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L-ALANINE DEHYDROGENASE FROM *THERMUS THERMOPHILUS*

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Summary

A heat-stable L-alanine dehydrogenase was isolated and purified from the extremely thermophilic microorganism, *Thermus thermophilus*, by affinity chromatography. The enzyme has a molecular weight of 290 000, as determined by the sedimentation equilibrium method, and is composed of six subunits of identical molecular weight as concluded from sodium dodecyl sulphate gel electrophoresis. The enzyme has been characterized in terms of pH- and substrate concentration-dependence of activity, substrate specificity, inhibition by D-alanine and D-cysteine and amino acid composition. The parameters obtained are very similar to those reported for L-alanine dehydrogenase from the mesophilic microorganism, *Bacillus subtilis* (Yoshida, A. and Freese, E. (1965) Biochim. Biophys. Acta 96, 248—262). The thermal stability of the *T. thermophilus* enzyme is much greater than that of the *B. subtilis* enzyme. Activation free energy ($\Delta G^{\ddagger}$), activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) values were determined for both the alanine deamination and for the heat inactivation reactions of the thermophilic and mesophilic enzymes. The values obtained for the catalytic reaction were practically equal. However, the two enzymes differed significantly in these parameters determined for the enzyme inactivation, which indicates that the factors ensuring the thermoresistance of the enzyme from *T. thermophilus* do not affect enzyme activity.

Introduction

L-Alanine dehydrogenase (L-alanine:NAD^+ oxidoreductase (deaminating), EC 1.4.1.1), which catalyses the following reaction:

L-alanine + H_2O + NAD^+ ⇌ pyruvate + NH_3 + NADH + H^+

has an important role in the carbon and nitrogen metabolism of various microorganisms by providing a link between carbohydrate and amino acid metabolisms. In contrast to glutamate dehydrogenase, alanine dehydrogenase occurs only in a limited number of bacterial species. Induction and repression of the enzyme production has been studied in several microorganisms [1—10]. In vitro it catalyses the reaction in both directions, however, in vivo the catabolic function of the enzyme is predominant [1,2,5,7—10]. The enzyme has been purified from *Bacillus subtilis* and *Bacillus sphaericus* and its enzymic properties have been determined [11—13]. In their recent report, Ohashima and Soda [13] have investigated a number of bacterial strains for their production of L-alanine dehydrogenase and have shown that all *Bacillus* strains tested possessed various amounts of this enzyme. No thermophilic bacteria were included in this survey. We have found that the extremely thermophilic bacterium, *Thermus thermophilus*, has high alanine dehydrogenase activity.

Studies on numerous enzymes from thermophilic bacteria have shown that these enzymes possess increased thermal stability compared to the enzymes from mesophilic species [14]. Detailed structural studies were undertaken with the aim of comparing the tertiary and quaternary structures of the same enzyme from a mesophilic and a thermophilic microorganism in order to be able to identify structural changes that confer thermal stability. In contrast to original expectations, the structural differences between thermophilic and mesophilic enzymes seem to be rather subtle and since these are part of the original overall species differences they are difficult to detect [15]. Still, it would be interesting to know whether these differences also influence enzyme activity or whether thermoresistance is established independently of the catalytic function.

We present here a detailed comparison of the thermophilic and mesophilic enzyme and show that by determining $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for both the enzyme reaction and for the inactivation process, it may be possible to decide whether structural features that bring about thermal stability also affect the catalytic reaction.

Materials and Methods

Sodium pyruvate was obtained from Merck (Darmstadt), Cibacron blue F3G A dye from Ciba-Geigy (Basel), L-amino acids from Cal-Biochem (Los Angeles), NAD and NADH from Reanal (Budapest). Sephadex G-200 was a product of Pharmacia (Uppsala). Protein Calibration Kit size II and *B. subtilis* L-alanine dehydrogenase were purchased from Boehringer-Mannheim GmbH. All other chemicals were analytical grade reagents.

Cultivation of the microorganism. A culture of *T. thermophilus* strain HB8 was kindly provided by Dr. Tairo Oshima, University of Tokyo. The bacteria were grown in a medium containing 0.8% peptone, 0.4% yeast extract, 0.3% NaCl, pH 7.5, at 75° C with an aeration of 1.5 l/min per l medium.

Enzyme assay. Alanine dehydrogenase activity was measured by recording the changes in absorbance at 340 nm due to the oxidation of NADH or the reduction of NAD using a Varian Techtron UV-VIS spectrophotometer Model 635. Unless otherwise noted all measurements were performed at 25° C. The

composition of the reaction mixture for the reductive amination of pyruvate was: 0.2 mol NH_4Cl; 4.5 mmol pyruvate; 0.27 mmol NADH; 0.2 mmol EDTA and 0.4 μg enzyme in 50 mM Tris-HCl buffer (pH 8.0) in a volume of 1 ml. For oxidative deamination of L-alanine: 25 mmol L-alanine; 1.4 mmol NAD; 0.2 mmol EDTA and 4 μg enzyme in 50 mM sodium carbonate/bicarbonate buffer (pH 10.0) in a volume of 1 ml. NAD and NADH concentrations were determined enzymatically. Specific activity is given in units per mg of protein; one unit catalyses the oxidation of 1 μmol of NADH per min at 25° C under the assay conditions.

