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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.

TABLE I: Purification of Enolase from *Thermus* X-1.

Fraction	Vol (ml)	Protein (mg)	Total Act. (IU)	Sp Act. (IU/mg)	Yield (%)
Crude extract	3020	24,160	214,524	8.9	100
Protamine sulfate supernatant	3370	10,450	217,307	20.8	101
$(NH_4)_2SO_4$ fraction	108	2,520	197,774	78.6	92
DEAE-cellulose eluate	765	788	176,741	224	82
Hydroxylapatite eluate	206	212	176,492	832	82
Agarose eluate	130	105	140,355	1,337	65

ml of 50 mM Tris-HCl (pH 7.5), 1 mM $MgSO_4$, and 0.1 mM PMSF solution (buffer B) and dialyzed against four 2-l. portions of the same buffer at 4°. The dialyzed ammonium sulfate fraction was diluted to 8 mg of protein/ml with buffer B, and 351 g of $(NH_4)_2SO_4$ was added per l. of protein solution. The pellet obtained by centrifugation at 27,000*g* for 20 min was discarded. Enolase was precipitated from the supernatant solution by further addition of 101 g of $(NH_4)_2SO_4$/l. of supernatant solution. The pellet obtained by centrifugation at 27,000*g* for 20 min was suspended in a minimal volume of 10 mM Tris-HCl (pH 7.5), 1 mM $MgSO_4$, and 0.1 mM PMSF solution (buffer C). The suspended pellet was dialyzed against four 2-l. portions of buffer C.

The ammonium sulfate fraction was applied to a 4 × 45 cm column of DEAE-cellulose equilibrated with buffer C. The column was washed with buffer C containing 0.15 M KCl at a flow rate of 120 ml/hr to remove contaminating proteins. Enolase was eluted using a linear gradient of 0.15–0.80 M KCl in buffer C. The pooled fractions containing activity were concentrated with an Amicon Diaflo unit using an XM-50 membrane and the concentrated solution was dialyzed against three 1-l. portions of 10 mM potassium phosphate (pH 7.1), 1 mM $MgSO_4$, and 0.1 mM PMSF solution (buffer D). This column chromatography and all subsequent ones in the isolation were performed at room temperature.

The dialyzed solution was applied to a 2 × 42 cm column of hydroxylapatite equilibrated with buffer D. The column was washed with 70 mM potassium phosphate (pH 7.1), 1 mM $MgSO_4$, and 0.1 mM PMSF solution to remove some contaminating proteins. Enolase was eluted using a linear gradient from 70 to 250 mM potassium phosphate (pH 7.1), containing 1 mM $MgSO_4$ and 0.1 mM PMSF. Pooled fractions containing activity were concentrated to 33 ml under 1 atm of N_2 in an Amicon Diaflo apparatus containing an XM-50 membrane.

The concentrated solution was applied to a 5 × 142 cm column of agarose (Bio-Gel A 1.5m) equilibrated with 50 mM Tris-HCl (pH 7.5) and 1 mM $MgSO_4$ solution (buffer E). Activity was eluted with the same buffer and the pooled activity was concentrated under nitrogen using a Diaflo unit. Purified *Thermus* X-1 enolase was filtered through a GS 0.22-μ sterilizing Millipore filter and stored in buffer E at 4°. Activity is stable for at least three months under these conditions.

Protein Determination. Procedure II according to Klungsöyr (1969) was utilized to determine protein concentrations during purification. *Thermus* X-1 enolase served for the standard curve once it has been purified. Measurement of the absorbancy at 280 nm and determination of the protein concentration by the refractive increment method described by Babul and Stellwagen (1969) were employed to determine $E^{1\%}_{280\ nm}$, and this value was routinely used for measuring purified enzyme.

Chemical Analyses. Purified *Thermus* X-1 enolase was dialyzed against H_2O containing anion- and cation-exchange resins external to the dialysis tubing. The dialyzed protein was lyophilized and dried in an Abderhalden apparatus over P_2O_5 *in vacuo* using boiling water as the solvent. The dried protein was cooled to room temperature in a vacuum desiccator over P_2O_5, and subsequently equilibrated with the atmosphere for 24 hr at room temperature. The moisture content was determined by weighing the protein before and after equilibration with the atmosphere. Protein prepared in this manner was utilized for chemical analysis and the results were corrected for the moisture content. The carbon, hydrogen, and nitrogen contents were determined using a Hewlett-Packard Model 185 F & M CHN analyzer with NBS samples serving as test standards. Solutions used for metal analysis were prepared from doubly distilled water which had been filtered through activated charcoal, a mixed-bed ion-exchange resin, and then a Millipore filter. Containers used during the metal analysis were soaked in 0.002% dithizone in CCl_4 and thoroughly rinsed with purified water. Polyethylene bottles were used whenever possible. Dialysis tubing was twice boiled in $NaHCO_3$ and Na_2EDTA, rinsed with water, and stored in 50% ethanol. It was thoroughly rinsed with purified water prior to use. *Thermus* X-1 enolase was dialyzed against 10 mM Tris-HCl (pH 7.5) prior to Mg analysis using a Perkin-Elmer Model 303 atomic absorption spectrophotometer. The dialysate was used to null the instrument. The dialysates had negligible absorption when compared to purified water. Magnesium turnings dissolved in approximately 10 ml of concentrated HCl and diluted to 1 l. with purified water were used to generate a standard curve. A portion of one sample dialyzed in this manner was lyophilized and sent to Stewart Laboratories (Knoxville, Tenn.) for elemental analysis by emission spectrography.

