11], the same enzyme and RNA-dependent RNA polymerase from *Halobacterium cutirubrum* [12,13] and glutamate dehydrogenase (EC 1.4.1.2) from a halobacteria species [14] have been obtained in pure or nearly pure states. In the present paper the purification and properties of alanine dehydrogenase (L-alanine:NAD^+ oxidoreductase (deaminating), EC 1.4.1.1) from *Halobacterium salinarium* are described.

Materials and Methods

Growth of cells. H. salinarium (ATCC 19 700) was grown aerobically at 37° C for 70—72 h as previously described [15].

Assay of alanine dehydrogenase. Unless otherwise stated, the activity of the enzyme was measured by recording the decrease of A_{366} nm during the NADH and NH_4^+-dependent reductive amination of pyruvate in an Eppendorf photometer at 37° C. The assay mixture contained 3.5 M NaCl; 40 mM Tris-HCl buffer (pH 9.0); 0.4 mM NADH; 14 mM sodium pyruvate; 150 mM NH_4Cl and enzyme in a final volume of 3.0 ml. The reaction was started by the addition of NH_4Cl.

The reverse reaction (NAD^+-dependent deamination of alanine, which proceeds about ten times slower) was determined under similar conditions. The assay mixture for this reaction contained 3.5 M KCl, 40 mM Tris-HCl buffer (pH 9.0), 10 mM NAD^+ and 40 mM L-alanine. The reaction was started by the addition of L-alanine. In both assays the initial rate of activity was linear with time over at least 10 min.

The specific activity in units (U) was defined as the amount of enzyme catalyzing either the oxidation or the reduction of one μmol of NADH or NAD^+, respectively, under the above assay conditions. Protein was determined by the method of Lowry et al. [16] with bovine serum albumin as standard.

Molecular weight determinations. SDS-polyacrylamide gel electrophoresis were carried out according to Weber and Osborn [18]; by gel filtration according to Andrews [19]. Isoelectric focusing of the purified enzyme was

performed by the method of Wrigley [20] as modified by Nees et al. [21].

Chemicals and enzymes. NAD^+, NADH, $NADP^+$, chymotrypsinogen, lactate dehydrogenase, ovalbumin, bovine serum albumin, and glycogen phosphorylase were obtained from Boehringer, Mannheim (F.R.G.). Yeast extracts, Bacto-tryptone and Bacto-agar were purchased from Difco-Laboratories, Detroit (U.S.A.). Ampholine was from LKB, Uppsala (Sweden). Alcoa A 305, DEAE-cellulose, Coomassie brilliant blue (R 250), 2-mercaptoethanol were from Serva Fine Chemicals, Heidelberg (F.R.G.) Agarose-hexane-nicotinamide adenine dinucleotide phosphate, AMP-agarose and ADP-agarose were products of PL-Biochemical Inc., Milwaukee, WI, U.S.A.

Results and Discussion

Purification of alanine dehydrogenase

All procedures, with the exception of step 1 and the centrifugations, which were done at 4°C, were carried out at room temperature. The following buffer solutions were used: buffer A: 50 mM Tris-HCl (pH 7.8), 4.3 M NaCl; buffer B: 50 mM potassium phosphate (pH 7.8), 3.1 M $(NH_4)_2SO_4$, 0.86 M NaCl; buffer C: 50 mM potassium phosphate (pH 7.8), 4.3 M NaCl; buffer D: 50 mM potassium phosphate (pH 7.8), 1.5 M Na_2SO_4. In addition, all buffers contained 10 mM 2-mercaptoethanol.

Preparation of cell-free extracts. 30 g cells (wet wt.) were suspended in 100 ml buffer A, and sonicated three times for 5 min. During the sonication periods and during intervals (10 min) the cells were kept in ice-bath. Centrifugation was carried out at 16 000 × *g* for 30 min. The resulting crude extract was diluted with the same buffer to a protein concentration of about 10 mg/ml.