Protein concentration. During purification of the enzyme the protein concentration was estimated from the absorbance at 280 nm. The extinction coefficient of the crystalline alanine dehydrogenase from *T. thermophilus* was determined by using the method of Jaenicke [16]; $E_{280}^{1\%} = 5.0\ cm^{-1}$. For the *B. subtilis* enzyme the value $E_{280}^{1\%} = 6.4\ cm^{-1}$ was used [11].

Preparation of the affinity matrix. Blue Sephadex G-200 was prepared according to the method of Böhme et al. [17].

Polyacrylamide gel electrophoresis. The method of Blatter et al. [18] was used in 7.5% gels at pH 8.3. Protein was stained with Coomassie brilliant blue G-250 [19].

Sodium dodecyl sulphate gel electrophoresis. SDS gel electrophoresis was performed as described by Weber and Osborn [20] in 7% gels. For molecular weight determination of the subunits the Protein Calibration Kit size II from Boehringer was used (subunit molecular weights ranged from 12 500 to 67 000).

Amino acid analysis. Protein samples were hydrolysed for 24 h in 6 M HCl at 110° C. The amino acid composition of the hydrolysate was determined on a Beckman Unichrome analyser.

Ultracentrifugal measurements. An MOM analytical ultracentrifuge type 3170/b equipped with Schlieren and interferometer optics was used. Sedimentation velocity experiments were run between 48 000 and 60 000 rev./min at different temperatures. Sedimentation coefficients were corrected to 20° C. The molecular weight of the native enzyme was determined by the meniscus-depletion sedimentation-equilibrium method of Yphantis [21] at a rotor speed of 8—14 000 rev./min for 10—20 h. The initial protein concentration varied between 0.15 and 0.30 mg/ml. The molecular weights of the samples were calculated from the interference fringe displacements characterizing the equilibrium concentration distribution.

Circular dichroism. CD spectra were recorded on a JASCO J 41A spectropolarimeter calibrated by using the method of Cassim and Yang [22]. The temperature of the cell compartment was controlled by a circulating water bath. Measurements were performed in a cell with a 0.5 mm light path. Sample concentration was 0.334 mg/ml. The mean residue weight of the amino acids was taken to be 115 in the calculation of molar ellipticity (θ), which is expressed as mean residue ellipticity in degrees/cm^2 per dmol. For monitoring the thermal denaturation of the enzyme the CD signal was recorded continuously at 197 nm while the temperature of the sample in the cuvette was raised to 90° C at a constant rate of 1° C/min.

Results and Discussion

Enzyme purification. Cells (25 g wet wt.) were suspended in 50-ml of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 14 mM magnesium acetate and 0.14 mM KCl, and were disrupted by sonication (three times for 30 s). Unbroken cells and cell debris were removed by centrifugation (39 000 × *g*, 45 min). To the supernatant, solid ammonium sulphate was added to bring the solution to 1.2 M. The precipitate was removed (11 000 × *g*, 30 min) and the supernatant was saturated to 2.1 M. The precipitated fraction contained all the alanine dehydrogenase activity. Although the enzyme content of the sonicates varied with different batches of cells, after the ammonium sulphate precipitation step the activity : protein ratio was always the same (Table I).

The 1.2—2.1 M ammonium sulphate fraction was dissolved in 5-ml of 20 mM sodium phosphate buffer, pH 7.5, containing 0.2 mM EDTA (referred to as standard buffer) and was dialysed against the same buffer overnight. The solution was applied to the affinity column (1 × 25 cm, 10 ml/h) which had been equilibrated with the same buffer. The bulk of the proteins was washed off with 100-ml of the standard buffer and then a protein fraction with alanine dehydrogenase activity was eluted with 50-ml of the standard buffer containing 0.1 M NaCl. The pooled active fractions were concentrated by saturation to 2.1 M with ammonium sulphate, dialysed against the standard buffer and then applied to a second affinity column (1 × 16 cm, 15 ml/h), equilibrated with the buffer. This column was then washed with 20-ml standard buffer containing 0.1 M NaCl and the enzyme was eluted by adding 1 mM NADH to the elution buffer. The fractions showing alanine dehydrogenase activity contained a single protein as judged from polyacrylamide gel electrophoresis in the presence and absence of SDS. Electrophoretic patterns after the first and second affinity chromatography are shown in Fig. 1. Purification steps are summarized in Table I.

Crystallization of the enzyme. To the pooled active fractions, solid ammonium sulphate was added slowly at 0°C to bring the solution to 1.2 M. Saturated ammonium sulphate was then added drop-wise until the solution became slightly turbid (pH 8.4). The next day the solution was brought to turbidity again by the addition of a few more drops of ammonium sulphate solution. Fine needle-like crystals developed after 2 or 3 days. Specific activities of these samples ranged from about 125 to 150 U/mg (2.08—2.5 kat/kg). The absorption spectrum of the enzyme is shown in Fig. 2. The spectrum indicates that there is probably no tryptophan in the enzyme.

