Partial Purification and Properties of *Halobacterium cutirubrum* L-Alanine Dehydrogenase

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(Received 5 July 1976)

1. *Halobacterium cutirubrum* L-alanine dehydrogenase was purified approx. 100-fold. 2. It has a mol.wt. of 72 500, about one-third that of two well-studied alanine dehydrogenases from non-halophiles. 3. The activity of the enzyme increases with temperature up to 70°C, but the protein itself is not thermostable. 4. In the reductive amination reaction, the enzyme is fully active in the presence of high concentrations of K⁺, Na⁺ or NH₄⁺ and partially active with Cs⁺ or Li⁺, but for oxidative deamination it has an absolute requirement for K⁺.

L-Alanine dehydrogenase [L-alanine-NAD⁺ oxidoreductase (deaminating), EC 1.4.1.1] was first isolated from *Bacillus subtilis* (Wiame & Piérard, 1955; Piérard & Wiame, 1960) and shown to catalyse the reversible, NAD⁺-dependent, oxidative deamination of L-alanine:

It has since been detected in many other bacteria, including *Bacillus cereus* (O'Connor & Halvorson, 1960), *Bacillus licheniformis* (Meers & Pedersen, 1972), *Desulfovibrio desulfuricans* (Germano & Anderson, 1968) and *Halobacterium salinarium* (Holmes *et al.*, 1965).

The enzyme appears to play an important role in alanine utilization by bacilli (O'Connor & Halvorson, 1959, 1960; McCowen & Pibbs, 1974), and the *B. cereus* and *B. subtilis* L-alanine dehydrogenases have been purified and studied in detail (O'Connor & Halvorson, 1961; McCormick & Halvorson, 1964; Yoshida & Freese, 1964, 1965). The 200-fold-purified *B. cereus* alanine dehydrogenase had a mol.wt. of 248 000 (McCormick & Halvorson, 1964), and the pure, crystalline *B. subtilis* enzyme had a mol.wt. of 228 000 (Yoshida & Freese, 1964) and appeared to consist of six identical subunits (Yoshida, 1965). These two enzymes had very similar enzymic properties.

The alanine dehydrogenase of *H. salinarium* has not been purified, but Keradjopoulos & Wulff (1974) have reported the interesting observation that the crude enzyme appears to have an unusually high optimum reaction temperature, and they have referred to it as thermophilic.

In the present paper, we describe the approximately 100-fold purification of *Halobacterium cutirubrum* L-alanine dehydrogenase and a study of its

molecular weight, enzymic properties and thermal stability.

Experimental

Materials

Materials were purchased from the following suppliers: standard laboratory chemicals, Fisher Scientific Co., Ottawa, Ont., Canada; Sepharose 6B, Pharmacia (Canada) Ltd., Montreal, P.Q., Canada; Schwarz-Mann protein molecular-weight standards, Picker Nuclear Engineering Co., Ottawa, Ont., Canada; cofactors, substrates and Tris (Trizma base), Sigma Chemical Co., St. Louis, MO, U.S.A.

Cell culture. *H. cutirubrum* NRC 34001 cells were grown at 37°C in the complex medium of Gochnauer & Kushner (1969), as previously described (Peterkin & Fitt, 1971).

Methods

Purification of *H. cutirubrum* L-alanine dehydrogenase. All operations were done at 0–5°C.

The bacteria were harvested after 72 h of growth by centrifugation at 27 000g_{max.} and resuspended in 4M-NaCl/10mM-Tris/HCl, pH 8.5 (3 ml/g wet wt. of cells), by slow addition of the buffer while the cells were stirred carefully with a bent glass rod. The suspension was stirred magnetically until smooth (1–2 h) and was then sonicated twice for 2 min, with a 1 min pause between successive sonications, by using a Bronwill Biosonik II ultrasonic desintegrator (large probe, setting 80). Unbroken cells and cell debris were removed by centrifugation at 46 000g_{max.} for 20 min and the supernatant was then centrifuged at 300 000g_{max.} (Beckman Ti60 rotor) for 1 h to give the crude extract (Table 1). The latter was diluted with 9 vol. of 4.2M-(NH₄)₂SO₄/10mM-imidazole/HCl