Fractionation with $(NH_4)_2SO_4$ and batch treatment with Sepharose 4B. The crude extract was slowly brought to 80% saturation with $(NH_4)_2SO_4$ by addition of a saturated solution of $(NH_4)_2SO_4$ (767 g/l at 4°C; adjusted to pH 7.8 by concentrated ammonia solution). After addition of the $(NH_4)_2SO_4$, the suspension was stirred for 15 min and centrifuged for 60 min at 16 000 × *g*. The pellet was discarded. The supernatant was stirred for 15 min with 150 g (wet wt.) of Sepharose 4B, which was previously three times equilibrated with 300 ml of buffer B. The enzyme was completely adsorbed to the Sepharose 4B. The Sepharose 4B was separated by filtration on a Buchner funnel. The filtrate contained most of the colored purple membrane and inactive protein. The enzyme was eluted from the Sepharose 4B by fine repeated washings each with 300 ml of buffer C. The combined eluates were concentrated eightfold by filtration with an Amicon filter PM10.

Sepharose 4B column chromatography. The concentrated solution of the preceding step was dialyzed for 16 h against buffer B. A small precipitate was removed by centrifugation at 40 000 × *g* for 60 min. The supernatant was applied to a column (5 × 30 cm) of Sepharose 4B, previously equilibrated with 2 l of buffer B. After application of the enzyme the column was washed with further 1.5 l of the same buffer. Elution of the enzyme was carried out with a linear gradient of decreasing concentrations of $(NH_4)_2SO_4$ and increasing concentrations of NaCl. The mixing vessel contained 1 l. The reservoir contained the same volume of buffer C (10-ml fractions, flow rate 120 ml/h). The active

fractions (fractions 100—180) were pooled and concentrated eight fold as in the preceding step.

DEAE-cellulose chromatography. A column (2.5 × 30 cm) of 100 g DEAE-cellulose (Whatman D 52) was equilibrated with 600 ml of buffer B was prepared. The concentrated enzyme of step 3 was dialyzed for 16 h against the same buffer and applied to the column and washed with further 250 ml buffer. Elution of the enzyme was performed by the same gradient as in step 3 (10-ml fractions; flow rate 70 ml/h). The active fractions (fractions 45—80) were pooled and concentrated by filtration with a Millipore filter type PTGS (molecular weight limit 10 000). Application of an Amicon filter with the same specifications as used in the previous steps was unsuccessful.

Hydroxyapatite chromatography. The preparation of step 4 was applied to a column (50 × 2.5 cm) of hydroxyapatite [22], which was equilibrated with 500 ml of buffer C. The enzyme was eluted with a linear gradient of 50—200 mM potassium phosphate (pH 7.8), containing 4.3 M NaCl. Fractions of 5.5 ml were collected with a flow rate of 22 ml/h. The enzyme appeared almost quantitatively in fractions with potassium phosphate buffer concentrations between 0.144 and 0.177 M. The active fractions were pooled and concentrated as in step 4.

Hydrophobic chromatography on butyl-Sepharose. A column of butyl Sepharose [23] (1.0 × 11.0 cm, capacity 32.2 μmol/ml) was equilibrated with 100 ml of buffer D. The preparation of step 5 was dialyzed for 16 h against the same buffer. A small precipitate was removed by centrifugation at 30 000 × *g* for 30 min. The supernatant was applied to the column and subsequently washed with 50 ml of the same buffer. Elution was done by a gradient with decreasing concentrations of Na_2SO_4 and increasing concentrations of NaCl. The mixing vessel contained 100 ml of buffer D. The reservoir contained 100 ml of buffer C (5.5-ml fractions; flow rate 26 ml/h). Active fractions (fractions 12—22) were pooled and concentrated as in step 4.

Chromatography on NADP-agarose. A column (1.0 × 6.0 cm) of agarose-hexane-nicotinamide adenine dinucleotide phosphate (AGNADP™; type 3) was equilibrated with 50 ml of buffer D. The enzyme solution of the preceding step was dialyzed for 16 h against the same buffer and applied to the column. Subsequently the column was washed with 50 ml of the same buffer (5.5-ml fractions; flow rate 25 ml/h). The active enzyme appeared in fractions 2—6. It should be noted that the enzyme is not specifically bound to the NADP-agarose but only eluted with buffer, not by a specific ligand. However, this procedure led to a two-fold increase of the specific activity by removal of contaminating proteins. This effect was not achieved by AMP-agarose or ADP-agarose. Neither was the enzyme bound by one of these gels. The active fractions were pooled and concentrated as in step 4. The enzyme was stable for several weeks when stored at 4°C. The entire purification procedure is summarized in Table I. All purification steps were performed under conditions which prevented inactivation of the enzyme by low salt concentrations. A similar pure enzyme was obtained by a second purification procedure with heat treatment, acetic acid precipitation at pH 4.4—3.8, fractionation with $(NH_4)_2SO_4$, chromatography on hydroxyapatite and DEAE-cellulose. In the course of this procedure several cycles of inactivation by removal of salt and reactivation by dialysis against

