A Thermostable Enolase from the Extreme Thermophile *Thermus aquaticus* YT-1†

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ABSTRACT: A procedure for the purification of enolase from the extreme thermophile *Thermus aquaticus* is described. The enzyme exhibits markedly enhanced stability to thermal inactivation and to inactivation by the denaturants guanidine hydrochloride and ethanol relative to rabbit muscle and yeast enolases. The optimum temperature for catalysis occurs at an assay temperature of 90°. The native protein has the hydrodynamic properties of a globular protein and consists of eight apparently identical polypeptide chains each having a molecular weight of 44,000. No significant changes in the sedimentation coefficient, near-ultraviolet absorption spectrum, far-ultraviolet optical rotatory dispersion, or the apparent K_M for the substrate or the cationic cofactor are detected in the temperature range 25–85°. Above 85°, increasing turbidity was observed indicative of protein aggregation.

Numerous reports have appeared in recent years describing the enhanced thermostability of the enzymic activity of proteins obtained from thermophilic microorganisms. We along with several other groups have chosen to purify a thermostable enzyme and to subject it to a detailed physical–chemical analysis in order to determine the structural basis for the enhanced thermostability. In order to magnify the degree of enhanced thermostability, we chose the extreme thermophile *Thermus aquaticus* as a source since fructose diphosphate aldolase partially purified from this organism has a temperature optimum for catalysis of about 90° (Freeze and Brock, 1970). The enzyme enolase was chosen for study since it is easily assayed at elevated temperatures and since the properties of enolases obtained from a variety of biological sources have been described (Wold, 1971), thereby facilitating comparative studies. This report describes the purification and gross properties of enolase obtained from *Thermus aquaticus* YT-1.

Materials and Methods

Materials. The trisodium salts of 2-phosphoglyceric acid and phosphoenolpyruvate were purchased from Boehringer and Soehne. The concentration of stock solutions of 2-phosphoglycerate were determined enzymically using rabbit muscle enolase, pyruvate kinase, and lactate dehydrogenase and measuring the oxidation of NADH spectrophotometrically. Reagent grade $MgSO_4$ was dried *in vacuo* over P_2O_5 prior to preparation of stock solutions. Ultra Pure guanidine hydrochloride was purchased either from Heico, Inc., or from Schwarz/Mann, and Ultra Pure Tris was obtained from Schwarz/Mann. Sodium dodecyl sulfate was obtained from Fisher Chem. Co. and recrystallized from ethanol. Hydroxylapatite, Bio-Gel HPT, was purchased from Bio-Rad. All proteins were purchased from the Sigma Chemical Co. except rabbit muscle aldolase and pyruvate kinase which were purchased from Boehringer and Soehne.

Growth of Thermus aquaticus. The organism was grown in a 14-l. fermentation Design Fermentor at 70°. The growth medium described by Brock and Freeze (1969) was used except that 4% soy broth was substituted for yeast extract to avoid contaminating organisms and 5–7 g of glucose/l. of medium was added to improve the yield. About 75–100 g wet weight of cells were harvested from 12 l. of growth medium within 8–12 hr after inoculation with an actively growing culture.

Purification of Enolase. All purification steps were performed at room temperature using a solvent consisting of 50 mM Tris-HCl buffer (pH 7.5) and 1 mM $MgCl_2$, denoted as standard buffer, unless noted otherwise. Protein concentrations were determined from the absorbancies at 260 and 280 nm (Warburg and Christian, 1942).

Cells were suspended in an equal volume of distilled water and homogenized in a Manton-Gaulin mill operated at 8000 psi. Cell debris and unbroken cells were removed by centrifugation at 27,300*g* for 1 hr at 4°. This method of cellular breakage liberated more enzymic units than procedures involving sonication or homogenization with glass beads in a micromill.

About 0.9 volume of 95% ethanol was added to the supernatant of the cellular homogenate at 4°. The resulting suspension was stirred gently for 0.5 hr and then centrifuged at 27,300*g* for 2 hr. Ethanol was found to be distinctly superior to other reagents such as protamine sulfate or ammonium sulfate for selective precipitation of the very viscous components in the centrifuged cellular homogenate. However, the volume fraction of ethanol required to achieve this fractionation varied among the homogenates processed and the volume fraction required for an aliquot of a given homogenate was found not to be necessarily appropriate for the whole homogenate. Therefore, each homogenate was titrated with ethanol to achieve the maximal purification.

A volume of 1-propanol equivalent to 40% the volume of the ethanol supernatant was added at 4°. The resulting sus-

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pension was stirred gently for 0.5 hr and then centrifuged at 7900*g* for 20 min. The precipitate was equilibrated overnight at 4° with a volume of standard buffer equal to the volume of the organism used for purification. Any insoluble material was removed by centrifugation. In contrast to the ethanol fractionation, the propanol fractionation was found to be quite reproducible.

The solubilized propanol precipitate was applied to a 6 × 55 cm DEAE-cellulose column equilibrated with standard buffer. The column was then washed with three to four column volumes of standard buffer and then developed with a linear 0–1 M KCl gradient contained in ten column volumes of standard buffer. Enolase activity eluted midway through the gradient and the fractions containing activity were pooled to give a total volume of about 1300 ml.

The pooled fractions were dialyzed against 10 mM phosphate buffer–1 mM $MgCl_2$ (pH 7.2) and then applied to a 4 × 45 cm hydroxylapatite column equilibrated with the same buffer. The column was washed with three column volumes of this buffer and then developed with a linear potassium phosphate gradient between 10 and 400 mM contained in ten column volumes. Enzymic activity eluted rather sharply near the beginning of the gradient. The active fractions were pooled and concentrated to 5 ml using an Amicon ultrafiltration cell and an XM-50 membrane.

The concentrated eluate was applied to a 2 × 90 cm column of Sepharose 6B equilibrated with standard buffer. Enolase activity emerged at approximately one-third of the total volume between the void volume and the included volume. The active fractions were pooled (approximately 150 ml) and dialyzed against 10 mM potassium phosphate buffer–1 mM $MgCl_2$ (pH 7.2).