buffer, pH 6.8, to give a final $(\text{NH}_4)_2\text{SO}_4$ saturation of approx. 72%, and the precipitate, containing about 35% (w/w) of the protein of the crude extract, was removed by centrifugation at $46000g_{\text{max}}$ for 30 min and discarded. The pH of the supernatant was adjusted to 3 with conc. HCl; the resulting precipitate, containing most of the L-alanine dehydrogenase activity, was collected by centrifugation at $46000g_{\text{max}}$ for 30 min and redissolved in 4M-NaCl/10mM-Tris/HCl, pH 8.5. The solution was dialysed for 4 h against two changes of 100 vol. of the latter to give the ' $(\text{NH}_4)_2\text{SO}_4$, pH 3' fraction (Table 1). The latter was then purified further by gel filtration through a column (2.5 cm $\times$ 85 cm) of Sepharose 6B previously equilibrated with 4M-NaCl/10mM-Tris/HCl buffer, pH 8.5. A maximum of 4.5 ml could be applied to the column without affecting the resolution. Elution was performed by upward flow by using the same buffer and a flow rate of 12 ml/h (hydrostatic pressure, 10 cm). Fractions (4 ml) were collected and assayed for enzymic activity and protein concentration. The L-alanine dehydrogenase activity emerged in a well-defined peak with a total volume of approx. 50 ml. Similar results were obtained by using 3M-KCl instead of 4M-NaCl in the buffers mentioned above. (It is inconvenient to use KCl concentrations above 3.5 M because crystallization of the KCl then occurs slowly at 0–5°C.)

Enzyme assays. L-Alanine dehydrogenase activity was assayed by using a Zeiss PMQ II spectrophotometer equipped with automatic zero, cell changer, transmission-extinction converter, recorder and thermostatically controlled cell holder. The standard assays I and II measured respectively the rates of decrease in A_{340} during NH_4^+ - and pyruvate-dependent oxidation of NADH and of increase in A_{340} during L-alanine-dependent reduction of NAD^+ . The assay I incubation medium (1 ml) contained: 0.1M-Tris/HCl, pH 9; 2.4M-NaCl; 0.5M- NH_4Cl ; 10mM-pyruvate; 0.2mM-NADH; enzyme, up to 10 units. The assay II incubation medium (1 ml) contained: 0.1M-Tris/HCl, pH 9; 1.8M-KCl; 50mM-L-alanine; 3mM- NAD^+ ; enzyme, up to 10 units. Incubation was at 37°C and the reactions were initiated by the addition

of the pyruvate or L-alanine respectively to the pre-incubated mixture after a stable baseline was obtained on the recorder. The linear change in absorbance between 5 and 10 min after initiation of the reaction was used for calculation of the activity. A unit of enzyme activity was defined as that amount of enzyme catalysing the transformation of 1 nmol/min of NADH in the standard assay I conditions. The ϵ of NADH was taken to be $6.22 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Horecker & Kornberg, 1948).

Malic dehydrogenase activity was assayed in the conditions of assay I with 10mM-oxaloacetate instead of pyruvate and without NH_4^+ .

Protein assay. Protein concentration was determined spectrophotometrically by the method of Warburg & Christian (1942).

Molecular-weight determination. The molecular weight of *H. cutirubrum* L-alanine dehydrogenase was determined by analytical gel filtration on a column (2.5 cm $\times$ 91 cm) of Sepharose 6B prepared and run in 4M-KCl/10mM-Tris/HCl buffer, pH 8.5, as described for preparative chromatography (see above). The column was calibrated with the following standard proteins: human γ -globulin (mol.wt. 160000); bovine serum album (mol.wt. 67000); and ovalbumin (mol.wt. 45000). The molecular weights of the standard proteins are those supplied by Schwarz-Mann with their kit. K_{av} values were calculated from the void volume (V_0 , determined with Blue Dextran, 186 ml), the calculated bed volume (V_t , 447 ml) and the measured elution volumes (V_e) by the standard procedure (Fischer, 1969). L-Alanine dehydrogenase was located by assay I. The V_e values for the standard proteins and the enzyme were: γ -globulin, 298 ml; serum albumin, 346 ml; ovalbumin, 364 ml; *H. cutirubrum* L-alanine dehydrogenase, 340 ml.

