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PURIFICATION AND PROPERTIES OF ISOCITRATE DEHYDROGENASE (NADP) FROM *THERMUS AQUATICUS* YT-1, *BACILLUS SUBTILIS*-168 AND *CHLAMYDOMONAS REINHARDTI*-Y-2

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SUMMARY

The NADP-specific isocitrate dehydrogenase (EC 1.1.1.42) was purified from the new extremely thermophilic gram-negative, non-spore-forming *Thermus aquaticus* YT-1, from the mesophilic spore forming bacterium *Bacillus subtilus*-168 and the mesophilic flagellated algae *Chlamydomonas reinhardti*. All three isocitrate dehydrogenases showed similar catalytic properties with respect to optimum pH, substrate and inhibitor specificity. However, the enzyme isolated from the extremely thermophilic microorganism was much more thermostable than the enzyme from either *B. subtilis* or *C. reinhardti*. Thus the enzyme from the extremely thermophilic bacteria catalyzed its enzymatic reaction *in vitro* at a higher "optimum temperature". The greater stability of enzymes from *T. aquaticus* suggest its general utility in the characterization of unusually labile cellular components.

INTRODUCTION

The enzymes from thermophilic microorganisms (microorganisms capable of growth at 55 °C or above) are more thermostable than the same enzymes from mesophilic or psychophilic microorganisms[1–3]. Extensive studies of the chemical and physical properties of homogeneous thermostable enzymes purified from *Bacillus stearothermophilus* by Pfueller and Elliott[4], Singleton *et al.*[5] and Howard and Becker[6] have failed to confirm previous proposals of different α helix content or extrinsic factors responsible for the observed greater thermal stability[3]. In fact, there appears to be no distinctive difference between the thermostable enzymes and similar enzymes purified from mesophilic microorganisms and the mechanism for thermostability remains to be elucidated.

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Bacillus stearothermophilus has been the organism of choice for the purification of thermostable enzymes because, until recently, it was the microorganism with the highest optimum growth temperature (55–65 °C depending on the strain)[7] and several enzymes have been extensively purified from *B. stearothermophilus* including α-amylase[4], glyceraldehyde-3-phosphate dehydrogenase[5,8], aspartokinase[9,10], malate dehydrogenase[11], amino peptidase[12] and pyrimidine nucleoside phosphorylase[13]. However, reliance on a single species of bacteria or a few less thermophilic species of the genus *Bacillus (e.g. Bacillus coagulans etc.)*[3] for the study of thermostable enzymes does not provide any possibility for the comparison of the same thermostable enzyme between widely differing microorganisms.

Thus the isolation of a Gram-negative, non-spore-forming, extremely thermophilic bacteria (*Thermus aquaticus* YT-1) by Brock and Freeze[14] and *Thermus* X-1 by Ramaley and Hixson[15] has permitted the purification and comparison of thermostable enzymes from apparently widely differing bacteria. The present report presents some of the properties of the highly purified NADP isocitrate dehydrogenase (EC 1.1.1.42) from *T. aquaticus* YT-1 and a comparison of its catalytic similarities with the highly purified enzyme from *Bacillus subtilis*-168 and partially purified enzyme from the photosynthetic flagellate algae *Chlamydomonas reinhardti* Y-2 as well as the NADP isocitrate dehydrogenase of *B. stearothermophilus* previously reported by Howard and Becker[6].

METHODS AND METERIALS

Organisms

T. aquaticus YT-1 was obtained from T. D. Brock (present address, University of Wisconsin, Madison, Wisc.). The *B. subtilis*-168 (wild type) was obtained from J. Spizizen (Scripps Clinic and Research Foundation, La Jolla, Calif.) and the *C. reinhardti* Y-2 from G. Hudock, Indiana University, Bloomington, Ind.

Media

The *T. aquaticus* cells were grown in a 0.3% (w/v) yeast extract, 0.3% (w/v) tryptone–salts medium[15] at 70 °C. *B. subtilis* cells were grown in a 0.02 M glucose, 0.05 M ammonium lactate–salt medium[16] at 37 °C. The cells were grown in a 189-l vat fermenter (Stainless and Steel Inc., St. Paul, Minn.) with vigorous agitation and aeration (6 ft^3 air per min). The cells were grown to the end of exponential growth, chilled to 10 °C and harvested by passage through a No. 61-G Cepa continuous centrifuge. The cells were suspended in 0.05 M potassium phosphate (pH 7.2), centrifuged (Serval refrigerated centrifuge, GSA rotor), and kept at −20 °C.

The *C. reinhardti* Y-2 cells were grown in the light at 25 °C in the high salt medium of Sueoka[17] supplemented with 0.2% sodium acetate, harvested by centrifugation, resuspended in 0.05 M potassium phosphate (pH 7.2), centrifuged again, and the cells stored at −20 °C.