Zinc was determined colorimetrically by complexation with diphenylthiocarbazone in CCl_4 according to Nelbach *et al.* (1972). The enzyme was dialyzed against 10 mM Tris-HCl (pH 7.5) in the presence and absence of 100 mM EDTA. The EDTA was subsequently removed by dialysis prior to zinc analysis. All samples were hydrolyzed in evacuated, sealed tubes in 6 N HCl at 110° for 16 hr. A stock zinc standard was prepared by dissolving granular zinc metal in HCl. Thirty nanomoles of zinc gave an absorbance of 0.87 at 525 nm.

Total cysteine and cystine were determined as cysteic acid after oxidation with performic acid (Hirs, 1967). Half-cystine was also analyzed as *S*-(2-aminoethyl)cysteine after aminoethylation with excess ethylenimine in the presence of 6 M guanidine hydrochloride and 2-mercaptoethanol (Cole, 1967). *Thermus* X-1 enolase was incubated with excess DTNB in the presence of 6 M guanidine hydrochloride, 0.2 M Tris-HCl (pH 8.2), and 0.02 M EDTA to determine the number of

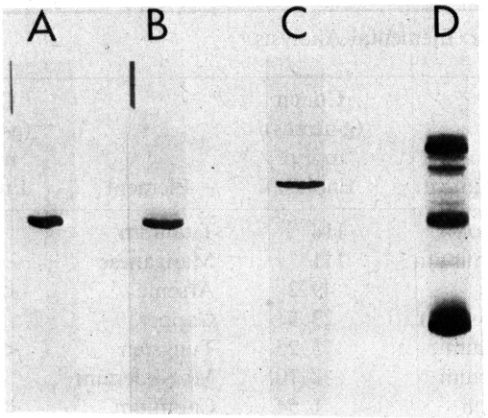

FIGURE 1: Polyacrylamide disc gel electrophoresis of *Thermus* X-1 enolase. (A) Native protein: about 55 μg of protein was applied to a 7% gel and electrophoresed at pH 9.5 using 2 mA/gel. (B) Dissociated protein in sodium dodecyl sulfate: *Thermus* X-1 enolase and the calibration proteins were incubated for 2 hr at 75° in the presence of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Aliquots of each protein containing 10 μg of protein were mixed with glycerol and Bromophenol Blue, applied to separate gels, and electrophoresed at 3 mA/gel until the dye migrated approximately 8 cm. Pyruvate kinase, fumarase, aldolase, carbonic anhydrase, and α-chymotrypsin served as the calibration proteins. (C) Dissociated protein in acidified urea: 20 μg of enolase was electrophoresed for 10 hr on a 15% acrylamide gel containing 6.25 M urea adjusted to pH 3.2 with acetic acid. (D) Cyanogen bromide digest: about 150 μg of enolase treated with cyanogen bromide was electrophoresed on a 15% acrylamide gel containing 6.25 M urea (pH 3.2).

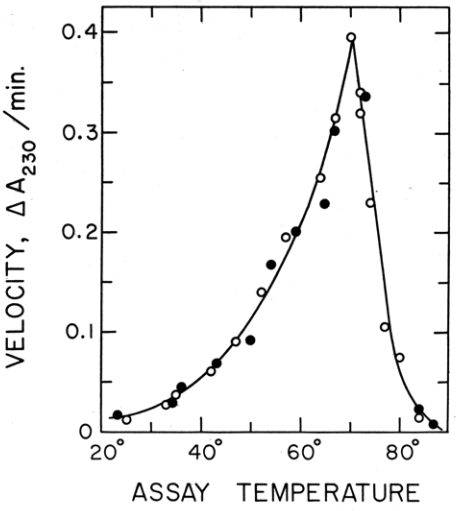

FIGURE 2: The effect of assay temperature on the rate of *Thermus* X-1 enolase catalysis. (○) Assay solution buffered with 39 mM phosphate, pH 7.25–7.42; (●) assay solution buffered with 33 mM Tris-HCl, pH 7.2–7.5. All assay solutions contained 2 mM 2-phosphoglycerate and 1 mM $MgSO_4$. A mixture of the buffer and $MgSO_4$ contained in 0.98 ml was placed in a cuvet and brought to the appropriate temperature in a thermostatable cell holder of a recording spectrophotometer. Ten microliters of enzyme solution was then added, mixed, and equilibrated for 1 min at which time the reaction was initiated by addition of 10 μl of substrate.