TABLE I

PURIFICATION OF ALANINE DEHYDROGENASE FROM *THERMUS THERMOPHILUS*

Purification step	Volume (ml)	Total protein (A_{280})	Total units (IU)	Purification (-fold)	Recovery (%)
Ammonium sulphate fractionation	15	474	1447	1	100
Blue Sephadex G-200 chromatography 1	10.5	5.76	914.8	52	63
Blue Sephadex G-200 chromatography 2	15	2.2	570	85	39

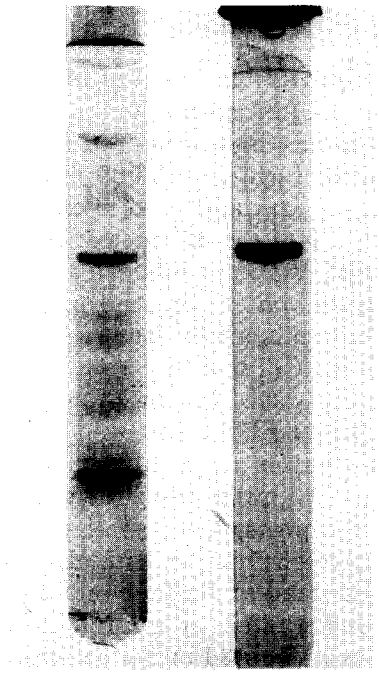

Fig. 1. Polyacrylamide gel electrophoretic pattern of the pooled samples showing A1DH activity after the first and second affinity chromatography. About 50 μg protein was applied to a 7.5%gel. Electrophoresis was run at pH 8.3.

Molecular properties of the T. thermophilus enzyme. The purified enzyme sediments as a single symmetrical boundary having a sedimentation coefficient $S_{20,w} = 9.26 \pm 0.10$ at a concentration of 3.0 mg/ml in 50 mM Tris-HCl buffer, pH 7.5. Variation of the rotor speed and of the temperature (4—20° C) did not

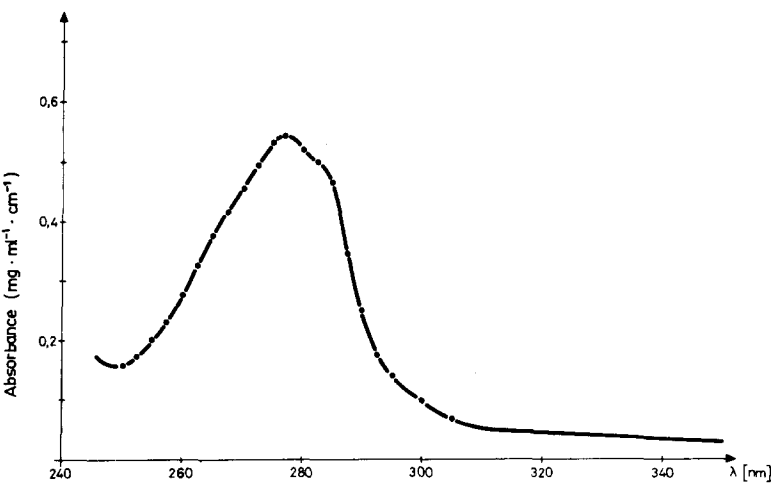

Fig. 2. Absorption spectra of the crystalline A1DH. 0.334 mg/ml protein in 20 mM sodium phosphate buffer at pH 6.9.

affect this value which shows that alanine dehydrogenase from *T. thermophilus* forms a homogeneous non-dissociable system at this concentration. The weight-average molecular weight of the enzyme is 290 000 as determined by the meniscus-depletion sedimentation-equilibrium method. The partial specific volume was calculated according to the method of Cohn and Edsall [23] on the basis of the amino acid composition and was found to be 0.745 ml/g. The subunit composition of the enzyme was estimated by SDS gel electrophoresis. The SDS-treated sample exhibited a single electrophoretic species with a molecular weight of 48 000. This indicates that the native enzyme is composed of six subunits with identical molecular weights.

Amino acid composition. The amino acid compositions of alanine dehydrogenase from *B. subtilis* and *T. thermophilus* are compared in Table II. It is obvious from previous studies on thermophilic enzymes that no generally valid correlation exists between the content of any particular amino acid or groups of amino acids and thermal stability, since it is not the overall composition but the location of particular residues in the three-dimensional structure that determines molecular stability [24]. Yet, it is interesting to note the lack of cysteine in the thermophilic enzyme. Cysteines can occur both in the interior and on the exterior of proteins and an external cysteine may be a potential source of instability [24].

TABLE II

AMINO ACID COMPOSITION OF ALANINE DEHYDROGENASE FROM *THERMUS THERMOPHILUS* AND *BACILLUS SUBTILIS*

n.d., not determined. Value of % amino acid given as mean value and deviations of complete duplicate analysis. Molar ratio of individual amino acids calculated on the basis of a subunit molecular weight of 48 000. The assumed molar ratio is the nearest integral number to the calculated molar ratio.