TABLE I

PURIFICATION OF ALANINE DEHYDROGENASE FROM *H. SALINARIUM*

Starting material 50 g wet cells.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
1. Crude extract	100	3500	1180	0.34	1	100
2. Fractionation with $(NH_4)_2SO_4$ and batch treatment with Sepharose 4B	200	850	1140	1.34	4	96
3. Sepharose 4B column chromatography	150	280	993	3.55	10	84
4. DEAE-cellulose chromatography	65	110	756	6.9	20	64
5. Hydroxyapatite chromatography	10	22	622	30	89	56
6. Hydrophobic chromatography on butyl-Sepharose	10	8.5	629	74	218	53
7. Chromatography on NADP-agarose	10	3.0	535	178	524	45

solutions with high salt concentrations had to be inserted. The yield was about 50% lower [24]. A comparison of kinetic and physical properties of the pure enzyme, obtained by the two purification procedures revealed no important differences.

The partial purification of the same enzyme from the closely related organism *H. cutirubrum* on the small scale and several properties of this enzyme have recently been reported by Kim and Fitt [25].

Properties of the enzyme

Molecular data. In polyacrylamide gel electrophoresis the enzyme moves as a sharp single band. The molecular weight of the pure protein was determined by gel filtration under non-denaturating conditions and by SDS-polyacrylamide gel electrophoresis. The first method gives a value of 60 000, the second method a value of 58 000. The similarity of the values obtained by the two different methods makes it likely, that the enzyme consists of only one polypeptide chain. This has to be confirmed by end group determinations and by sedimentation studies. Kim and Fitt [25] found a molecular weight of 72 500 for the alanine dehydrogenase of the closely related organism *H. cutirubrum*. The alanine dehydrogenase from *Bacillus cereus* has a molecular weight of 248 000 [26], the enzyme from *Bacillus subtilis* has a molecular weight of 228 000. The latter enzyme consists of six identical or nearly identical subunits with a molecular weight of 38 000 [27]. Thus the alanine dehydrogenases of non-halophilic bacteria have a considerably higher molecular weight and a more complex structure than the enzymes from halophilic bacteria. It seems to be of interest to compare the molecular weight and other properties of the halophilic enzyme to the thermophilic alanine dehydrogenase, described by Epstein and Grossowicz [28]. The isoelectric point determined by isoelectric focusing is at

pH 6.3. The protein has an ultraviolet spectrum with a symmetric maximum at 280 nm. The ratio E_{280}/E_{260} is 1.5. The pH optimum for the catalyzed reaction in both directions is about 9.0. NAD^+ and NADH cannot be replaced by $NADP^+$ resp. NADPH as coenzymes. L-Alanine as a substrate can be replaced by L-α-aminobutyric acid. The relative reaction rates for the two substrates (in both cases 40 mM) are 100 for L-alanine and 170 for L-α-aminobutyric acid. The corresponding D-amino acids, D- and L-α-aminoisobutyric acid, L-glycine, L-leucine, L-isoleucine, L-lysine, L-methionine, L-threonine, L-valine, and L-serine are not deaminated. In the reductive amination reaction pyruvate (14 mM) can be replaced by α-oxobutyric acid, which is 1.2 faster aminated than pyruvate. Less than 10% activity is observed with oxaloacetic acid, hydroxypyruvate, or glyoxylic acid as substrates. These values are almost identical with the data reported by Kim and Fitt [25] for alanine dehydrogenase from *H. cutirubrum*.