Results and Discussion

Purification and molecular weight of *H. cutirubrum* L-alanine dehydrogenase

Table 1 summarizes a typical purification of *H. cutirubrum* L-alanine dehydrogenase by the method described in the Experimental section. The enzyme

Table 1. Purification of *H. cutirubrum* L-alanine dehydrogenase

A crude extract (110 ml) was prepared from 50 g wet wt. of bacteria and portions (0.9–1 ml) were purified as required by the method described in the Experimental section. The bulk crude extract could be stored at –20°C with little change in activity for up to 6 months. Enzymic activity was determined by assay I (reductive amination of pyruvate).

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Sp. activity (units/mg)	Yield (%)
Crude extract	0.9	4.5	7605	1690	100
$(\text{NH}_4)_2\text{SO}_4$, pH 3	4.5	1.35	5445	4033	72
Sepharose 6B					
(a) Total	48		4604		61
(b) Three best fractions	12	0.019	3192	168000	42

activity emerged from the Sepharose 6B column in a sharply defined peak that was well separated from the bulk of the protein, so that the best fractions, containing about 40% of the original activity, were purified approx. 100-fold. The method is simple, but the size of sample (about 4–5 ml) that can be applied to the gel-filtration column without a loss of resolution severely limits the amount of enzyme that can be processed at one time.

The approximate molecular weight of the enzyme was determined by analytical gel filtration through a standardized Sepharose 6B column and found to be 72 500. The mol.wts. of the L-alanine dehydrogenases of *B. cereus* (McCormick & Halvorson, 1964) and *B. subtilis* (Yoshida & Freese, 1964) have been shown to be 248 000 and 228 000 respectively, so that the *H. cutirubrum* enzyme appears to be significantly smaller than these two well-characterized L-alanine dehydrogenases, even though the difference is not so great as that between the molecular weights of certain extreme-halophile enzymes and those of their non-halophile counterparts (Louis *et al.*, 1971; Peterkin & Fitt, 1971; Fitt *et al.*, 1975; Fitt & Peterkin, 1976). Yoshida (1965) found that *B. subtilis* L-alanine dehydrogenase consists of six identical subunits, so if sufficient of the *H. cutirubrum* enzyme can be purified it will be interesting to establish if it contains more than one polypeptide chain.

The partially purified enzyme was free from the glutamate dehydrogenase activity present in the crude

extract, but was not completely separated from malate dehydrogenase (Holmes & Halvorson, 1965; Higa & Cazzulo, 1973) during the Sepharose 6B gel-filtration step of the purification procedure. However, the distribution of activities at this stage (Fig. 1) proves that the two activities are associated with different proteins, and the molecular weight of the malate dehydrogenase, estimated from the position of the peak fraction in Fig. 1, was about 100 000, significantly larger than that of the L-alanine dehydrogenase.

Enzymic properties

A detailed study of the requirements of the 100-fold-purified enzyme was made for both the reductive amination of pyruvate and the oxidative deamination of alanine.

Changes in the pH of the assay medium had a similar effect on both the forward and backward reactions, with the enzyme having a well-defined optimum around pH 9 in both cases. This is in good agreement with those previously reported for the amination reaction with the enzyme from other bacteria, but significantly lower than the values of pH 10–10.5 found for the deamination reaction by earlier workers (O'Connor & Halvorson, 1960; Yoshida & Freese, 1965; Germano & Anderson, 1968).