Assay of isocitric dehydrogenase

Isocitric dehydrogenase was assayed with the substrate concentrations and pH (7.4) given by Hanson and Cox[18] for the *B. subtilis* isocitrate dehydrogenase. The assay was conducted at 25 °C for the *C. reinhardti* and *B. subtilis* enzymes and at

either 25 or 70 °C for the *T. aquaticus* isocitrate dehydrogenase. The assay was carried out in 1-ml cuvettes in a Gilford Model 2000 recording spectrophotometer at 340 nm with its thermal spacers connected to a Haake circulating water bath. The cuvettes were raised to the desired temperature by placing them in a thermal block heater prior to transfer to the spectrophotometer and the temperature inside the cuvette prior and following the reaction was measured with a Yellow Spring thermister probe. NADP was added just prior to the enzyme and the data reported are the initial velocities of the enzymatic reaction. One unit of enzyme is defined as that amount of enzyme causing the reduction of 1 μmole of $NADP^+$ per min. Protein was measured by the method of Lowry *et al.*[19] with bovine serum albumin as a standard.

Purification of T. aquaticus isocitrate dehydrogenase

250 g (wet wt) of *T. aquaticus* cells were suspended in an equal volume of 0.05 M potassium phosphate (pH 7.2). Acid-washed glass beads (75–150 μm Sigma Type I) were added to make a thick paste (2 vol. of bead to 1 vol. of suspended cell). The mixture was placed in the 200-ml beaker of a Vibrogen cell mill (Rho Scientific Inc.) and the cells disrupted at 4 °C by the vibratory action of the Vibrogen cell mill at its maximum speed (4500 strokes/min with an amplitude of 7 mm). The cell paste–glass beads were removed from the Vibrogen and 1–2 vol. of 0.05 potassium phosphate (pH 7.2) was added. The glass beads, unbroken cells and some cell debris were removed by centrifugation (7500 × *g* for 5 min in a Serval refrigerated centrifuge). The supernatant fraction was centrifuged for 120 min at 30 000 rev./min in a Spinco ultracentrifuge (No. 30 rotor, 78 000 × *g* average) and the precipitate fraction was discarded.

90% (v/v) acetone (cooled to −72 °C by passage through a dry ice–acetone bath) was added dropwise to the above supernatant fraction (in 0.5 (v/v) amounts). The material was stirred with a magnetic stirrer and the temperature maintained at −20 °C after the first addition of acetone. 30 min after each addition the precipitate was removed by centrifugation at 10 000 × *g* for 10 minutes at −20 °C (Servall refrigerated centrifuge, GSA rotor). The bulk of the carotenoid membrane fraction, that had not been previously removed by centrifugation, was rendered insoluble and removed by centrifugation at the low acetone concentrations whereas the isocitric dehydrogenase was precipitated at higher acetone concentrations (63–75% (v/v) 90% acetone).

The precipitates were dissolved in a minimal volume of 0.01 M potassium phosphate (pH 7.2) and dialyzed overnight against 2 l of the same buffer containing 1 mM mercaptoethanol. The fractions containing the isocitric dehydrogenase with a high specific activity were applied to a 6 cm × 90 cm column of fibrous DEAE-cellulose connected in series to a 5 cm × 125 cm column of fibrous DEAE-cellulose. Both columns had been preequilibrated with the above buffer. The isocitrate dehydrogenase was eluted with a 4-l linear gradient of potassium phosphate (pH 7.2) from 0.01 M to 0.5 M containing 1 mM 2-mercaptoethanol. The isocitrate dehydrogenase eluted as a single peak between 0.07 and 0.09 M potassium phosphate.

Solid $(NH_4)_2SO_4$ was added to the pooled fractions from the DEAE-cellulose containing the bulk of the isocitrate dehydrogenase activity while the pH was maintained at 7.2 by the addition of 50% (v/v) $(NH_4)_2SO_4$. (The pH was determined by the use of a glass electrode on a 1/20 dilution). The precipitates were dissolved in a

minimal volume of 0.05 M potassium phosphate (pH 7.2), and fractions containing the higher specific activities (60–85% saturated $(NH_4)_2SO_4$) were combined and passed through a 2.5 cm × 35 cm column of Sephadex G-25 containing 0.05 M Tris (pH 7.2), 0.05 M KCl, 1 mM 2-mercaptoethanol and 5 mM potassium phosphate.

The enzyme was then placed on a 2.5 cm × 50 cm column of calcium phosphate (brushite form) and eluted with a 2-l gradient of potassium phosphate (pH 7.2) from 5 mM to 0.2 M in the above Tris–KCl–mercaptoethanol buffer. The isocitrate dehydrogenase eluted at a phosphate concentration of 0.02 M.

The fractions from the calcium phosphate column containing more than 30% of the activity of the peak tube were combined and the enzyme concentrated by Lyphogel (Gelmann Co.). The enzyme solution was applied to a 2.5 cm × 50 cm column of Sephadex G-200 containing 0.05 M potassium phosphate (pH 7.2) and 0.05 M KCl and eluted with the same buffer.