Kinetic data. The apparent K_m values for the substrates of the reaction in both directions in the presence of several salts were determined by the methods of Lineweaver and Burk [29] from the initial reaction rates. The results are summarized in Table II. For the reductive amination of pyruvate the K_m values in the presence of 3.5 M NaCl or KCl are almost identical. In the presence of the same concentration of $NaNO_3$ the apparent K_m values are about ten times higher. The K_m values are not influenced by the increase of the salt concentration over the range from 1.0 M to 3.5 M NaCl or KCl neither by changes of the temperature between 37°C and the respective optima of temperature (see below!).

The effect of different anions and cations on the enzyme activity. The enzyme is active only in the presence of high concentrations of NaCl or other salts. In order to elucidate the effect of different ions further, the standard assays for the oxidative amination of L-alanine (forward reaction) and for the reductive amination of pyruvate (back reaction) were performed in the presence of increasing concentrations of different cations and anions (Figs. 1A, B and 2).

The increase of activity in the back reaction with increasing concentrations of NaCl, KCl and CsCl is almost identical (Fig. 1A). In all three cases the linear plot of enzyme activity against salt concentrations gives sigmoidal curves. At lower concentrations the stimulatory effect of RbCl is remarkably higher than the effect of identical concentrations of the other salts. Due to their limited

TABLE II

APPARENT K_m VALUES OF ALANINE DEHYDROGENASE IN THE PRESENCE OF DIFFERENT SALTS

Salt	Reductive animation of pyruvate			Oxidative deamination of L-alanine	
	NADH (M)	Pyruvate (M)	NH_4Cl (M)	NAD^+ (M)	L-Alanine (M)
3.5 M NaCl	$7.2 \cdot 10^{-5}$	$9.0 \cdot 10^{-4}$	$6.0 \cdot 10^{-1}$	no reaction detectable	
3.5 M KCl	$3.1 \cdot 10^{-5}$	$9.5 \cdot 10^{-4}$	$5.0 \cdot 10^{-1}$	$1.5 \cdot 10^{-3}$	$5.3 \cdot 10^{-3}$
3.5 M $NaNO_3$	$5.3 \cdot 10^{-4}$	$4.7 \cdot 10^{-3}$	1.4	no reaction detectable	

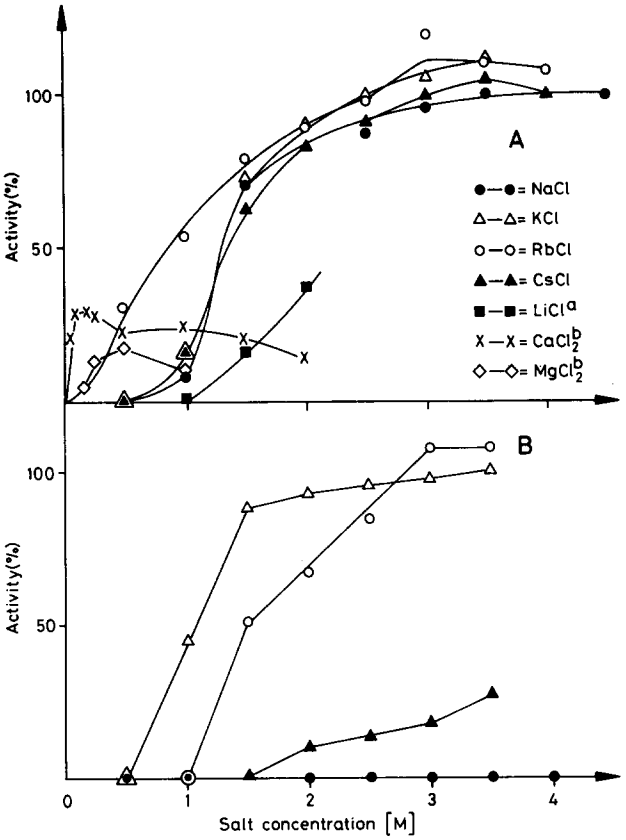

Fig. 1. The effect of monovalent and divalent cations on the reductive amination of pyruvate (A) and on the oxidative amination of L-alanine by alanine dehydrogenase (B). In the absence of other cations the reductive amination of pyruvate is stimulated to some extent by ammonium ions, which are required as a substrate. This 'basal' activity was subtracted from the values given in Fig. 2. (a) Protein is precipitated at higher concentrations; (b) insoluble at higher concentrations.