In common with other halophile enzymes, *H. cutirubrum* L-alanine dehydrogenase has an absolute requirement for a high ionic strength for stability and optimum activity. Dialysis of the enzyme solution against 10 mM-Tris/HCl, pH 8.5, led to a complete loss of the activity of the enzyme as measured in the standard conditions at high ionic strength. Partial re-activation of the crude enzyme occurred during prolonged re-dialysis of the salt-free solution against 4.5 M-NaCl/10 mM-Tris/HCl, pH 8.5, to give 35% of the original activity. Complete re-activation occurred if 10 mM-2-mercaptoethanol was present at all times during the two dialyses, in agreement with the results obtained by Keradjopoulos & Wulff (1974) with the crude *H. salinarium* enzyme. However, the partially purified *H. cutirubrum* enzyme was much less stable in these conditions and a maximum re-activation of 50% was obtained even in the presence of the thiol.

Fig. 2 shows that the requirement of the enzyme for NaCl (or KCl) in reductive amination was dependent on the NH_4^+ concentration. A plateau of activity was attained at any given NH_4Cl concentration when the sum of the NH_4Cl and NaCl (or KCl) concentration was approx. 1.2 M, but this plateau was displaced to progressively higher values as the NH_4Cl concentration increased (Fig. 2). Clearly, NH_4^+ is at least as effective an activator of *H. cutirubrum* L-alanine dehydrogenase as KCl or NaCl, in addition to being an essential co-substrate; the fact that during the purification (Table 1) precipitation at pH 3 can be performed with good recovery of activity in the presence of approx. 3.8 M-(NH_4)₂SO₄ suggests that NH_4^+ is also

Fig. 1. Separation of *H. cutirubrum* L-alanine dehydrogenase and malate dehydrogenase by gel chromatography

A sample of an '(NH_4)₂SO₄, pH 3' fraction from a standard purification was passed through a column (2.5 cm × 85 cm) of Sepharose 6B prepared and run as described in the Experimental section. Fractions (4 ml) were collected and assayed for L-alanine dehydrogenase (assay I) and malate dehydrogenase activities (see the Experimental section). A_{280} , ○; alanine dehydrogenase activity, ●; malate dehydrogenase activity, ○.

Fig. 2. Effect of K^+ , Na^+ and NH_4^+ concentration on the rate of reductive amination of pyruvate by *H. cutirubrum* L-alanine dehydrogenase

The rate of the reductive amination reaction was determined as described under assay I (see the Experimental section) at the indicated concentrations of KCl, NaCl and NH_4Cl . NH_4Cl concentration: ○, 0.1M; ●, 0.25M; ▲, 0.5M; ■, 0.75M.

Fig. 3. Effect of K^+ , Na^+ and NH_4^+ concentration on the rate of oxidative deamination of L-alanine by *H. cutirubrum* L-alanine dehydrogenase

The rate of the oxidative deamination reaction was measured as described under assay II (see the Experimental section) at the indicated concentrations of KCl(●), NaCl(Δ) and NH_4Cl (□).

able to stabilize the enzyme. The latter had 86 and 64% of its maximum activity in the presence of 1.5M-CsCl or -LiCl respectively, so that the requirement for a monovalent cation during reductive amination is relatively non-specific.

In contrast, the enzyme had a remarkable specificity for K^+ in the oxidative deamination reaction with L-alanine (Fig. 3) and was completely inactive with Na^+ or NH_4^+ alone. Further, Na^+ did not inhibit the reaction in the presence of K^+ and neither Cs^+ nor Li^+ could replace the latter. Although Baxter & Gibbons (1957) have described a cysteine desulphhydrase from an extreme halophile that was only 12% as active with NaCl as with KCl at their respective optimum concentrations and Cazzulo & Vidal (1972) found that the crude 'malic' enzyme of *H. cutirubrum* was inactive with NaCl and active in the presence of either KCl or NH_4Cl , to our knowledge this is the first case of an enzyme from an extreme halophile that has been shown to have different univalent cation requirements for the forward and backward reactions.

The cofactor and substrate specificities of the *H. cutirubrum* L-alanine dehydrogenase were studied in both the amination and deamination reactions. The enzyme was absolutely specific for NAD^+ or NADH, according to the direction of the reaction, and they could not be replaced by $NADP^+$ or NADPH respectively. The kinetics of alanine deamination appeared to be Michaelis-Menten with a K_m (apparent) for NAD of 0.5 mM. Similarly, the rate of pyruvate amination increased to a short plateau at 0.2–0.4 mM-NADH, but higher concentrations of this cofactor were inhibitory.