The enzyme was applied to a 2.5 cm × 80 cm column of DEAE-Sephadex A-50 equilibrated with the above buffer and eluted with a KCl gradient to 0.5 M in 0.05 M potassium phosphate (pH 7.2). The fractions containing the highest activity were pooled, the enzyme concentrate by Lyphogel and stored at −20 °C.

Purification of the B. subtilis isocitrate dehydrogenase

The *B. subtilis* isocitrate dehydrogenase was purified by DEAE-cellulose chromatography, $(NH_4)_2SO_4$ fractionation, Sephadex G-200 gel filtration, DEAE-Sephadex and brushite chromatography as previously reported for *B. subtilis* nucleoside diphosphokinase[20] except that fractions containing the isocitrate dehydrogenase were combined and subjected to each subsequent step. The purified *B. subtilis* isocitric dehydrogenase was stored at −20 °C.

Purification of C. reinhardti isocitrate dehydrogenase

5–10 g of *C. reinhardti* cells were suspended in 2 vol. of 0.05 M potassium phosphate (pH 7.2) containing 1 mM 2-mercaptoethanol and disrupted by 1 min sonic treatment at 5 °C until microscopic observation showed no intact cells (3–4 treatments). Cell debris was removed by centrifugation at 105 000 × *g* for 90 min at 5 °C (Spinco refrigerated centrifuge).

The cell free extract (supernatant) was applied to a 2.5 cm × 50 cm G-200 Sephadex column containing 0.05 M potassium phosphate[1], 0.05 M KCl and 1 mM 2-mercaptoethanol. The enzyme was eluted with the same buffer and applied to a 0.9 cm × 50 cm column of DEAE-Sephadex A-50 with the same buffer and eluted with a 1-l gradient of KCl from 0.05 M to 0.5 M potassium phosphate with 1 mM mercaptoethanol. The fractions containing the highest activity of isocitric dehydrogenase were pooled, concentrated by Lypogel treatment and stored at −20 °C.

Because of the lability of the *C. reinhardti* isocitrate dehydrogenase the above purification was also carried out with 30% (w/v) glycerol and 1 mM citrate added to the buffers.

Molecular weight

The apparent molecular weight of the isocitrate dehydrogenase was determined by the Stokes radii obtained from Sephadex G-200 gel filtration of the purified enzymes in a 2.5 cm × 35 cm gel column[20]. The Sephadex G-200 was prepared in 0.05 M

potassium phosphate (pH 7.2, 0.05 M KCl), the fine particles were removed, and the column precalibrated with 5 mg of aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease, all added separately.

Determination of the michaelis constants

The apparent Michaelis constants (K_m values) and standard deviation of error of the three isocitrate dehydrogenases for substrates were calculated from velocity *vs* substrate data by the use of a Wang 700 computer and the manufacturer's suggested program. The data were also examined by preparation of Lineweaver–Burk double reciprocal plots ($1/v$ *vs* $1/S$, not shown) for the absence of substrate inhibition.

Synthesis and purification of oxalomalate (α-hydroxy-β-oxalosuccinate) and γ-hydroxy-α-ketoglutarate

0.1 mmole of oxaloacetate (13.2 mg) and 0.1 mmole of glyoxylate (92 mg) were dissolved in 10 ml of distilled–deionized water and the pH adjusted to 6.8 with 1 M KOH[21]. The solution was kept at 37 °C for 2 h, diluted to 400 ml at 4 °C and placed on a 1 cm × 30 cm column of Dowex-1 in the carbonate form (prepared by washing with 1 M NH_4HCO_3 containing 15 ml of NH_4OH per l and then washed until the effluent was neutral). The oxalomalate was eluted with a linear gradient of triethylamine removed by evaporation *in vacuo* at 4 °C. The oxalomalate was measured by formation of the 2,4-dinitrophenylhydrazone of its decarboxylated product γ-hydroxy-α-ketoglutarate and identified by paper chromatography in butanol saturated with 3% (v/v) NH_3 (ref. 22). The γ-hydroxy-α-ketoglutarate was prepared by acidification of the oxalomalate to pH 1.5 followed by adjustment of the pH to 7.0 (ref. 21).

Chemicals

Fibrous DEAE-cellulose (Whatman DE-23) was obtained from Reeve Angel. DEAE-Sephadex A-50, Sephadex G-25 and G-200 were obtained from Pharmacia Fine Chemicals. Isocitrate, NAD and NADP were obtained from Sigma Chemical Co. $(NH_4)_2SO_4$ (enzyme grade) was from Mann Research Laboratories. The yeast extract and tryptone or Peptone No. 50 for growth of the *T. aquaticus* was obtained from Difco and General Biochemicals. All other chemicals were of reagent grade and water used was building distilled water which was passed through two Continental deionizers in series.