Amino acid	*Th. thermophilus* alanine dehydrogenase			*B. subtilis* alanine dehydrogenase (Assumed molar* ratio)
	% of amino acid	Calculated molar ratio	Assumed molar ratio	
Lys	2.76 ± 0.01	23.13 ± 0.42	23	28
His	1.00 ± 0.01	8.38 ± 0.08	8	9
Arg	2.22 ± 0.15	18.60 ± 1.26	19	14
Asx	5.05 ± 0.06	42.32 ± 0.84	42	43
Thr	3.13 ± 0.06	26.23 ± 0.50	26	34
Ser	2.10 ± 0.04	17.60 ± 0.33	18	20
Glx	5.12 ± 0.19	42.91 ± 1.59	43	51
Pro	2.75 ± 0.04	23.04 ± 0.33	23	25
Gly	6.55 ± 0.09	54.89 ± 0.75	55	51
Ala	6.75 ± 0.18	56.56 ± 1.50	57	65
Val	4.88 ± 0.24	40.89 ± 2.01	41	49
Met **	1.67 ± 0.01	14.00 ± 0.08	14	11
Ile	3.40 ± 0.01	28.49 ± 0.08	28	28
Leu	4.96 ± 0.10	41.56 ± 0.84	42	42
Tyr	1.48 ± 0.02	12.40 ± 0.16	12	12
Phe	1.00 ± 0.01	8.38 ± 0.08	8	7
Cys **	0	0	0	1
Trp	n.d.	n.d.	n.d.	1

* Data from Yoshida and Freese [11]. The molar ratio was calculated assuming that cysteine residue is 1.00 [11].

** Determined on hydrolysates of performic acid-oxidized samples.

Comparison of the thermophilic and mesophilic enzymes. We have compared the kinetic parameters determined for the thermophilic enzyme with those reported for the *B. subtilis* alanine dehydrogenase [12].

The pH dependence of activity. The pH dependence of activity is shown in Fig. 3a and b. The pH optimum for the reductive amination of pyruvate is at about 8.5 for the mesophilic and at 8.0 for the thermophilic enzyme. For L-alanine oxidation the optimum is higher than pH 10.5. It is interesting to note that the shapes of the curves are also very similar for the two enzymes.

Substrate saturation curves. Curves were determined at 25° C. The Michaelis constants were obtained from the double-reciprocal plots, which gave straight lines for each substrate. K_m values are practically equal to those of the mesophilic enzyme (see Table III).

Alanine analogs as competitive inhibitors. The rate of L-alanine deamination was measured in the presence of D-cysteine and D-alanine. Both amino acids are competitive inhibitors as is concluded from Fig. 4a and b and from the Lineweaver-Burk plots (not shown). Inhibitor constants (K_i) were determined

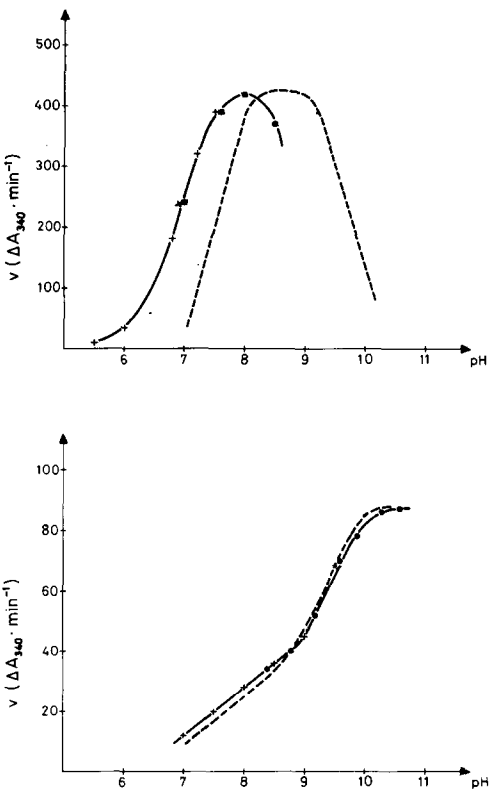

Fig. 3. (a) Effect of pH on the rate of amination. Data for the mesophilic enzyme are taken from Ref. 12 (- - - - -). The reaction mixture in the case of the *T. thermophilus* enzyme contained the amount of substrates specified in Enzyme assay in 50 mM sodium phosphate (+———+) (pH 5.2—7.4) and in 50 mM Tris-HCl (•———•) (pH 7.0—8.4. (b) Effect of pH on the rate of deamination. Data for the mesophilic enzyme are obtained from Ref. 12 (- - - - -). The reaction mixture for the thermophilic enzyme is specified in Enzyme assay. The buffers used are: 50 mM Tris-HCl (+———+) (pH 7.0—9.0) and 50 mM sodium carbonate/bicarbonate (•———•) (pH 8.4—10.6).

TABLE III

K_m AND K_i VALUES FOR ALANINE DEHYDROGENASE FROM *B. SUBTILIS* AND *T. THERMOPHILUS*

K_m and K_i values for the *B. subtilis* enzyme were obtained from Ref. 12. The rate of pyruvate reduction and L-alanine deamination were measured as specified in Materials and Methods with varying substrate and inhibitor concentrations. K_m is determined from the corresponding Lineweaver-Burk plots, K_i from Dixon plots. Values are expressed in mM.