The role of NH_4^+ as an activating univalent cation in reductive amination has been discussed above. However, it is also an essential co-substrate in this reaction and it is clear from Fig. 2 that at KCl or NaCl concentrations above 1.2M the rate of amination was dependent on NH_4^+ concentration. A detailed study of this requirement was made in the presence of 1.5M-NaCl and Fig. 4 shows that the enzyme was not fully saturated with NH_4^+ even when the concentration of the latter was 3.4M. In these conditions, there was a considerable loss of NH_3 from the reaction mixture and the baseline extinctions on the recorder were high; it was also inconvenient to achieve high concentrations of both Na^+ and NH_4^+ : 0.5M- NH_4^+ was therefore used in the routine assay I.

The activity of the enzyme in either the amination or de-amination reactions was unaffected by the presence of either Mg^{2+} (5, 10 or 20mM) or EDTA (disodium salt) (1, 2 or 4mM). The effect of several other cations on the reductive amination reaction only was also studied in the conditions of assay I (see the Experimental section) at a final concentration of 10mM. Ca^{2+} had no effect and the following cations all inhibited the reaction to the extent indicated: Cd^{2+} , 36%; Co^{2+} , 60%; Cu^{2+} , 60%; Fe^{2+} and Fe^{3+} , 85%; Mn^{2+} , 60%; Ni^{2+} , 60%.

In the oxidative deamination reaction only L-alanine and L-α-aminobutyric acid were found to be

Fig. 4. Effect of NH_4^+ concentration on the rate of the reductive amination reaction in the presence of 1.5M-NaCl

The rate of the reductive amination reaction was determined in the presence of 1.5M-NaCl and with the indicated NH_4Cl concentrations as described under assay I (see the Experimental section). To attain final NH_4Cl concentrations of at least 1 M conveniently, all the other components of the reaction were dissolved in a solution of the enzyme in 3M-NaCl/0.2M-Tris/HCl, pH9. Equal volumes of the latter and of NH_4Cl solutions, pH9, of double the required concentration were warmed to 37°C and mixed in the spectrophotometer cuvette immediately before assay. Each point on the graph represents the average of five successive determinations of the rate, because the loss of NH_3 from the solutions and the high baseline values on the recorder at high concentrations of NH_4Cl caused variations of up to $\pm 10\%$ in individual results.

substrates (see Table 3); there was no reaction with the D-forms of these compounds, but they inhibited deamination of the corresponding L-forms by about 10% when added to the appropriate reaction mixtures at a concentration of 50mM. The enzyme had a slightly broader substrate specificity in the reductive amination reaction (Table 2), but it should be noted that α -oxoglutarate was not a substrate, so that the enzyme was free from glutamate dehydrogenase. The reaction with oxaloacetate was dependent on NH_4^+ , and therefore not due to malate dehydrogenase, whose absence was confirmed by the failure of either L- or D-malate to serve as a substrate in the conditions of the deamination reaction (Table 2); as shown below, it is probably caused by conversion of the oxaloacetate into pyruvate by a contaminating oxaloacetate decarboxylase (see below). These results show that the *H. cutirubrum* L-alanine dehydrogenase has a restricted substrate specificity compared with those of the *B. cereus* (O'Connor & Halvorson, 1961) or *B. subtilis* (Yoshida & Freese, 1965) enzymes and, in this respect, is more like the *D. desulfuricans* enzyme that was reported by Germano & Anderson (1968) to be highly specific for L-alanine in the deamination reaction.

The K_m (apparent) values of *H. cutirubrum* L-alanine dehydrogenase for (i) pyruvate, NH_4^+ and NADH in the reductive amination reaction (assay I) and (ii) L-alanine and NAD^+ in oxidative deamination (assay II) were determined from plots of $[S]/v$ versus $[S]$ by standard methods. The results were: pyruvate 0.8mM; NADH, 0.2mM; L-alanine, 7mM;

Table 2. Substrate specificity of *H. cutirubrum* L-alanine dehydrogenase

The substrate specificities of *H. cutirubrum* L-alanine dehydrogenase in the reductive amination and oxidative deamination reactions were determined in the conditions of assay I and assay II (see the Experimental section) respectively. The substrate concentrations were 10mM for reductive amination and 50mM for oxidative deamination. The results are expressed as a percentage of the activity observed with the appropriate standard substrate (pyruvate or L-alanine).