RESULTS

Purification of the NADP isocitrate dehydrogenases

Tables I and II show a summary of the purification of NADP-specific dehydrogenase from *T. aquaticus* YT-1 and *C. reinhardti* Y-2. The *T. aquaticus* enzyme was purified 224-fold from the cell free extract. However, there are still several minor additional protein bands seen after polyacrylamide disc-gel electrophoresis. The *C. reinhardti* enzyme was purified 34-fold and represents only a partially purified preparation. The *C. reinhardti* enzyme was quite labile and further purification resulted in considerable loss of activity. Isocitrate dehydrogenase was purified 500-fold from *B. subtilis*-168 during the purification of nucleoside diphosphokinase[20] and co-

TABLE I

SUMMARY OF THE PURIFICATION OF *T. aquaticus* ISOCITRATE DEHYDROGENASE

Procedure	*Units (μmoles/min)*	*Protein (mg)*	*Spec. act. (units/mg protein)*	*Yield (%)*
76 000 × *g* supernatant	591	9.700	0.061	100
63–75% (v/v) acetone	412	2.180	0.19	70
DEAE-cellulose	309	150	2.1	52
$(NH_4)_2SO_4$ (60–85% satd)	213	89	2.4	36
Brushite	109	14	7.8	18
Sephadex G-200	81	7.4	10.9	14
DEAE-Sephadex	52	3.8	13.7	8.8

TABLE II

SUMMARY OF THE PURIFICATION OF *C. reinhardti* ISOCITRATE DEHYDROGENASE

Procedure	*Units (μmoles/min)*	*Protein (mg)*	*Spec. act. (units/mg protein)*	*Yield (%)*
105 000 × *g* supernatant	14.8	197	0.075	100
Sephadex G-200	10.2	20	0.512	69
DEAE-Sephadex	5.08	2.5	2.58	34

migrated with the major protein band during gel electrophoresis. However, it is not completely homogeneous.

Apparent molecular weight

Fig. 1 shows the results of Sephadex G-200 gel filtration of the three isocitrate dehydrogenases and indicates an apparent molecular weight of 60 000–70 000 for the YT-1 enzyme, 75 000–80 000 for the *B. subtilis* enzyme and 90 000–100 000 for the *C. reinhardti* enzyme.

Optimum reaction temperature

Fig. 2 shows the effect of temperature on the initial velocity of the reaction catalysed by the NADP isocitrate dehydrogenases purified from the three microbial sources. The "optimum reaction temperature" of each of the isocitrate dehydrogenases is a reflection of the optimum growth temperature of the microorganism from which it was purified with the "optimum reaction temperature" of the partially purified enzyme higher than the optimum growth temperature of the organism. However the term optimum reaction temperature is misleading because at higher temperatures, thermal denaturation of the enzyme results in an overall decrease in the apparent enzyme velocity and thus the actual value of the "optimum temperature" for an individual enzyme has no quantitative significance[23].

The apparent Arrhenius activation energies for the three isocitrate dehydrogenases are similar; 10 000 cal for the *C. reinhardti* enzyme, 11 000 cal for the *B. subtilis* enzyme and 12 500 calories for the *T. aquaticus* enzyme.

Enzyme thermostability

The *T. aquaticus* isocitrate dehydrogenase is much more thermostable than the

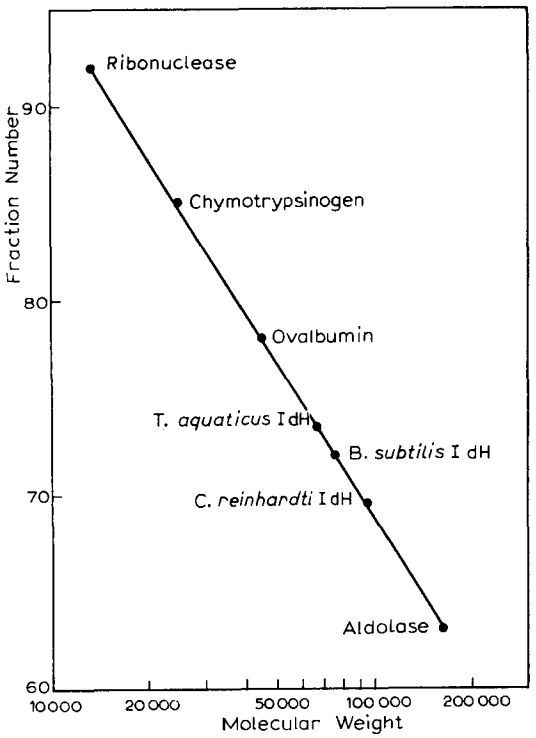

Fig. 1. Determination of apparent molecular weight of the *T. aquaticus*, *B. subtilis* and *C. reinhardti* isocitrate dehydrogenase by Sephadex G-200 gel permeation column chromatography.