The dialyzed eluate was applied to a 1.5 × 20 cm hydroxylapatite column equilibrated with 10 mM phosphate buffer–1 mM $MgCl_2$ (pH 7.2). The column was washed with three column volumes of this buffer and then developed with a linear phosphate gradient between 10 and 400 mM in 1 mM $MgCl_2$ contained in ten column volumes. Fractions containing >90% maximal activity were pooled and the phosphate was rapidly removed by exclusion chromatography using a column equilibrated with standard buffer. Removal of the phosphate was critical to inactivation of the purified enzyme by microbiological contaminants.

Enzymic Activity. The catalytic activity of *T. aquaticus* enolase was routinely measured by a continuous spectrophotometric assay at 70° using an assay solution containing 33 mM Tris-HCl buffer adjusted to pH 8.8 at 25° (pH 7.5 at 70°), 2 mM 2-phosphoglycerate, and 1 mM $MgCl_2$. Assays were performed in 1-ml Teflon-stoppered cuvets placed in a thermostatable cell holder in either a Cary Model 14 or Gilford Model 2000 recording spectrophotometer. The temperature of assay solutions was measured with a calibrated Yellow Spring Instrument thermister probe. The increase in absorbance at 230 nm due to formation of the product, phosphoenolpyruvate, was recorded for at least 2 min. The absorbance change per minute was converted to International enzyme units using an extinction for phosphoenolypyruvate at 70° of 2.90×10^3 M^{-1} cm^{-1}. The absorbance change at 230 nm exhibited a linear dependence on protein concentration up to at least 0.8 absorbance unit/min.

Either Tris-HCl or ammonium hydroxide buffers were used as indicated for enzymic assays at temperatures other than 70°. The pH of these buffers was adjusted at 22° such that the desired pH would be achieved at the assay temperature using a $\Delta pK/°C$ of 0.028 for Tris-HCl and 0.031 for ammonium hydroxide (Dawson *et al.*, 1969). In those measurements employing phosphate buffers, no pH correction was made since the p*K* of phosphate does not change more than 0.1 pH unit over the temperature range investigated. The extinction of the catalytic product, phosphoenolpyruvate, decreases nearly linearly with increasing temperature from 3.52×10^3 M^{-1} cm^{-1} at 10° to 2.81×10^3 M^{-1} cm^{-1} at 90°. Correction was also made for the pH dependence of the extinction of phosphoenolpyruvate. Cells with path lengths up to 50 mm were used to increase the observed absorbance changes at low substrate or $MgCl_2$ concentrations.

The enzyme activity of rabbit muscle enolase was assayed as described by Winstead and Wold (1966) and that of yeast enolase as described by Westhead and McLain (1966).

Amino Acid Analysis. Aliquots of protein were dialyzed exhaustively against distilled water and then hydrolyzed in 6 N HCl for 110° *in vacuo* for the indicated times. Performic acid oxidations were done using the procedure of Hirs (1967). Basic hydrolysis was done for 24 hr in 5 N NaOH as described by Hugli and Moore (1972). A Spinco Model 120C amino acid analyzer was used for all measurements.

Sedimentation Measurements. Sedimentation velocity measurements at room temperature were made using 12-mm double-sector aluminum-filled Epon centerpieces, sapphire windows, and a Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner and operated at 60,000 rpm. Photographs were taken with Kodak metallographic plates and analyzed with a Nikon microcomparator. Sedimentation coefficients were calculated as described by Schachman (1957).

Sedimentation velocity measurements at elevated temperatures were made using a titanium rotor and a Spinco high-temperature accessory. A double-sector aluminum centerpiece and high-temperature cell gaskets were employed. The rotor was preheated in an air oven to approximately the desired temperature prior to insertion of the cell and coupling to the drive motor.

Sedimentation equilibrium measurements were made using the meniscus depletion method of Yphantis (1964). Photographs were taken with Kodak II-G spectroscopic plates using interference optics. Molecular weights were calculated by a computer program which fits the data points by least squares to the standard expression for apparent weight-average molecular weight.

Electrophoresis. All electrophoretic measurements were done at room temperature using 9.5-cm polyacrylamide gels and a Buchler Polyanalyst. Stacking gels were omitted unless noted otherwise. Between 5 and 50 μg of purified enolase was subjected to electrophoresis at pH 7 using the procedure of Orr *et al.* (1972) and at pH 9.5 using the method of Davis (1965). The electrophoresis of dissociated enolase was measured in 0.1% sodium dodecyl sulfate as described by Laemmli (1970). Gels were stained for protein by immersion in Coomassie Blue and destained electrophoretically in methanol–acetic acid according to Weber and Osborn (1969). Relative mobilities in detergent gels were calculated according to Weber and Osborn (1969) using vernier calipers to measure gel dimensions.

Viscometry. Viscometric measurements were made at 22° with a Cannon-Ubbelohde 1-ml dilution viscometer with outflow times of about 80 sec for dilute aqueous solutions. Outflow times were measured to the nearest 0.01 sec, and measurements were continued until at least five consecutive times were within ±0.05 sec of the average of these readings. The viscometer and solution were equilibrated for at least 20 min

TABLE I: Purification of Enolase from *T. aquaticus*.

Fraction	Vol (ml)	Total Protein (mg)	Total Units (IU)	Sp Act. (IU/mg)	Purifcn (-fold)	Recov (%)
Homogenate	1540	63,900	60,000	0.95	1	100
Ethanol supernatant	2220	26,700	48,800	1.8	1.9	81
Propanol precipitate	500	11,900	47,500	4.0	4.2	79
DEAE-cellulose eluate	1315	1,190	36,300	30.5	32.1	60
Hydroxylapatite eluate I	166	138	23,800	173	182	40
Sepharose 6B eluate	156	24.7	18,000	727	765	30
Hydroxylapatite eluate II	28	17.7	15,900	900	947	26.5

before starting measurements. A Neslab TV 70/150 water bath was used, which maintained the temperature within ±0.02°.