Substrate	Activity (%)
(a) Oxidative deamination	
L-Alanine	100
D-Alanine	0
L- α -Aminobutyric acid	170
D- α -Aminobutyric acid	0
L- and D-Malic acid	0
L- and D-Aspartic acid, L-glutamic acid, L-glycine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-serine, L-threonine, L-tryptophan, L-valine	0
(b) Reductive amination	
Pyruvic acid	100
α -Oxobutyric acid	220
α -Oxovaleric acid	11
Glyoxylic acid	4
Oxaloacetic acid	50
α -Oxoglutaric acid	0

Table 3. Amino acid analysis of reductive amination reaction mixtures containing pyruvate or oxaloacetate as substrates

Individual assay I reaction, mixtures (3 ml) containing pyruvate (Expt. 1) or oxaloacetate (Expt. 2) and the partially purified enzyme (138 units/assay) were incubated as usual (see the Experimental section), and the amount of NADH oxidized was determined spectrophotometrically. At zero time and after 5 and 10 min of incubation, 20% (w/v) sulphosalicylic acid (0.2 ml) was added to the appropriate reaction mixture. The mixtures were then cooled in ice for 10 min and centrifuged to remove precipitated protein. Portions (0.7 ml) of the supernatants were mixed with 1 mM-norleucine (0.2 ml) and the mixtures analysed with a Technicon TSM amino acid analyser. Actual yields of alanine in the reaction mixtures were calculated from the ratio of alanine to norleucine in the samples and the known concentration of the latter. Theoretical yields of alanine were calculated from the measured consumption of NADH. No product other than alanine could be detected in either case.

Substrate	Time (min)	Alanine yield ($\mu\text{mol/ml}$)	
		Theoretical	Found
Expt. 1, pyruvate	0	0.00	0.00
	5	0.38	0.35
	10	0.61	0.65
Expt. 2, oxaloacetate	0	0.00	0.00
	5	0.29	0.32
	10	0.51	0.49

NAD⁺, 0.5 mM; NH₄⁺, 0.82 M. The values are similar to those reported for the *B. cereus* and *B. subtilis* enzymes (O'Connor & Halvorson, 1960; Yoshida & Freese, 1965) except in the case of NH₄⁺. The K_m value for the latter is about 20 times that reported for the other two alanine dehydrogenases, a difference that is presumably related in some as yet unknown way to the halophilic character of the *H. cutirubrum* enzyme. Reductive amination of pyruvate was approximately ten times as fast as oxidative deamination of L-alanine, as measured in the conditions of assays I and II respectively, by using equal amounts of partially purified enzyme.

The nature and rates of the reductive amination reactions with pyruvate and oxaloacetate were studied quantitatively by using an amino acid analyser (Table 3). In both cases, L-alanine was the only product detected. The amount formed from pyruvate agreed well with the expected yield calculated from the amount of NADH oxidized during the reaction. When the substrate was oxaloacetate, there was also good agreement with the yield of L-alanine, predicted on the assumption that the measured oxidation of NADH was solely due to reactions leading to alanine formation. Two reaction sequences could explain the formation of alanine from oxaloacetate in these conditions. (1) The reductive amination of oxaloacetate to aspartate, by alanine (or an aspartate) dehydrogenase, followed by decarboxylation of aspartate to alanine by aspartate decarboxylase (Tate & Meister, 1971). (2) Formation of pyruvate from oxaloacetate catalysed by oxaloacetate decarboxylase (Plaut & Lardy, 1949; Horton & Kornberg, 1964), followed by reductive amination of the pyruvate to alanine by alanine dehydrogenase. Since L-aspartate was not a substrate for the partially purified enzyme

Fig. 5. Effect of assay temperature on the rate of the reductive amination reaction

The rate of reductive amination of pyruvate by *H. cutirubrum* L-alanine dehydrogenase was determined at the indicated temperatures in the conditions of assay I (see the Experimental section) in the presence of either 2.6 M-KCl or 2.75 M-NaCl. The broken and solid lines show the results of experiments performed on separate occasions with different samples of the enzyme. The results are expressed as a percentage of the activity at 37°C. NaCl, ●; KCl, △; Expt. 1, —; Expt. 2, ----.