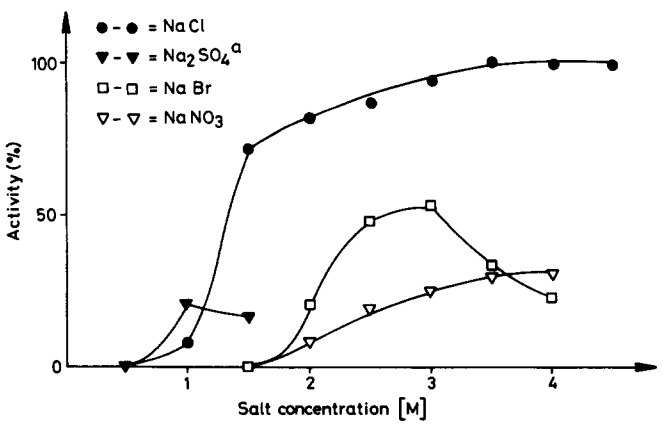

Fig. 2. The effect of different anions on the reductive amination of pyruvate by alanine dehydrogenase. (a) Not soluble at higher concentrations.

solubility the effects of LiCl and of earth alkali chlorides can only be studied in the lower concentration ranges. These salts (Fig. 1A) stimulate the enzyme activity much less than for the above-mentioned alkali chlorides. In the forward reaction the enzyme is stimulated by KCl and RbCl. In the presence of NaCl or LiCl and of several earth alkali chlorides no activity can be detected at all. CsCl has a low stimulatory effect. At present there is no plausible explanation for the different effects of the salts on the reaction in both directions. As the cations also the anions greatly differ in their effects on the enzyme activity. The largest effect is caused by chloride (Fig. 2). Much smaller effects occur in the presence of bromide, nitrate, or sulfate. Replacement of sodium chloride by the corresponding fluorides, iodides, perchlorates or thiocyanates abolishes the enzyme activity completely.

The effect of temperature on the enzyme activity in the presence of different salts. The enzyme shows a remarkable grade of thermophilicity. The highest reaction rates are observed in the presence of 3.5 M KCl at 70°C. In the presence of identical concentrations of NaCl or $NaNO_3$, the optimum of temperature is 5°C and 25°C, respectively, lower. Beyond the optima of temperatures sharp decreases of the reaction rates are caused by thermal denaturation of the protein (see below). The thermal stability was determined (Fig. 3) in the presence of 3.5 M KCl for both the reductive amination of pyruvate and the oxidative deamination of alanine. As the activity of oxidative deamination in the presence of NaCl is extremely low, the thermal stability in the presence of NaCl (3.5 M) was only determined for the reductive amination of pyruvate (Fig. 3). The stability of the enzyme is considerably lower in the presence of KCl (Fig. 3A and B) than in the presence of NaCl. However, in the presence of both salts thermal denaturation starts at temperatures below the optima for catalytic activity. A shift of the thermal inactivation curves to higher temperatures is caused by the substrates (data not shown in Fig. 3).

Inactivation and reactivation of alanine dehydrogenase by changes of the salt concentration. The active enzyme is completely inactivated by dialysis against salt-free Tris-HCl buffer (50 mM; pH 7.8) containing 10 mM 2-mercaptoethanol at 4°C within 120 min. The inactivation occurs nearly parallel to the decrease of the salt concentration. The inactivation of alanine dehydrogenase was not prevented by the presence of its substrates and coenzymes. Complete reactivation of the inactivated enzyme is achieved by dialysis against the same buffer containing high salt concentrations. The reactivation, however, proceeds one magnitude slower than the inactivation. The pH optimum for the reactivation is 7.8, the optimal temperature is 30°C. Thus the optima of pH and temperature for reactivation differ markedly from the corresponding values for catalytic activity. As previously reported thiol groups are essential for inactivation and reactivation [15,24]. In Fig. 4 the dependence of inactivation and reactivation on different salt concentrations is shown. Dialysis of the active enzyme against decreasing concentrations of NaCl for 14 h results in a progressive decrease of activity beginning at a concentration of 2.0 M. Complete inactivation is achieved at 0.5 M NaCl. Under identical conditions reactivation of completely inactivated enzyme is not observed at NaCl concentrations below 2.5 M. Full activity is reached at 4.0 M NaCl. Thus the curves of inactivation and reactivation show a characteristic hysteresis loop [8]. The same enzyme sample can be