Spectral Measurements. Difference absorption measurements between aliquots of an enolase solution at room temperature and at elevated temperatures were made over the range 250–350 nm using a Cary Model 14 recording spectrophotometer equipped with a thermostatable sample cell holder. The same solution was heated to increasingly higher temperatures and difference spectra were recorded at appropriate intervals using a full-scale range of 0.1 absorbance unit.

Optical rotatory dispersion measurements were made over the range 200–250 nm at approximately 10° intervals between 25 and 95°. Solutions and solvents were placed in thermostatable cylindrical cells and equilibrated at each temperature prior to measurements. Similar measurements were made in circular dichroism mode but were limited to 25° since the design of the Cary Model 60 CD spectropolarimeter precludes circular dichroic measurement above 50°.

Results

The analytical details of a purification of enolase from 250 g wet weight of *T. aquaticus* are shown in Table I. The purified protein was usually homogeneous as judged by the appearance of a single band when examined by polyacrylamide gel electrophoresis at pH 7.0 and at pH 9.5, by a single sedimenting component when examined by sedimentation velocity and by a linear ln d *vs.* r^2 plot after equilibrium sedimentation. In some preparations it was found necessary to repeat the second hydroxylapatite chromatographic step in order to attain this homogeneity. While the cell preparations appeared homogeneous by the electrophoretic and sedimentation criteria, their specific activities ranged from about 450 to 950 IU/mg of protein.

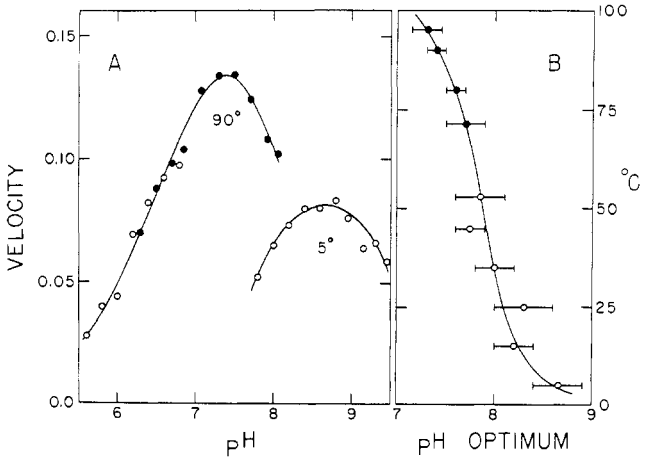

FIGURE 1: Effect of pH on the catalytic activity of *T. acquaticus* enolase: (A) the dependence of the catalytic rate on pH at the temperature extremes; (B) the effect of temperature on the pH optimum for catalysis. The bars indicate the pH range over which the rate of catalysis was essentially maximal at each temperature. All assay solutions contained 1 mM $MgCl_2$ and 2 mM 2-phosphoglycerate. The open circles refer to assay solutions buffered with 33 mM Tris-HCl and the filled circles represent assay solutions buffered with 33 mM NH_4OH-HCl.

Catalytic Measurements. The rate of catalysis of *T. aquaticus* enolase increases with increasing assay temperatures at pH 7.9 between 10 and 90°. Above 90° the rate of catalysis decreases sharply with increasing assay temperatures indicating the onset of rapid thermal inactivation. By contrast, rapid thermal inactivation of rabbit muscle enolase occurs at temperatures in excess of 44° (Bernfeld and Bieber, 1969) demonstrating the greatly enhanced thermostability of the enolase from the thermophile.

The pH optimum of *T. aquaticus* enolase was measured at a series of different assay temperatures in order to compare the effect of temperature on catalytic parameters of the enzyme. A typical bell-shaped dependence of the catalytic rate on pH was observed at all assay temperatures; the pH profiles obtained at 5 and 90° are illustrated in Figure 1A. For assay temperatures greater than 70° it was necessary to use NH_4OH–HCl buffers in order to maintain the pH in the range of interest. Assay solutions containing Tris-HCl or NH_4OH–HCl buffers gave comparable results as shown in Figure 1A in the pH range 6.3–6.8 at 90°. At each temperature the maximum catalytic rate was essentially constant over a limited pH range. The pH range of maximal activity decreased in a regular manner from about pH 9 to 7 as the assay temperature was increased from 5 to 95°, as shown in Figure 1B. All subsequent catalytic measurements were done within the pH optimum range for the assay temperature utilized.

The dependence of enzymic activity on the concentration of both substrate, 2-phosphoglycerate, and cofactor, Mg^{2+}, was measured at a series of assay temperatures. Metal-free enzyme was obtained by dialysis against several changes of 10 mM EDTA followed by dialysis against several changes of buffer; such enzyme preparations were inactive in the absence of added Mg^{2+}. The rate of catalysis was measured for a minimum of eight different ligand concentrations distributed equally about the apparent K_M value. The data were analyzed using a computer program which determined the weighted least-squares fit of the experimental values to a rectangular hyperbola as described by Lehner and Barker (1970). The kinetic data collection at all temperatures investigated de-