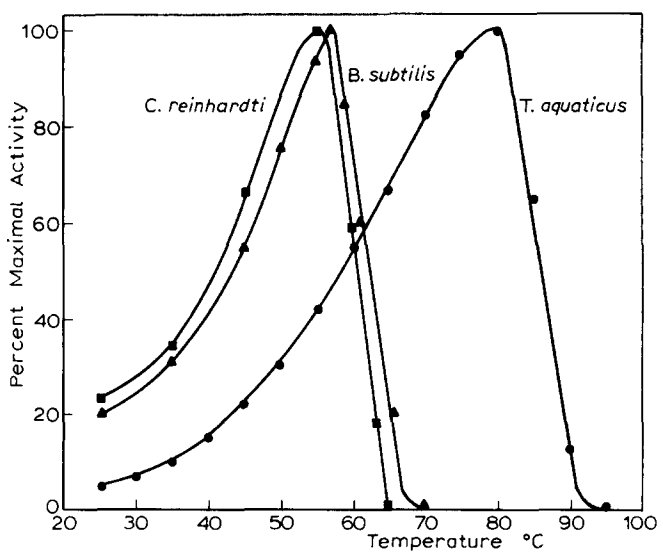

Fig. 2. Effect of temperature on the rate of reaction catalyzed by the *T. aquaticus*, *B. subtilis* and *C. reinhardti* isocitrate dehydrogenase.

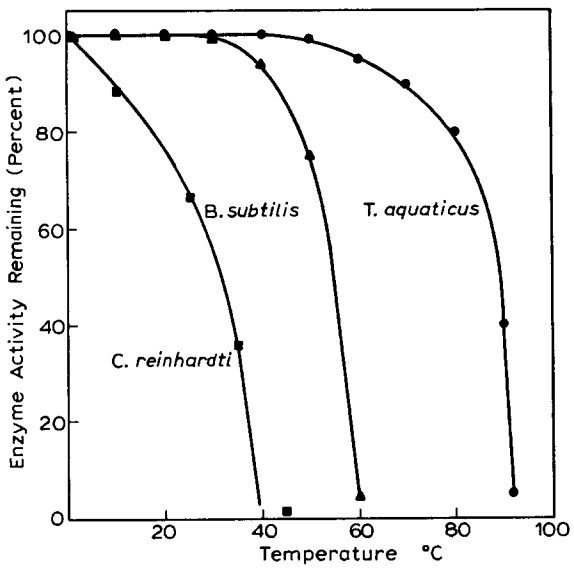

Fig. 3. Stability of the *T. aquaticus*, *B. subtilis* and *C. reinhardti* isocitrate dehydrogenase. The purified enzyme was incubated for 1 h at the indicated temperature in 0.05 M potassium phosphate (pH 7.2) at a protein concentration of 10 μg/ml protein for the *B. subtilis* and *T. aquaticus* enzyme and 75 μg/ml protein for the *C. reinhardti* enzyme. 0.01–0.10 ml was removed after one hour cooled to 25 °C and assayed for enzyme activity at 70 °C for the *T. aquaticus* enzyme and 25 °C for the *B. subtilis* and *C. reinhardti* enzyme.

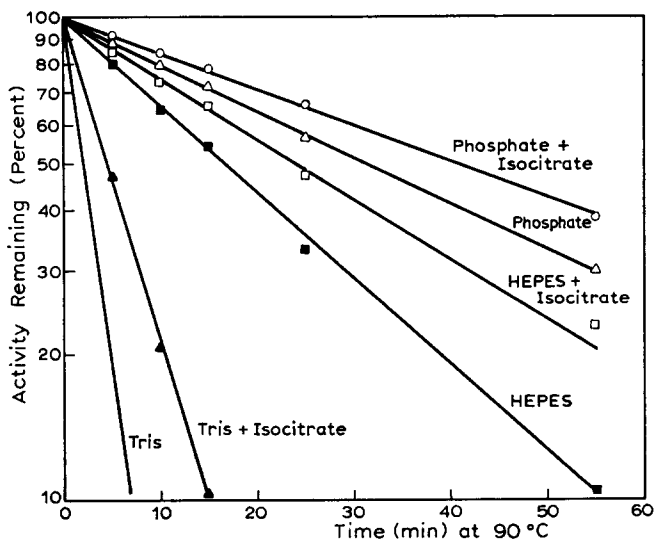

Fig. 4. Thermostability of *T. aquaticus* isocitrate dehydrogenase activity at 90 °C and protection by phosphate and isocitrate 10 μg/ml of purified *T. aquaticus* (2 ml) were incubated at 90 °C. 0.1-ml samples were removed at the times indicated, cooled to 2 °C and assayed for enzyme activity.

enzyme from either *B. subtilis* or *C. reinhardti*. Fig. 3 shows the enzyme activity remaining after 1 h incubation in 0.05 M potassium phosphate buffer at the indicated temperatures. The temperature at which 50% of the activity was lost was 30 °C for the *C. reinhardti* enzyme, 54 °C for the *B. subtilis* enzyme and 88 °C for the *T. aquaticus*. As has been observed for a number of other enzymes the rates of thermal inactivation of these three isocitrate dehydrogenases are strongly influenced by the composition of the incubation mixture as well as the protein concentration. Fig. 4 shows that isocitrate decreases the rate of thermal inactivation of the *T. aquaticus* enzyme at 90 °C and that thermal inactivation occurs much more rapidly in Tris buffer than in HEPES or phosphate buffer. Similar results have been obtained with the *B. subtilis* and *C. reinhardti* enzyme.