(Table 2) and no aspartic acid could be detected by amino acid analysis of the reaction mixture, the second alternative is more probable.

Fig. 6. Thermal stability of *H. cutirubrum* L-alanine dehydrogenase

Samples of a solution of the enzyme in 3M-KCl/0.01M-Tris/HCl, pH 8.5, were heated at 55°C (▲), 60°C (○) or 70°C (●) for the indicated time. They were then cooled in ice and assayed for reductive amination activity at 37°C in the conditions of assay I (see the Experimental section). The results are expressed as a percentage of the activity of the unheated enzyme assayed in the same conditions.

Thermal stability of *H. cutirubrum* L-alanine dehydrogenase

Keradjopoulos & Wulff (1974) found that the crude L-alanine dehydrogenase of *H. salinarium*, an organism closely related to *H. cutirubrum* (Kushner, 1968), had an optimum reaction temperature of 70°C in 3.4M-KCl and 60°C in 3.4M-NaCl. They considered the enzyme to be thermophilic, but did not present any evidence that the protein was thermally stable in the absence of its substrates.

We have now determined the optimum reaction temperature (Fig. 5) and examined the thermal stability (Fig. 6) of the 100-fold purified *H. cutirubrum* L-alanine dehydrogenase. The results in Fig. 5 confirm Keradjopoulos & Wulff's (1974) observation that the optimum reaction temperature of the enzyme is high. The optimum temperature appeared slightly higher with K⁺ than with Na⁺ as the principal univalent cation in the assay mixture in one series of experiments, but no difference was observed on another occasion (Fig. 5): this variability was due to the fast thermal inactivation of the enzyme at higher temperatures (see below), with a consequent imprecision in the measurement of initial velocities arising from the short time of reaction. However, Fig. 6 shows that the thermal stability of *H. cutirubrum* L-alanine

dehydrogenase is in fact not significantly greater in the absence of substrate than that of most well-studied enzymes from mesophiles (e.g. *Escherichia coli*) and quite unlike the remarkable resistance to heat-inactivation of truly thermophilic enzymes from organisms such as the moderate thermophile *Bacillus stearothermophilus* (see, e.g., Amelunxen & Lins, 1968; Saunders *et al.*, 1969) or the extreme thermophile *Thermus aquaticus* (see, e.g., Freeze & Brock, 1970; Higa & Ramaley, 1973).

Conclusions

The results presented above show that *H. cutirubrum* contains an active L-alanine dehydrogenase that requires a high ionic strength for optimum activity and stability. Its activity in the standard reductive-amination assay increases with temperature up to about 70°C, but the enzyme itself is not thermostable.

The molecular weight of the enzyme is approx. 72500. Thus, although it is not an unusually small protein, it is less than one-third the size of the two well-studied *B. cereus* and *B. subtilis* L-alanine dehydrogenases.

H. cutirubrum L-alanine dehydrogenase has a much more restricted substrate specificity than the corresponding *B. cereus* and *B. subtilis* enzymes, especially in the deamination reaction. However, its most remarkable property is its absolute requirement for K⁺ for oxidative deamination compared with its ability to catalyse the reverse reaction, reductive amination, equally well in presence of K⁺, Na⁺ or NH₄⁺, and to a lesser extent with Cs⁺ or Li⁺, as the univalent cation. This is the first case to be described of an enzyme from an extreme halophile whose alkali-metal-cation specificity differs for the forward and backward reactions.

We thank the Laboratory Centre for Disease Control, Health and Welfare Canada for the grant of study leave (to E. K. K.), the Medical Research Council for an operating grant (to P. S. F.) and Dr. P. J. Anderson for performing the amino acid analyses of reaction mixtures.

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