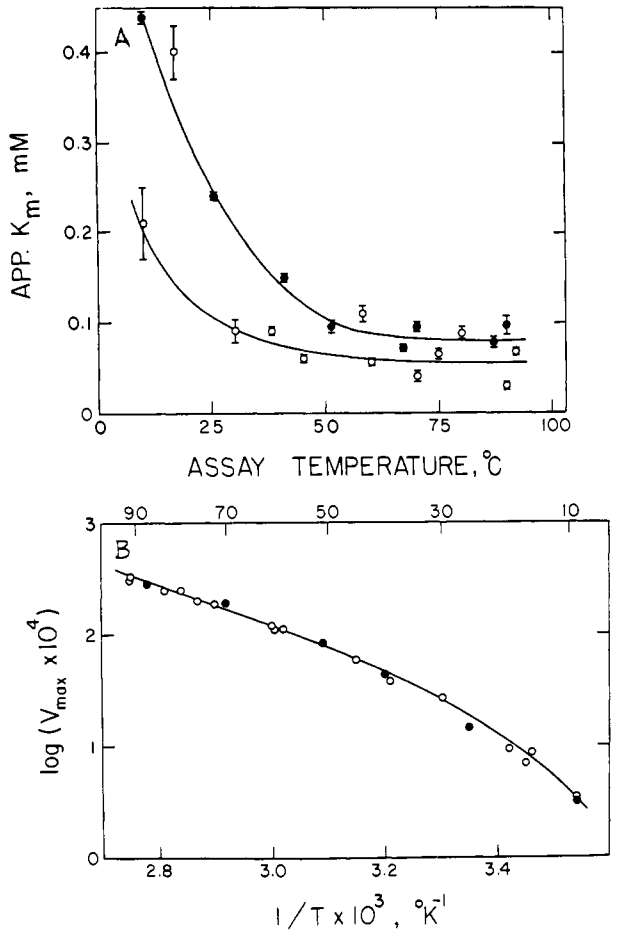

FIGURE 2: Effect of temperature on the Michaelis constants and maximum velocity of *T. aquaticus* enolase. The data points were derived from double-reciprocal plots of the dependence of rates of catalysis on ligand concentrations. The error bars indicate the standard estimate of error: (A) variation of the apparent K_M values with assay temperature; (B) Arrhenius plot of V_{max} values. The open circles refer to measurements with 2-phosphoglycerate as the variable ligand and the filled circles refer to Mg^{2+} as the variable ligand.

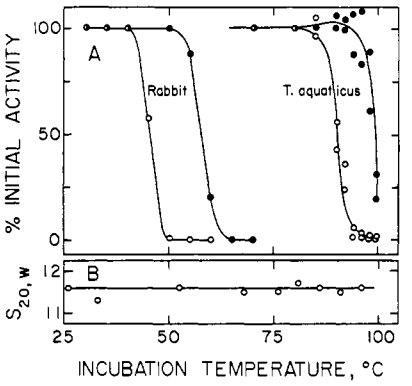

FIGURE 3: Effect of temperature on some properties of rabbit muscle and *T. aquaticus* enolase. (A) Irreversible thermal inactivation. Solutions of rabbit muscle and *T. aquaticus* enolases were each dialyzed at 4° against three changes in buffer solutions containing 10 mM EDTA followed by three or four changes of buffered solutions containing no EDTA or added metal ions. The buffer for rabbit muscle enolase was 30 mM potassium phosphate (pH 6.9) and that for *T. aquaticus* enolase was 33 mM NH_4OH–HCl (pH 9.3) (25°). Neither solution exhibited any catalytic activity in assay solutions devoid of $MgCl_2$. Aliquots of each dialyzed enzyme solution containing 15 μg/ml of protein in their respective buffers were incubated for exactly 5 min in screw-capped vials at the indicated temperatures and then quickly cooled in an ice–water bath. A parallel set of incubations was performed with each dialyzed enzyme in buffered solutions containing either 2 mM $MgCl_2$, 2 mM 2-phosphoglycerate, or both. Rabbit muscle enolase was assayed at 25° while *T. aquaticus* enolase was assayed at 70°. The open circles refer to preincubation solvents containing buffer with and without 2-phosphoglycerate. The filled circles refer to preincubation solvents containing buffer and $MgCl_2$ both with and without 2-phosphoglycerate. (B) Effect of temperature on the sedimentation coefficient of *T. aquaticus* enolase. The solvent contained 10 mM potassium phosphate buffer (pH 7.5) and 100 mM KCl. The protein concentration was 4.5 mg/ml. Four different aliquots of the protein solution were used to collect the data points shown. Each aliquot was sedimented initially at a temperature below 80°. The rotor was then moved from the centrifuge, inverted to remix the solution, reequilibrated at a temperature above 80°, and spun at that temperature.

scribed linear double-reciprocal plots and the standard error of the K_M values was less than ±10% in all measurements except one. As shown in Figure 2A, the calculated K_M values for both 2-phosphoglycerate and $MgCl_2$ decrease with increasing assay temperatures up to 50° and then remain constant above this temperature.

Maximal velocity values were also calculated from the rate data described above and normalized to a constant enzyme concentration. These values describe a curvilinear Arrhenius plot over the temperature range 10–50° which becomes linear above 50° as shown in Figure 2B. The limiting slope at temperatures above 50° corresponds to an activation energy of 5.8 kcal/mol which increases by a factor of 3 as the assay temperature is reduced to 20°.

The resistance of the catalytic activities of *T. aquaticus*, rabbit muscle and yeast enolase to conditions commonly used to denature proteins were compared. These comparisons were made by exposing aliquots of each enzyme to increasingly rigorous denaturing conditions and then measuring the catalytic activity remaining under standard assay conditions. The denaturant which produces a 50% irreversible loss of the initial catalytic activity is termed the $A_{1/2}$ value.

The response of the catalytic activity of rabbit muscle and *T. aquaticus* enolase to preincubation of each enzyme for 5 min in various solvents at various temperatures is shown in Figure 3A. While the presence of 2-phosphoglycerate offered no protection to irreversible inactivation, Mg^{2+} increased the $A_{1/2}$ temperature between 10 and 13°. The initial activity of *T. aquaticus* enolase is completely retained after incubation in Tris-HCl buffer for at least 2 hr at 75 and 85°. This result does not necessarily mean that no conformational changes occurred during these incubations but that any changes affecting the catalytic site are reversed upon cooling to 70°. Preincubations of the enzyme at the $A_{1/2}$ temperature, 90°, or at 95° resulted in a progressive irreversible inactivation with increasing incubation time. Within the experimental variation, ±5%, the irreversible inactivation at 90 and 95° is first order.