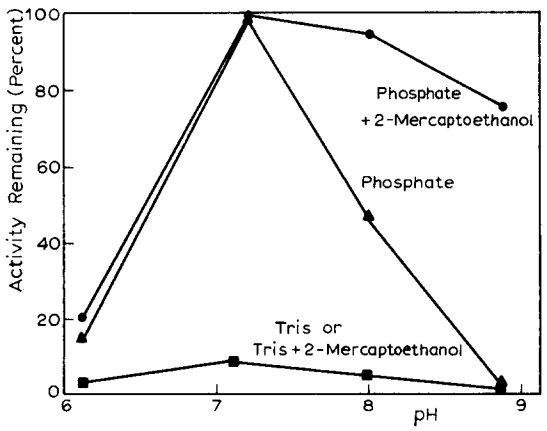

Fig. 5. Stability of *B. subtilis* isocitrate dehydrogenase at 4 °C and protection by phosphate and 2-mercaptoethanol. 10 μg of purified *B. subtilis* enzyme was diluted into 1 ml of the indicated buffer *plus* components. This 1-ml solution was placed in dialysis tubing and dialyzed for 24 h against 25 ml of the same buffer (wrist action shaker) for 24 h at 6 °C.

The *T. aquaticus* enzyme is also more stable to a wider range of pH values than is the *B. subtilis* or *C. reinhardti* enzyme. Fig. 5 shows *B. subtilis* enzyme activity remaining after dialysis of very low protein concentrations of enzyme for 24 h at 6 °C. There was almost complete loss of activity (98%) in the sample dialyzed against Tris. In the presence of phosphate, there was appreciable loss of activity at pH 6 and 8. 2-Mercaptoethanol provided some protection at the higher pH values. Table III shows the protective effect of isocitrate with the *B. subtilis* enzyme (pH of 8.5–8.8) and isocitrate and 2-mercaptoethanol were used in all of the buffers employed for the purification of all three enzymes. Fig. 6 shows the results of a similar experiment with the *C. reinhardti* and showed an appreciable loss of activity at dilute enzyme concentrations in the presence of phosphate even in the presence of isocitrate and 2-mercaptoethanol. Fig. 7 shows the results of the same experiment with the *T. aquaticus* enzyme. There was no detectable loss in enzyme activity between pH 6 and 9 after dialysis for 24 h at 6 °C. The effect of buffer and pH on the inactivation of the enzyme was demonstrated by heating the samples at 70 °C for 180 min to obtain partial inactivation. Under these conditions it is observed that a pH of 7 and phos-

TABLE III

EFFECT OF ISOCITRATE AND CITRATE ON THE STABILITY OF *B. subtilis* ISOCITRATE DEHYDROGENASE AT pH 8.5

B. subtilis isocitrate dehydrogenase was dialyzed 24 h at a protein concentration of 10 μg/ml as indicated in Fig. 5. 10^{-4} M 2-mercaptoethanol was present in all samples and 10^{-3} M isocitrate or citrate was added where indicated.

Buffer	*Addition*	*Relative activity*
0.1 M phosphate	—	0.560
0.1 M phosphate	isocitrate	1.00
0.1 M phosphate	citrate	0.860
0.1 M HEPES	—	0.13
0.1 M HEPES	isocitrate	0.34
0.1 M HEPES	citrate	0.28
0.1 M Tris	—	< 0.02
0.1 M Tris	isocitrate	< 0.02
0.1 M Tris	citrate	< 0.02

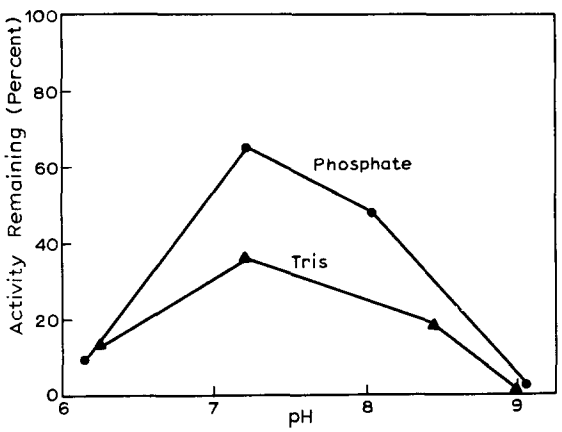

Fig. 6. Stability of *C. reinhardti* isocitrate dehydrogenase at 4 °C. Conditions of dialysis prior to assay where the same as in Fig. 5 except that the protein concentration was 1 mg/ml.

phate buffer provide the best buffer and pH for retention of *T. aquaticus* enzyme isocitrate dehydrogenase activity and thus all three enzymes are quite similar in that respect.