cysteine residues (Vanaman and Stark, 1970). Between 11 and 12 cysteine residues per mole of rabbit muscle enolase were measured by aminoethylation and by DTNB titration as reported previously (Wold, 1971).

Total phosphorus content of the protein was determined by a modified Fiske–Subbarow procedure (Bartlett, 1959) after digestion in H_2SO_4 at 150° for 4.5 hr. Potassium dihydrogen phosphate served as the phosphorus standard. The neutral hexose and pentose content of enolase was measured by the phenol–sulfuric acid procedure (Dubois *et al.*, 1956). Mannose and xylose were used as standards.

Other Measurements. Procedures for measurement of enzymic activity, hydrodynamic and electrophoretic properties, amino acid composition, and cyanogen bromide cleavage have been described previously (Stellwagen *et al.*, 1973). Enolase activity was detected on polyacrylamide gels using the procedure of Dave *et al.* (1966). Norleucine was added to some protein samples prior to hydrolysis as an internal standard for amino acid analysis.

Diffusion measurements were made with a double-sector synthetic boundary centerpiece and the analytical ultracentrifuge as described by Chervenka (1970). Changes in the protein gradient with time were measured with interference optics at a rotor velocity of 5600 rpm.

Results

The summary of a typical purification is given in Table I. This procedure has been repeated three times giving a preparation with an average specific activity of 1344 ± 24 units/mg. The homogeneity of enolase purified by this procedure was examined by disc gel electrophoresis at two values of pH and at two acrylamide concentrations. A single protein band was observed on 5 and 7% acrylamide gels at pH 9.5 (Figure 1) and 8.2. The position of enolase activity corresponded to the position at which protein stained. A control gel, incubated in the absence of 2-phosphoglycerate, did not exhibit the dark band against a greenish fluorescent background characteristic of enolase activity. In one preparation, a slower migrating minor band constituting about 1% of the total protein was observed.

Catalytic Measurements. The effect of increasing assay temperature on the rate of catalysis of *Thermus* X-1 enolase is shown in Figure 2. A temperature optimum of 70° is indicated using either Tris-HCl or phosphate buffered assay solutions. The values shown in Figure 2 give a linear Arrhenius plot whose slope corresponds to an activation energy of 14.5 ± 0.6 kcal/mol.

The increase in the rate of catalysis at 70° with increasing concentration of substrate, 2-phosphoglycerate, gives a linear double-reciprocal plot whose intercept corresponds to an apparent K_M of 0.15 mM. A similar value could only be estimated for K_A of $MgSO_4$ since inhibition was observed at $MgSO_4$ concentrations in excess of 1 mM.

The effect of preincubation of various enolases at increasing temperatures on their catalytic activity is shown in Figure 3. Preincubation of rabbit muscle, yeast, and *Thermus* X-1 enolase for 5 min in solutions free of Mg^{2+} give $A_{1/2}$ values, the temperature at which 50% of the initial activity is lost, of 40, 45, and 74°, respectively. Preincubation in the presence of 1 mM $MgSO_4$ increased the $A_{1/2}$ of rabbit muscle enolase 18°, that of yeast enolase 10°, and that of *Thermus* X-1 enolase 14°.

Hydrodynamic Measurements. Native *Thermus* X-1 enolase sediments as a single symmetrical boundary. The dependence of the sedimentation coefficient on protein concentration can be expressed by the equation, $s_{20,w} = 13.2\ S\ (1 - 0.016c)$, where 13.2 S is the sedimentation coefficient at infinite dilution and c is the protein concentration in milligrams per milliliter. In the presence of 6 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol the sedimentation coefficient of the protein is reduced from 12.3 to 1.3 S at a concentration of 5.9 mg/ml.