The catalytic activity of *T. aquaticus* enolase is also more stable in the presence of protein denaturants at 25° than is either rabbit muscle or yeast enolase. As shown in Figure 4A, the $A_{1/2}$ for both rabbit muscle and yeast enolase occurs in 0.5 M guanidine hydrochloride while the $A_{1/2}$ for *T. aquaticus* enolase requires 1.8 M guanidine hydrochloride. It should be noted that only 75% of the yeast enolase can be irreversibly inactivated by guanidine hydrochloride while both rabbit muscle and *T. aquaticus* enolase are totally irreversibly inactivated by this solvent.

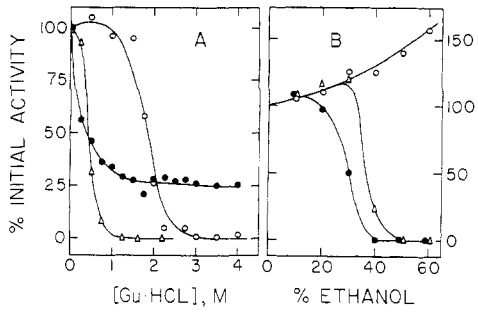

FIGURE 4: Effects of denaturants on the activity of enolases at 25°. (A) Guanidine hydrochloride: enolase solutions were incubated in buffered solutions containing the indicated concentrations of guanidine hydrochloride for 1 hr. Aliquots were then removed for enzymic assay. Sufficient guanidine hydrochloride was added to each assay solution so that the final concentration was 90 mM. (B) Ethanol: enolase solutions were incubated in buffered solutions containing the indicated volume per cent ethanol for 2–16 hr. (○) *T. aquaticus* enolase was incubated in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MgCl_2$. (●) Yeast enolase was incubated and assayed in 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM $MgCl_2$. (△) Rabbit muscle enolase was incubated and assayed in 50 mM imidazole buffer (pH 6.9) containing 1 mM $MgCl_2$.

As shown in Figure 4B, *T. aquaticus* enolase is increasingly activated by exposure to increasing concentrations of ethanol up to at least 60 vol %, while both yeast and rabbit muscle enolase are irreversibly inactivated in this concentration range of ethanol. The $A_{1/2}$ values for yeast and rabbit muscle enolases occur at 30 and 37% ethanol, respectively. Both rabbit muscle and yeast enolase are completely irreversibly inactivated by preincubation for 2 hr in a 0.3% solution of the nonionic detergent Brij (poly(oxyethylene lauryl alcohol)). By contrast, *T. aquaticus* enolase remains fully active after preincubation for up to 4 hr in 1% Brij 35.

Physical–Chemical Measurements. T. aquaticus enolase sediments as a single apparently symmetrical boundary in 50 mM Tris-HCl and 1 mM $MgCl_2$ (pH 7.5), over the protein concentration range 0.5– 4.5 mg/ml. The dependence of the sedimentation coefficient on concentration can be expressed as: $s_{20,w} = 12.7\ S\ (1 - 0.02c)$, where c is the protein concentration in milligrams per milliliter and $s_{20,w}$ is the sedimentation coefficient in Svedbergs. The $s_{20,w}$ at infinite dilution, $s^0_{20,w}$ is 12.7 ± 0.3 S, a value obtained by least-squares analysis of the experimental values. The effect of increasing temperature on the sedimentation coefficient of the native protein is shown in Figure 3B. In calculating these sedimentation coefficients, it was assumed that the $\bar{v}$ of the protein is independent of temperature. If however, it is assumed that the $\bar{v}$ of enolase has a thermal coefficient of 4.35×10^{-4} ml/deg, the average value measured for three other proteins (Bull and Breese, 1968), the calculated $s_{20,w}$ would then increase in a linear fashion from 11.6 S at 20° to 13.6 S at 96°. Only one sedimenting boundary was observed throughout the temperature range examined. However, turbidity was visible in the protein solutions at temperatures of 86° and above.

Acidification of a solution of enolase containing 1.5 mg/ml protein in 50 mM KCl at neutral pH by addition of HCl to pH 2.0 lowers the sedimentation coefficients of the protein from 12.3 to 3.3 S. Rapid neutralization of an aliquot of this acidified solution produced a turbid suspension which was enzymically inactive. Although dialysis of another aliquot of the acidified protein solution for 2 days against several charges of 50 mM KCl–5 mM phosphate buffer (pH 7.3) also produced turbidity, the soluble protein which accounted for less than 50% of the total protein had a specific activity and sedimentation coefficient characteristic of the native enzyme.

Solid guanidine hydrochloride was added to a solution of the native protein to give a final concentration of 6 M after adjustment of the pH to 7.5. In this solvent the protein had a sedimentation coefficient of 1.7 S at a concentration of 1.5 mg/ml.

The molecular weight of native *T. aquaticus* enolase was measured by equilibrium sedimentation using the meniscus depletion method of Yphantis (1964). A total of six measurements were made using aliquots from five different enzyme preparations. The initial protein concentration was 0.25 mg/ml, the temperatures ranged from 20 to 22°, the rotor speed ranged from 11,000 to 14,000 rpm, the sedimentation time from 20 to 24 hr and the solvent was either 75 mM potassium phosphate buffer (pH 7.5) or 33–50 mM Tris-HCl buffer (pH 7.5) containing 100–200 mM KCl and 1 mM $MgCl_2$ (pH 7.5). In all six measurements, the protein gradient at equilibrium described a linear ln d *vs.* r^2 plot. An average molecular weight of $3.28 \pm 0.12 \times 10^5$ g/mol was calculated from the six equilibrium gradients. In making these calculations, a $\bar{v}$ of 0.728 ml/g derived from the amino acid composition was used.

The molecular weight of the native enzyme was also measured by electrophoresis of the protein in polyacrylamide gels having different porosities according to the procedure described by Hedrick and Smith (1968). The variation in the mobility of enolase in gel concentrations of 4, 5, 6, and 7% at pH 9.5 corresponded to a molecular weight of $3.55 \pm 0.05 \times 10^5$ g/mol in two measurements. Xanthine oxidase, urease, and phosphorylase *a* were used as calibration proteins for this procedure.