Catalytic properties of the enzyme

Table IV shows the apparent Michaelis constants (K_m values) of the three isocitrate dehydrogenases for D-*threo*-isocitrate and NADP at the indicated temperatures and concentration of the fixed substrate used in the standard assay. The enzymes used Mn^{2+} in preference to Mg^{2+} and there was no detectable activity with NAD. The pH optimum of the three enzymes was similar when corrected for the decrease in pH due to increase in temperature[24] and was 7.5. HEPES appeared to give a slightly higher rate of enzyme activity than did Tris and seemed to shift the pH optimum slightly to a more alkaline pH.

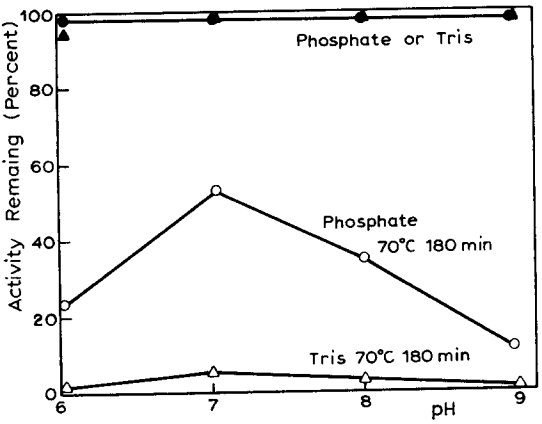

Fig. 7. Stability of *T. aquaticus* isocitrate dehydrogenase at 4 °C. Conditions of dialysis prior to assay were the same as in Fig. 5 except that the protein concentration was 5 μg/ml.

TABLE IV

APPARENT MICHAELIS CONSTANTS (K_m VALUES) FOR ISOCITRATE AND NADP

Enzyme source	*Assay temperature (°C)*	K_m *(μM)*	
		Isocitrate	*NADP*
T. aquaticus	25	9.7 ± 1.4	16.5 ± 4.7
	70	18.1 ± 4.7	31.0 ± 8.0
B. subtilis	25	9.1 ± 3.0	27.4 ± 5.7
C. reinhardti	25	12.5 ± 4.0	10.0 ± 3.2

TABLE V

OXALOACETATE *plus* GLYOXYLATE INHIBITION OF ISOCITRATE DEHYDROGENASE ACTIVITY

The *T. aquaticus* enzyme inhibition was determined at 70 °C. The *B. subtilis* and *C. reinhardti* enzyme inhibition were determined at 25 °C.

Concentration of inhibitor (mM)	*Inhibition (%)*		
	Oxaloacetate only	*Glyoxylate only*	*Oxalacetate + glyoxylate*
(a) T. aquaticus			
10	34.3	15.4	93.5
1.0	1.4	1.4	80.5
0.5	0	1.4	47.8
0.1	0	0	9.6
(b) B. subtilis			
10	34.6	12	89.7
1.0	2.0	4	61.5
0.5	0	0	38.0
0.1	0	0	5.0
(c) C. reinhardti			
10	32.6	8.3	100
1.0	18.2	0	68.6
0.5	6.4	0	48.6
0.1	0	0	22.7

Inhibition by glyoxylate and oxaloacetate

Table V shows the inhibition of the three isocitrate dehydrogenases by glyoxylate and oxaloacetate. However, it is not certain whether the oxaloacetate and glyoxylate act as concerted inhibitors of isocitrate dehydrogenase[25] or whether the inhibition is due to condensation and formation of oxalomalate[21-22] which is formed non-enzymatically from oxaloacetate and glyoxylate or its decarboxylated product γ-hydroxy-α-ketoglutarate[28]. Table VI shows that oxalomalate and γ-hydroxy-α-ketoglutarate do inhibit the reaction. Oxalomalate is almost as effective an inhibitor as the combination of oxalacetate and glyoxylate, while α-hydroxy-α-ketoglutarate is not as inhibitory.

TABLE VI

INHIBITION OF *C. reinhardti* ISOCITRATE DEHYDROGENASE BY OXALOMALATE AND α-HYDROXY-α-KETOGLUTARATE

The inhibition was determined at 25°C.

Concentration of inhibitor (mM)	*Inhibition (%)*		
	Oxalacetate + glyoxylate	*Oxalomalate*	*γ-Hydroxy-α-ketoglutarate*
10	100%	100%	70.1%
1	68.6	52.3	24.7
0.5	48.6	46.6	6.2
0.1	22.7	11.4	0

DISCUSSION

The purification of the NADP isocitrate dehydrogenase from *T. aquaticus*, *B. subtilis* and *C. reinhardti plus* the characterization of the *B. stearothermophilus* NADP isocitrate dehydrogenase by Howard and Becker[6] permits a comparison of a single enzyme from organisms with different optimum growth temperatures. However a direct comparison is limited to some extent by the fact that these are different species of organisms and differences interpreted as being due to their growth temperatures may in fact be the result of some other evolutionary or physiological constraint.