	K_m					K_i	
	Pyr	NADH	NH_3	L-Ala	NAD	D-Ala	D-Cys
B. subtilis	0.54	0.023	38.0	1.7	0.18	20.0	0.64
T. thermophilus	0.75	0.035	59.0	4.2	0.12	5.0	0.90

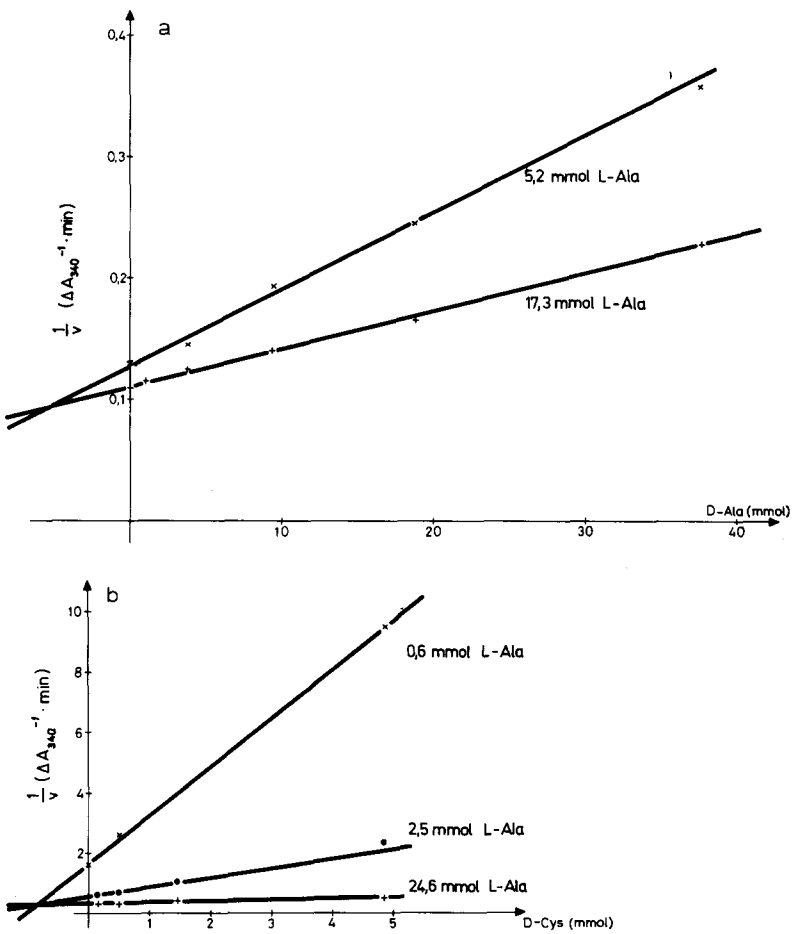

Fig. 4. Competitive inhibition by L-alanine analogs. (a) Effect of D-alanine on the reaction rate of deamination. The reaction mixture specified in Enzyme assay was used with the variation of the L-alanine and D-alanine concentrations (5.2 or 17.3 mM L-alanine and 4—40 mM D-alanine, respectively. (b) Effect of D-cysteine on the reaction rate of deamination. The reaction mixture is specified in Enzyme assay but contained different concentrations of L-alanine (24.6, 2.5 and 0.6 mM) and various amounts of D-cysteine (0.14—4.9 mM).

from the Dixon plots and are compared to the values reported for the *B. subtilis* enzyme (Table III). D-Cysteine appears to be a more effective inhibitor for both enzymes than D-alanine. The significantly smaller K_i value for D-alanine in the case of the *T. thermophilus* enzyme might be a further indication of the higher substrate specificity of this enzyme (see below).

Substrate specificity. Deamination of a variety of L-amino acids was also tested. The results are summarised in Table IV. β-Alanine, L-lysine, -asparagine, -cysteine, -glutamine, -threonine, -proline, -phenylalanine, -histidine, -methionine, -tyrosine and -tryptophan were not deaminated. Data show that the *T. thermophilus* alanine dehydrogenase has narrower substrate specificity than the mesophilic enzyme.

Temperature dependence of the catalytic reaction. To determine the activation free energy ($\Delta G^{\ddagger}$), activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) for the alanine deamination we have measured V values as a function of temperature between 20 and 60° C for both the thermophilic and mesophilic enzymes. Results are given in the form of Arrhenius plots which are linear for both enzymes. This indicates that there is probably no change in enzyme conformation or mechanism in this temperature range (Fig. 5). $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are practically the same for the mesophilic and thermophilic enzymes (see Table V).

Heat stability. Heat denaturation of the thermophilic enzyme was detected by CD measurements. The CD spectra of the *T. thermophilus* enzyme were first recorded at 12° C (Fig. 6) and the sample was then heated to 90° C at a constant rate of 1° C/min and kept there for 10 min. The CD spectrum taken at this temperature shows the complete denaturation of the sample (Fig. 6). Precipitation was not detected. The sample was then cooled back to 12° C, but the CD spectrum did not change showing that denaturation was irreversible.