Four equilibrium sedimentation measurements of enolase obtained from three different enzyme preparations were made in the presence of 6 M guanidine hydrochloride adjusted to pH 7.5. Solutions containing 0.25 mg/ml of protein were sedimented for 22–24 hr at either 36,000 or 40,000 rpm at 22°. The protein gradient observed at equilibrium described a linear ln d *vs.* r^2 plot in each measurement. Assuming no preferential binding of solvent, the molecular weight was calculated to be $4.36 \pm 0.52 \times 10^4$ g/mol. A single equilibrium sedimentation measurement of the enzyme in 5 mM potassium phosphate and 50 mM KCl adjusted to pH 2 with HCl gave a molecular weight of 4.40×10^4 g/mol. In this measurement, a 0.26-mg/ml of protein solution was sedimented for 25 hr at 34,000 rpm and 22°.

The molecular weight of the dissociated protein was also determined by measuring its electrophoretic mobility in calibrated polyacrylamide gels containing sodium dodecyl sulfate. Both the continuous phosphate buffer system described by Weber and Osborn (1959) and the discontinuous Tris–glycine buffer system described by Laemmli (1970) were employed. The protein migrated as a single sharp band whose mobility corresponded to a molecular weight of $4.55 \pm 0.05 \times 10^4$ g/mol in five measurements. Bovine serum albumin, pyruvate kinase, pig heart fumarase, rabbit muscle aldolase, carbonic anhydrase, and myoglobin were used to calibrate the gels.

The reduced viscosity of native *T. aquaticus* enolase measured in 100 mM KCl, 50 mM Tris-HCl buffer, and 1 mM $MgCl_2$ (pH 7.5) is virtually independent of protein concentration over the range 1.0–4.5 mg/ml, giving an intrinsic viscosity of 6.0 ml/g. In the presence of 6 M guanidine hydrochloride adjusted to pH 7.5, the reduced viscosity exhibits a slight negative dependence on protein concentration in the range 0.5–2.0 mg/ml. Because

TABLE II: Amino Acid Analyses of *T. aquaticus* Enolase.

Amino Acids	Residues/10^5 g of Protein			
	24 hr[a]	48 hr[a]	72 hr[a]	Av
Alanine	95.5	92.0	93.7	93.7
Arginine	54.2	59.6	56.8	57.0
Aspartic acid	68.9	60.7	64.2	64.6
1/2-Cystine	nd[e]	nd	nd	0
Glutamic acid	128.5	127.4	125.1	127.0
Glycine	139.3	141.5	139.3	140.0
Histidine	14.8	16.1	15.4	15.3
Isoleucine	30.7	31.8	31.5	31.3
Leucine	79.4	78.6	79.9	79.3
Lysine	54.6	56.2	55.7	55.5
Methionine	10.2	7.3	10.3	10.2[c]
Phenylalanine	26.1	26.6	26.1	26.3
Proline	33.0	33.9	32.4	33.3
Serine	82.5	73.2	67.6	90.7[b]
Threonine	32.6	37.2	34.8	39.7[b]
Tryptophan	9.2[d]	na[e]	na	9.2
Tyrosine	15.4	14.2	13.2	16.4[b]
Valine	61.3	61.1	61.6	61.3

[a] Hydrolysis times in 6 N HCl. [b] Value obtained by extrapolation to zero hydrolysis time. The threonine analysis after 24-hr hydrolysis was not used. [c] Average of 24- and 72-hr hydrolysis values. The 48-hr hydrolysate contained some methionine sulfoxide. [d] Alkaline hydrolysis. [e] nd, none detected; na, not analyzed.

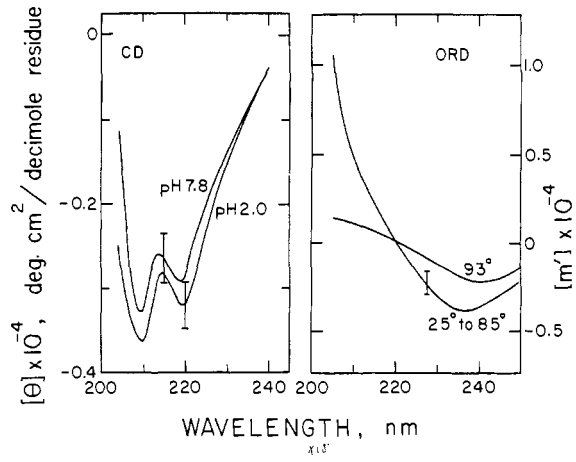

FIGURE 5: Spectropolarimetric measurements of *T. aquaticus* enolase. (A) Circular dichroic measurements: the solvent contained 2 mM Tris-HCl buffer and 6 mM KCl adjusted to either pH 2.0 or 7.5. The protein concentration was 140 μg/ml. Solutions were placed in a thermostatable cylindrical quartz cell having a path length of 10 mm and scanned at the rate of 4 nm/min using a full-scale range of 0.1°. The molecular ellipicity, $[\theta]$, has the units (deg cm²)/dmol of amino acid residue. (B) Optical rotatory dispersion measurements: the solvent contained 10 mM phosphate buffer and 100 mM KCl adjusted to pH 7.0. The protein concentration was 74 μg/ml. Measurements were made as above except using the optical rotatory dispersion mode. The reduced mean residue rotation, $[M']$, has the units (deg cm²)/dmol of amino acid residue.

of the limited protein available for irreversible denaturation studies the protein concentration range could not be extended to include higher values. An intrinsic viscosity of 34 ml/g was estimated from the measured values.

The absorbance spectrum of *T. aquaticus* enolase exhibits a maximum at 279 nm. At 1% solution of the protein has an extinction coefficient of 9.18 cm^{-1} at this wavelength. The differential refractiometric procedure of Babul and Stellwagen (1969) was used to measure the protein concentration. An $A_{280}:A_{260}$ ratio of 1.8 was routinely observed. No protein absorbance was detected between 310 and 800 nm. Increasing the temperature of a solution of enolase containing 0.82 mg/ml of protein 50 mM phosphate buffer (pH 7.5) resulted in a small uniform increase in the near-ultraviolet absorbance over the temperature range 25–85°. This increase could be accounted for by the thermal coefficient for the extinction of the constituent aromatic amino acid residues (Lehrer and Barker, 1971) Above 85° a marked increase in absorbance was observed which was characteristic of scattering.