The apparent molecular weight of the three isocitrate dehydrogenases are 60 000–70 000 for *T. aquaticus*, 75 000–80 000 for *B. subtilis* and 90 000–10 000 for *C. reinhardti*, compared with 87 000–92 600 for *B. stearothermophilus*, 80 000 for *Azotobacter vinelandii*[29-30] and *Escherichia coli*[31]. In our examination of other enzymes of *T. aquaticus* the molecular weight of the enzymes tends to be slightly less than the molecular weight of the same enzymes from mesophilic organisms.

The most significant difference between the isocitric dehydrogenases of *T. aquaticus*, *B. subtilis* and *C. reinhardti* was the greater thermostability of the *T. aquaticus* enzyme compared to the *B. subtilis* enzyme and the lability of the *C. reinhardti* enzyme. This is in agreement with previous studies with *B. stearothermophilus* showing that enzymes from this moderate thermophile were more stable than the enzymes of the mesophile *Bacillus cereus*[1]. Thus enzymes from *T. aquaticus* (optimum growth temperature 70 °C) are more stable than enzymes from *B. subtilis* (optimum growth temperature 37 °C) or *C. reinhardti* (recent observation suggests that *C. reinhardti* may have an optimum growth temperature of 30–35 °C rather than 25 °C

commonly used; Togasaki, R., unpublished). However, it should be noted that the enzyme from *C. reinhardti* is much less pure than the other two enzymes and hence, since higher protein concentrations are present in these incubations, the comparative stability for the *C. reinhardti* enzyme may be even less than presently reported.

Isocitrate, but not NADP, showed protection against enzyme inactivation for all three isocitrate dehydrogenases as was observed for the *B. stearothermophilus* enzyme[6]. (The protection in phosphate buffer or with 2-mercaptoethanol appears to be a general protection and we have observed this with other enzymes). The rate of thermal inactivation of the isocitrate dehydrogenase *in vitro* is dependent on the presence of substrates and other components, as well as protein concentration, during the inactivation. Thus the temperature of 50% inactivation should probably be regarded only as an *in vitro* estimate of the enzyme's relative thermostability[32].

The three isocitrate dehydrogenases have similar Michaelis substrate constants (K_m values) and the K_m of the *T. aquaticus* enzyme for isocitrate and NADP was higher at 70 °C than at 25 °C as was reported for the *B. stearothermophilus* isocitrate dehydrogenase when the temperature was increased from 36 to 50 °C (ref. 6). All three enzymes showed inhibition by oxaloacetate and glyoxylate only when they were added together as has been reported for the isocitrate dehydrogenase from protozoa[26,27] and other bacteria[25,33,34]. Aside from the increased thermostability, the *T. aquaticus* YT-1 isocitrate dehydrogenase does not seem to differ from previously isolated isocitrate dehydrogenase.

It is not completely clear that the inhibition of the NADP isocitrate dehydrogenase is due to concerted inhibition by oxaloacetate and glyoxylate. DePamphilus[35] has studied the kinetics of the *B. subtilis* enzyme in some detail and has reached the conclusion that there is an ordered enzyme reaction sequence in which NADP binds preferentially to the free enzyme. Glyoxylate *plus* oxaloacetate acts as an inhibitor for the NADP enzyme and the inhibition is reversed by isocitrate. There was no evidence of an allosteric type of inhibition. The three isocitrate dehydrogenase in the present report all showed an appreciable inhibition by oxalomalate or γ-hydroxy-α-ketoglutarate when low levels of isocitrate were used and acted as competitive inhibitors for isocitrate.

Although only a few enzymes have been purified from *T. aquaticus* (aldolase[36], ω-amidase[37] and amino acid-activating enzymes[32]) or the *Thermus* X-1 isolate[15] (threonine deaminase[39], lactic dehydrogenase and fumarase[40]), it appears that these new bacteria will provide a valuable additional source of "thermostable" enzymes for comparative enzyme studies[41].

The essential problem in the study of thermobiology and these more thermostable enzymes is the physical–chemical basis for their thermostability. Since studies to date have not elucidated the basis for thermostability of enzymes from thermophilic bacteria[4–6], we have begun to approach the problem through the study of possible regulatory enzymes in these thermophiles and hence our interest in the NADP isocitrate dehydrogenase as a regulatory enzyme.

Brock in 1969 (ref. 42) suggested "it is possible that induced fit and allosteric interactions between proteins and small molecules require a certain flexibility of structure with a highly cross-linked, rigid and hence heat-stable protein—thus thermophiles may have sacrificed efficiency and control of enzyme function in order to grow at high temperatures" and indeed the aspartokinase[9] and threonine de-

aminase[10] of *B. stearothermophilus* shows less regulation at higher temperatures. However other enzymes (*e.g.* ribonucleotide reductase from *Thermus* X-1; Sando, G. and Hogenkamp, H., personal communication) show increased regulation at 70 °C.

Thus detailed physical and conformational study of the regulatory enzymes of these new extremely thermophilic gram-negative bacteria will be of extreme interest not only in the study of regulatory proteins but perhaps also in providing a clue to the basis for their thermostability.

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