The kinetics of denaturation were followed at a constant wavelength, at 197 nm. The loss of secondary structure of the protein is accompanied by a decrease in the intensity of the CD signal at this wavelength. The temperature of the sample was raised at a constant rate of 1° C/min to 90° C where the CD signal reached zero value in 10 min indicating the complete denaturation. However, up to 80° C the CD signal did not change significantly, which shows that no major change occurs in the structure up to this temperature (Fig. 7).

Heat inactivation. To characterise quantitatively the heat stability of the *T. thermophilus* and *B. subtilis* enzymes we have determined their 'melting

TABLE IV

SUBSTRATE SPECIFICITY OF ALANINE DEHYDROGENASE IN THE DEAMINATION REACTION

The rate of deamination was measured under the conditions used to determine the rate of L-alanine deamination but instead of L-alanine the reaction mixtures contained 100 mM of the amino acid indicated (except L-Tyr, 10 mM; and L-Trp, 50 mM). The amino acids not listed in the table were not deaminated at any detectable rate under these reaction conditions. Data are given in the Table in $v/v_{\text{L-Ala}} \times 100$. The data for *B. subtilis* were obtained from Yoshida and Freese [12].

	L-Ala	L-Val	L-Ile	L-Ser	Gly
B. subtilis	100	9	5	5	0
T. thermophilus	100	0.2	0.1	2.2	0.1

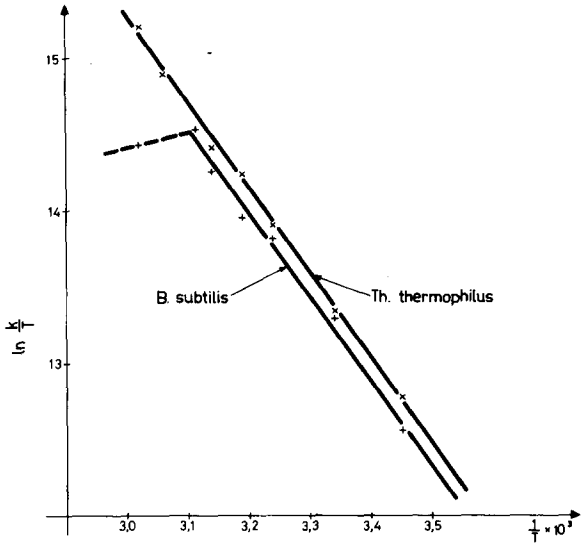

Fig. 5. Arrhenius plot for the deamination reaction. Effect of temperature on the activity of A1DH from *B. subtilis* and *T. thermophilus* was measured in the presence of various amounts of substrates in 50 mM cyclohexylaminopropanesulphonic acid buffer, pH 10.0. No pH correction was made since the pH of the buffer does not change by more than 0.1 between 25 and 60°C. The pH dependence of the reaction rate was determined at 25 and 50°C and found to be the same. *V* values were determined from the double-reciprocal plots of the dependence of rate of catalysis on ligand concentrations at each temperature. *T. thermophilus* enzyme (X———X), *B. subtilis* enzyme (+———+).

temperatures' (50% loss of activity in 5 min under the reaction conditions described in Materials and Methods), which have been suggested to express thermal stability [25]. The data obtained show that the enzyme from *T. thermophilus* exhibits remarkable thermal stability. Its melting temperature is 86°C, whereas that of the *B. subtilis* enzyme is only 63°C (Fig. 8). Enzyme concentration in the range of 1 to 100 μg/ml did not affect the inactivation rates.

The time course of heat inactivation shows first-order kinetics (Fig. 9). From the first-order rate constants and from their temperature dependence $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were determined and these data are summarized in Table VI. It can be seen that the enthalpy contribution to $\Delta G^{\ddagger}$ is about 4 times as large in the case of the *T. thermophilus* enzyme as compared to the *B. subtilis* enzyme.

TABLE V

THERMODYNAMIC PARAMETERS OF THE ENZYME REACTION

$\Delta G^{\ddagger}$ was determined from the first-order rate constants of the deamination reaction, $\Delta H^{\ddagger}$ from their temperature dependence (Fig. 5) and $\Delta S^{\ddagger}$ was calculated from $\Delta G^{\ddagger}$ and $\Delta H^{\ddagger}$.

	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol per K)	$\Delta G^{\ddagger}$ (25°C) (kJ/mol)
B. subtilis	46.1	65.7	26.5
T. thermophilus	46.1	66.9	26.1