The far-ultraviolet optical rotatory dispersion and circular dichroic spectra of enolase are shown in Figure 5. While both measurements give evidence of the presence of secondary structure, quantitative estimates of fractions of α-helical and β structure could not be made owing to excessive noise below 205 nm. The mean residue rotation observed at 233 nm corresponds (Chen and Yang, 1971) to a helical content of about 10%. As shown in Figure 5, the secondary structure of native enolase largely persists at pH 2.0 and at 85° at pH 7.5. Above 85° there was a progressive diminution in the optical rotatory dispersion.

Chemical Measurements. Aliquots of purified *T. aquaticus* enolase each containing 150 μg of protein were hydrolyzed in duplicate for 24, 48, or 72 hr in 6 N HCl at 110° *in vacuo* and then subjected to amino acid analysis. The duplicate analyses which generally varied by less than ±3% were averaged and are shown in Table II as the number of residues per 10^5 g of protein.

The methionine content was confirmed by oxidation of a protein sample with performic acid prior to acid hydrolysis and amino acid analysis. Using this procedure 10.0 ± 0.9 methionine sulfone residues were detected per 10^5 g of protein in two analyses. No evidence of the presence of cysteic acid was detected in the performic acid oxidized hydrolysates. Similarly, no cysteinyl residues could be detected by reaction of the protein with 5,5′-dithiobis(2-nitrobenzoic acid) in 6 M guanidine hydrochloride at pH 8.5 as described by Vanaman and Stark (1970). Sufficient protein was used in this reaction so that one residue of cysteine per 10^5 g of protein would have been detected.

Cyanogen Bromide Cleavage. About 1 mg of *T. aquaticus* enolase was reacted with an excess of cyanogen bromide in 1 ml of 70% formic acid for 24 hr at room temperature. The reaction was terminated by dilution with ten volumes of water. The reaction mixture was lyophilized, dissolved in 0.9% acetic acid containing sufficient urea to solubilize the lyophilized material, and subjected to polyacrylamide electrophoresis in 15% gels containing 0.9% acetic acid and 6.25 M urea as described by Panyim and Chalkley (1969). Five major peptide bands were observed after staining with Coomassie Brilliant Blue.

Discussion

Both electrophoretic and sedimentation measurements indicate that the polypeptide chains constituting *T. aquaticus* enolase are the same size. The equilibrium sedimentation measurements made in the presence of either guanidine hydro-

chloride or a low pH gave an average molecular weight of $43.6 \pm 5.2 \times 10^3$ while the electrophoretic mobility of the protein in detergent corresponded to an average molecular weight of $45.5 \pm 0.5 \times 10^3$. By contrast the molecular weight of native enolase was $328 \pm 12 \times 10^3$ as measured by equilibrium sedimentation and $355 \pm 5 \times 10^3$ as measured by zone electrophoresis. Taken together, these measurements indicate that *T. aquaticus* enolase consists of eight polypeptide chains each having a molecular weight of about 44×10^3. The number of peptides produced by cyanogen bromide cleavage suggests that the polypeptide chains are identical. This is, however, a tentative conclusion because aggregation of the peptides may have occurred. The size of the polypeptide chains of *T. aquaticus* falls within the range of $41–50 \times 10^3$ measured for enolases purified from rabbit muscle, yeast, *E. coli*, trout muscle and salmon muscle (Wold, 1971). However, the native form of all these enolases is dimeric while that of *T. aquaticus* is octameric.

The intrinsic viscosity of native *T. aquaticus* enolase, 6.0 ml/g, suggests that the protein is compact and rather symmetrical. Combining the intrinsic viscosity with the sedimentation coefficient 12.7 S, the molecular weight of 3.52×10^5 and a $\bar{v}$ of 0.782 ml/g, for the native protein in the Scheraga–Mandelkern equation (1953) gives a value for the constant, β, of 2.19×10^6 which corresponds to an axial ratio for an equivalent prolate ellipsoid of revolution of 4. Such an axial ratio is characteristic of four globular subunits arranged in a square-planar geometry suggesting that the eight polypeptide chains of *T. aquaticus* enolase form a cubic array of eight globular subunits. The magnitude of the sedimentation coefficient of the dissociated protein at low pH, 3.3 S, is consistent with the proposed globular conformation of the polypeptides in the native enzyme. Reduction of the sedimentation coefficient to 1.7 S in guanidine hydrochloride suggests that the conformation of the polypeptide is more random in this solvent.

The amino acid composition of *T. aquaticus* enolase is characteristic of a globular protein except for the absence of cysteine. The protein must then be free of any intra- or interchain disulfide cross-links. However, the intrinsic viscosity of 34 ml/g estimated for the protein in 6 M guanidine hydrochloride is less than that calculated (Tanford *et al.*, 1967) for a randomly coiled polypeptide chain containing 419 residues, 38.5 ml/g. Since this solvent is presumed to cleavage all intrachain noncovalent bonds, the disparity of the observed and calculated values allows the presence of a small number of covalent or coordinate-covalent inter-chain cross-links.

At 25°, the pH optimum and Michaelis constant for the substrate 2-phosphoglycerate of *T. aquaticus* enolase lie within the range of values measured for enolases obtained from 20 different biological sources, pH 6.8–8.1 and 0.04–2.8 mM, respectively (Wold, 1971). Similarily, the maximum specific catalytic activity of *T. aquaticus* enolase at 25°, which ranges from 50 to 103 μmol of substrate dehydrated per min per mg of protein, overlaps the range of 88–164 μmol of substrate per min per mg measured at optimal pH, ionic strength, and Mg^{2+} concentration at 25° for purified trout muscle, rabbit muscle, and yeast enolases (Cory and Wold, 1966). However, in contrast to rabbit muscle enolase which is inactivated at assay temperatures above 44° (Bernfeld and Bieber, 1969), *T. aquaticus* enolase is not inactivated until the assay temperature exceeds 90°. As shown in Figure 2, the apparent K_M and the enthalpy of activation remain essentially constant over the assay temperature range of 50–90°. We are of the opinion that the catalytic site of *T. aquaticus* enolase is in common with those of mesophilic enolases and that one or more structural features of the protein maintains the site at elevated temperatures.