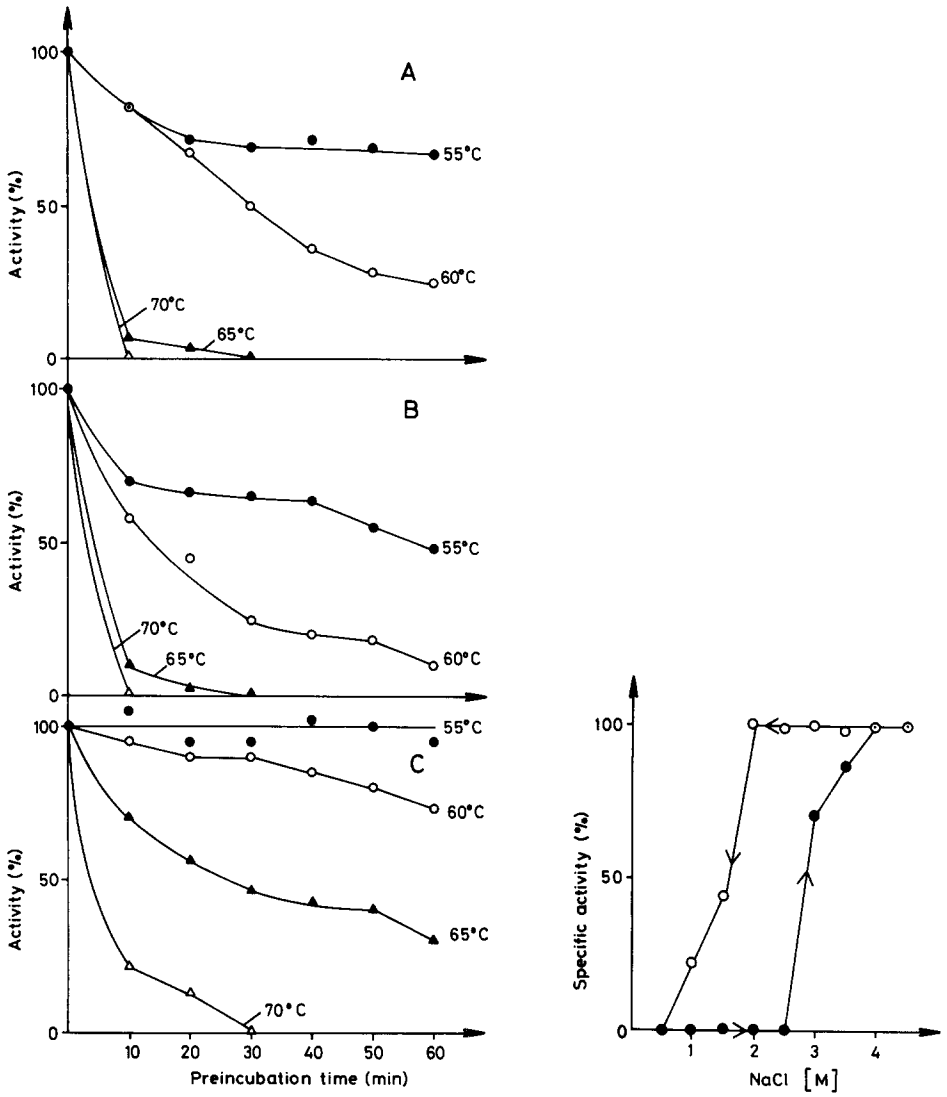

Fig. 3. Thermal stability of alanine dehydrogenase in the presence of NaCl and KCl. (A) Oxidative deamination of alanine in the presence of 3.5 M KCl. (B) Reductive amination of pyruvate in the presence of 3.5 M KCl. (C) Reductive amination in the presence of 3.5 M NaCl. Active enzyme was preincubated at the different temperatures for the times as indicated. Incubation was stopped by chilling in an ice-bath. The activity was determined in the standard assays.

Fig. 4. Subsequent inactivation and reactivation of alanine dehydrogenase by withdrawal and addition of NaCl.

subjected to several cycles of inactivation and reactivation without any loss of activity. The changes of protein conformation in the course of these transitions are at present studied.

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