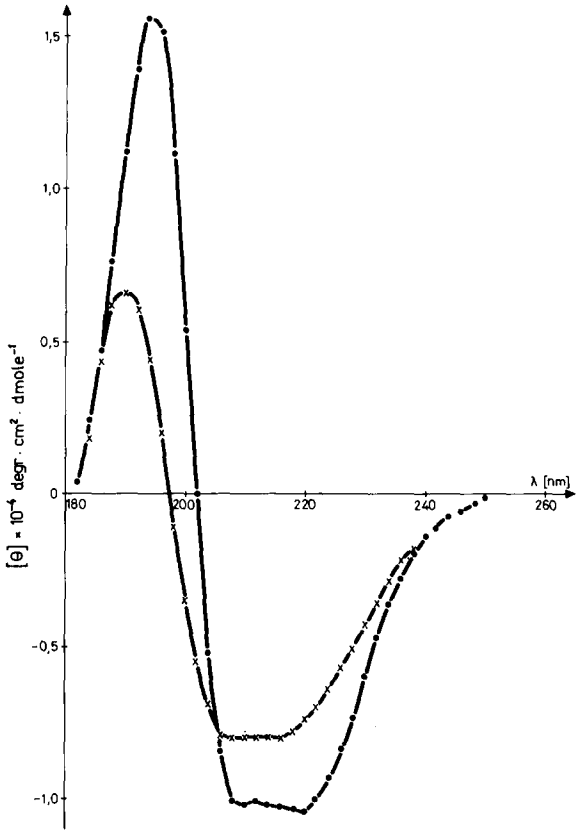

Fig. 6. CD spectra of the *T. thermophilus* A1DH were taken at 12° C in 20 mM sodium phosphate buffer, pH 6.9. The enzyme concentration was 0.334 mg/ml (●———●). The sample was then heated to 90° C at a constant rate of 1° C/min, and the CD spectra were taken again (X———X).

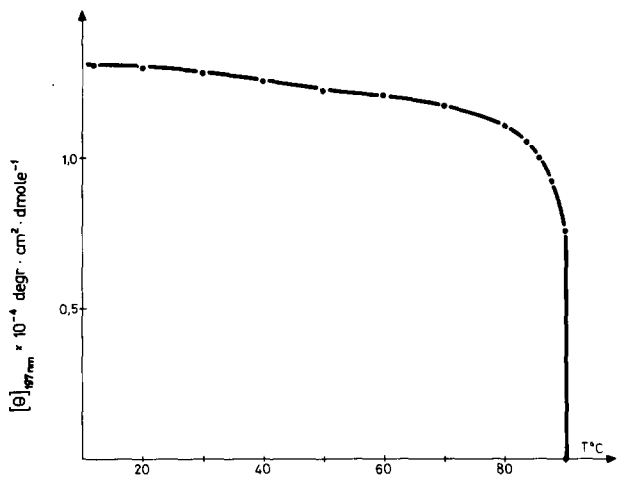

Fig. 7. Kinetics of heat denaturation. CD signal of the thermophilic enzyme was detected at 197 nm while the sample (0.334 mg/ml protein in 20 mM sodium phosphate buffer) was heated at a constant rate of 1° C/min. The CD signal reached zero value at this wavelength at 90° C in 10 min.

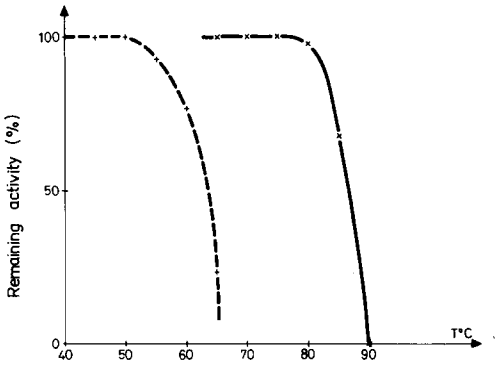

Fig. 8. Melting temperatures for irreversible inactivation were determined by incubating aliquots of the enzymes in 10 mM triethanolamine-HCl buffer, pH 8.0, for 5 min (enzyme concentration was 10 μg/ml) at the selected temperature followed by rapid cooling to 0° C and subsequent enzymic assay at 25° C. *B. subtilis* enzyme (+———+), *T. thermophilus* enzyme (X———X).

Because of the large difference in $\Delta S^{\ddagger}$ between the two enzymes, $\Delta G^{\ddagger}$ decreases faster with increasing temperature in the case of the thermophilic enzyme than with the mesophilic one. If we could assume that Arrhenius plots are linear up to at least 94° C, at this temperature the rate of inactivation would be the same for the thermophilic and mesophilic enzymes, not allowing us to distinguish between them on the basis of thermal stability.

$\Delta G^{\ddagger}$ can be considered as a measure of thermoresistance, since it is logarithmically proportional to the rate constant of inactivation. A considerable increase in heat resistance requires only a slight increase in $\Delta G^{\ddagger}$. The observed differences in $\Delta G^{\ddagger}$ between thermophilic and mesophilic enzyme pairs can be gained in a variety of ways, just by the addition of a few of the commonly observed bonds that stabilize proteins, as suggested by Hocking and