As shown in Figures 3B and 5, the octameric folded conformation of *T. aquaticus* enolase persists over the temperature range 25–85°. Above 85°, increasing turbidity develops indicative of a conformational change which exposes sites for aggregative reactions. The persistence of conformation over this temperature range correlates with the retention of catalytic activity as shown in Figure 3A. Since activity measurements are not obscurred by the turbidity, the irreversible loss of enzymic activity was used to determine conformational transitions that occur above 85°. Using this parameter, *T. aquaticus* was found to undergo a transition whose midpoint occurs at 90°. This transition is 45° higher than that observed with rabbit muscle enolase as shown in Figure 3A. Using activity as an index of irreversible conformational change, *T. aquaticus* enolase was found to be significantly more stable to inactivation by guanidine hydrochloride or by ethanol as shown in Figure 4. Thus, those structural features of *T. aquaticus* enolase which stabilize it to thermal inactivation provide stability to other denaturants as well. Speculations as to the structural basis of the enhanced stability of *T. aquaticus* enolase will be considered in the discussion of the following article in order to incorporate the properties of an enolase with intermediate thermostability.

Finally, the apparent increased thermostability of enolases in the presence of $MgCl_2$ merits comment. As shown in Figure 3A, the $A_{1/2}$ value for rabbit muscle enolase is increased from 45 to 58° by preincubation in buffered solutions containing 1 mM $MgCl_2$ while that of *T. aquaticus* enolase is increased from 90 to 99°. By contrast, the sharply defined temperature optimum for rabbit muscle enolase measured in assay solutions containing 1 mM $MgCl_2$ occurs at 45° and that for *T. aquaticus* enolase at 90°. This suggests that the presence of 1 mM $MgCl_2$ in the preincubation solutions of both enzymes delays the onset of irreversible inactivating by introducing a limited thermal range of reversible inactivation.

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Enolase from the Thermophile *Thermus* X-1†

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ABSTRACT: A procedure for the purification of enolase from the thermophilic bacterium *Thermus* X-1 is described. Hydrodynamic and electrophoretic measurements indicate that the native enzyme is globular and consists of eight apparently identical polypeptide chains each having a molecular weight of 48,000. Viscosity measurements in concentrated guanidine hydrochloride indicate the absence of inter- and intra-chain covalent cross-linkages. The K_M for 2-phosphoglycerate, the K_A for Mg^{2+} and the energy of activation are comparable to those reported for enolase obtained from a variety of biological sources. However, the optimum temperature for catalysis by *Thermus* X-1 enolase is about 25° higher than measured for other enolases. The noncovalent residue interactions within *Thermus* X-1 enolase must provide the enhanced thermostability since the enzyme is free of significant amounts of organic phosphate, carbohydrate, and metallic cations. The thermostability of four enolases, rabbit muscle, yeast, *Thermus* X-1, and *T. aquaticus* YT-1, exhibits a positive correlation with the content of residues capable of forming side-chain hydrogen bonds and a negative correlation with the average hydrophobicity.

Because of the limited quantities of purified enolase obtained from the extreme thermophile *Thermus aquaticus* YT-1 and because of the wide variation in the specific activity of the final product (Stellwagen *et al.*, 1973), an alternative source of a thermostable enolase was sought. A nonpigmented thermophilic bacterium *Thermus* X-1 (Ramaley and Hixson, 1970) was selected since this bacterium is similar to *Thermus aquaticus* YT-1 and is free of both the cartenoid pigments and an unidentified slime that makes enzyme purification from *T. aquaticus* extracts rather difficult. This report describes a purification procedure for enolase from *Thermus* X-1 and compares the properties of this enzyme with enolase obtained from rabbit muscle, yeast, and *Thermus aquaticus* YT-1.

Materials and Methods

Materials. Chemicals and biopolymers were purchased from the sources listed previously (Stellwagen *et al.*, 1973). Slants of *Thermus* X-1 were provided by Dr. R. F. Ramaley.

Growth of Thermus X-1. The organism was grown aerobically at 69–70° in a 30-l. Fermentation Design bench top fermentor. The growth medium described by Ramaley and Hixson (1970) was modified by adjustment of the Castenholz basal salt solution to pH 7.2 with 1 N NaOH prior to autoclaving. Soy broth and pancreatic digest of casein, both in a final concentration of 0.2% (w/v), and glucose, at 0.33% (w/v), were employed as carbon and nitrogen sources. Bacteria were harvested in late-log phase with a Sharples centrifuge, and the cell paste was stored at −15°.

Purification of Thermus X-1 Enolase. About 425 g of *Thermus* X-1 were suspended in 800 ml of 10 mM Tris-HCl (pH 7.5), 1 mM $MgSO_4$, and 1 mM PMSF[1] solution (buffer A) at 4° using a blender. Cells were ruptured by treatments with a Manton–Gaulin homogenizer at 550 atm. The homogenate was diluted to 3000 ml with buffer A, and centrifuged at 27,000*g* for 90 min. A 1% protamine sulfate solution, adjusted to pH 6, was added dropwise to the crude supernatant solution until a ratio of 0.20 mg of protamine sulfate/mg of protein was attained. A clean, yellow supernatant solution was obtained after centrifugation at 27,000*g* for 15 min.

Most of the protein was precipitated from the protamine sulfate supernatant solution by addition of 561 g of $(NH_4)_2SO_4$ per l. of solution. The pH was maintained at 6.5–6.6 during the $(NH_4)_2SO_4$ addition by adding NaOH. The pellet obtained by centrifugation at 27,000*g* for 20 min was suspended in 150

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMSF, phenylmethyl sulfonyl fluoride.