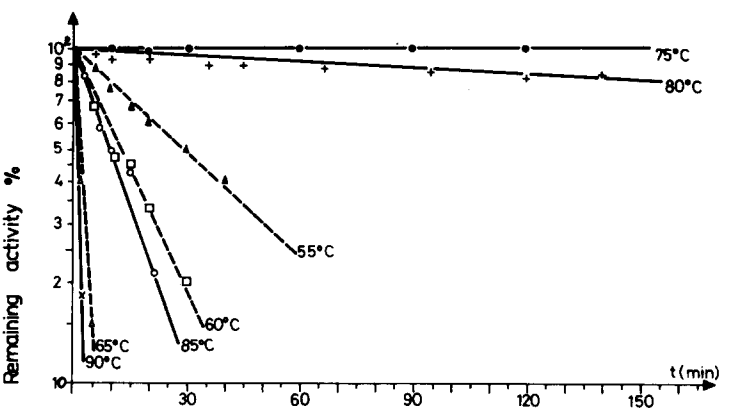

Fig. 9. Kinetics of heat inactivation. Aliquots of the enzymes were incubated at different temperatures for various time intervals in 10 mM triethanolamine-HCl buffer, pH 8.0. Enzyme concentration was 1, 10 and 100 μg/ml. After rapid cooling to 0° C, remaining activity was measured at 25° C. *B. subtilis* enzyme (- - - - -), *T. thermophilus* enzyme (———). (90° C (X———X), 85° C (○———○), 80° C (+———+), 75° C (●———●), 65° C (△———△), 60° C (□———□), 55° C (▲———▲).

TABLE VI

THERMODYNAMIC PARAMETERS OF HEAT INACTIVATION

The parameters were determined for the thermophilic and mesophilic enzymes from the temperature dependence of the first-order rate constants of heat inactivation.

	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol per K)	$\Delta G^{\ddagger}$ 63°C (kJ/mol)	$\Delta G^{\ddagger}$ 86°C (kJ/mol)
B. subtilis	171.8	247.2	89.4	—
T. thermophilus	736.3	1788.2	—	94.7

Harris [24]. Various proteins from various sources may have acquired thermal stability by different means. In the case of alanine dehydrogenase the increased heat stability is a consequence of structural features which are reflected in an increased $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. This later partly compensates the effect of the high $\Delta H^{\ddagger}$ in $\Delta G^{\ddagger}$. In contrast to this, the comparison of the data published recently for pig muscle and *Thermus aquaticus* lactate dehydrogenase [26] show that the increased heat resistance of the *T. aquaticus* enzyme is gained by interactions which practically do not influence the value of $\Delta H^{\ddagger}$ but considerably decrease $\Delta S^{\ddagger}$.

Although in the catalytic reaction $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are identical for the thermophilic and mesophilic alanine dehydrogenases, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ differ significantly in the heat inactivation processes. This suggests that in this case the factors which endow the *T. thermophilus* enzyme with the greater thermoresistance do not affect the enzyme action.

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References

1 Hong, M.M., Shen, S.C. and Braunstein, A.E. (1959) Biochim. Biophys. Acta 36, 288—189
2 O'Connor, R.J. and Halverson, H.O. (1961) J. Bacteriol. 82, 648—656
3 McCormick, N.G. and Halverson, H.O. (1964) J. Bacteriol. 87, 68—74
4 Freese, E. (1964) Biochim. Biophys. Acta 81, 442—447
5 Raunio, R.P. and Jenkins, W.T. (1973) J. Bacteriol. 115, 560—566
6 Nitta, Y., Yasuda, Y., Tochikubo, K. and Hachisuka, Y. (1974) J. Bacteriol. 117, 588—592
7 McCowen, S.M. and Phibbs, J.P.V. (1974) J. Bacteriol. 118, 590—597
8 Berberich, R., Kaback, M. and Freese, E. (1968) J. Biol. Chem. 243, 1006—1011
9 Freese, E. and Oosterwyk, I. (1963) Biochemistry 2, 1212—1216
10 Epstein, I. and Grossowicz, N. (1969) J. Bacteriol. 99, 414—417
11 Yoshida, A. and Freese, E. (1964) Biochim. Biophys. Acta 92, 33—43
12 Yoshida, A. and Freese, E. (1965) Biochim. Biophys. Acta 96, 248—262
13 Ohashima, T. and Soda, K. (1979) Eur. J. Biol. 100, 29—39
14 Singleton, R. and Amelunxen, R.E. (1973) Bacteriol. Rev. 37, 320—342
15 Schär, H.P. and Zuber, H. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 795—807
16 Jaenicke, R. (1974) Anal. Biochem. 61, 623—627
17 Bohme, M.J., Kopperschlager, G., Schulz, J. and Hoffman, E. (1972) J. Chromatogr. 69, 209—214
18 Blatter, D.P., Garner, F., van Slyke, K. and Bradley, A. (1972) J. Chromatogr. 64, 147—155
19 Diezel, W., Kopperschläger, G. and Hofmann, E. (1972) Anal. Biochem. 48, 617—620
20 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412

21 Yphantis, D.A. (1964) Biochemistry 3, 297—317
22 Cassim, J.Y. and Yang, J.T. (1969) Biochemistry 8, 1946—1951
23 Cohn, E.J. and Edsall, J.T. (1943) Proteins, Amino Acids and Peptides, p. 370, Reinhold, New York
24 Hocking, J.D. and Harris, J.I. (1976) in Enzymes and Proteins from Thermophilic Microorganisms (Zuber, H., ed.), Experimentia Supplementum 26, pp. 121—133, Birckhäuser Verlag, Basel
25 Amelunxen, R.E. and Murdock, A.L. (1977) Development in Industrial Microbiology, Vol. 18, p. 238
26 Lakatos, S., Halász, G. and Závodszky, P. (1978) Biochem. Soc. Trans. 6, 1195—1197