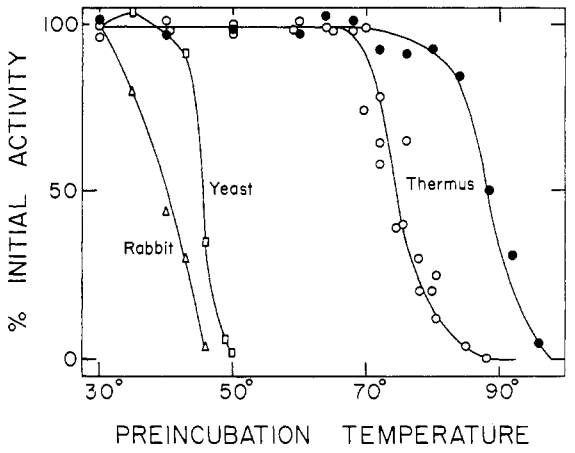

FIGURE 3: Thermal stability of various enolases. Aliquots of concentrated enzyme solutions were diluted with solvent equilibrated at the desired temperature. The diluted enzyme solution was incubated at that temperature for exactly 5 min and then rapidly cooled to 0° in an ice–water bath. A 10-μl sample of chilled preincubated enzyme was assayed as indicated. (○) *Thermus* X-1 enolase incubated in either 39 mM phosphate buffer or 50 mM Tris-HCl buffer (pH 7.4). The enzyme preparation was depleted of Mg^{2+} and Zn^{2+} by dialysis against 100 mM phosphate–100 mM EDTA (pH 7.2) followed by dialysis against 100 mM phosphate (pH 7.2); (●) *Thermus* X-1 enolase incubated in 50 mM Tris–1 mM $MgSO_4$ (pH 7.4). *Thermus* X-1 enolase catalytic activity was assayed at 70° as described in methods; (△) rabbit muscle enolase incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA; (□) yeast enolase incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA. The pH of the Tris-HCl buffers was adjusted to have a pH of 7.4 at the indicated temperatures. Rabbit muscle and yeast enolases were assayed at 30° in 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM $MgSO_4$ and 2 mM 2-phosphoglycerate.

The dependence of the diffusion coefficient of native enolase on protein concentration can be expressed by the equation, $D_{20,w} = D^0_{20,w}(1 + 0.021c)$, where c is the protein concentration in milligrams per milliliter. A value for $D^0_{20,w}$ of $3.42 \pm 0.02 \times 10^{-7}$ cm²/sec was estimated. Combination of the values derived for $D^0_{20,w}$, $s^0_{20,w}$, and $\bar{v}$ in the Svedberg equation gives a molecular weight of 3.58×10^5 g/mol. The values for $D_{20,w}$, M_W, and $\bar{v}$ are characteristic of a particle having a frictional ratio of 1.30 relative to an equivalent anhydrous sphere. This frictional ratio is characteristic for an ellipsoid of revolution having an axial ratio of 6, indicating that native *Thermus* X-1 enolase is rather symmetrical.

Linear ln d *vs.* r^2 plots are obtained after equilibrium sedimentation of each of three preparations of native enolase. The initial protein concentrations ranged from 0.28 to 0.50 mg per ml. Their slopes correspond to an average apparent molecular weight of $3.82 \pm 0.05 \times 10^5$ g/mol. Linear ln d *vs.* r^2 plots are also observed for two samples of protein sedimented in 6 M guanidine hydrochloride–0.1 M 2-mercaptoethanol. The slopes correspond to an average apparent molecular weight of $4.52 \pm 0.07 \times 10^4$ g/mol assuming no preferential binding of the solvent.

The linear dependence of the reduced viscosity of enolase in 6 M guanidine hydrochloride–0.1 M 2-mercaptoethanol on protein concentration gives a Huggins constant of 0.80. The measured values extrapolate to an intrinsic viscosity of 39.6 ± 0.2 ml/g.

Electrophoretic Measurements. The molecular weight of dissociated enolase was estimated by gel electrophoresis in the presence of sodium dodecyl sulfate. A single protein band was observed, as shown in Figure 1, whose mobility in eight measurements corresponded to a molecular weight of $4.86 \pm 0.27 \times 10^4$ g/molecule. The same value was obtained when the protein was preincubated in 1% sodium dodecyl sulfate in the presence or absence of 0.1 M 2-mercaptoethanol. A single electrophoretic band was also observed after incubation of the enzyme in 9 M urea (pH 3.5), followed by electrophoresis in 6.25 M urea (pH 3.2) as shown in Figure 1. Only one band was observed after electrophoresis from 5 to 34 hr in this solvent.

TABLE II: Elemental Analysis.

Element	Concn (g-atoms)/mol of Enolase)	Element	Concn (g-atoms/mol of Enolase)
Silicon	114	Titanium	0.18
Aluminum	111	Manganese	0.14
Zinc	49.2	Arsenic	<0.10
Potassium	23.5	Copper	0.05
Sodium	3.25	Tungsten	<0.04
Calcium	2.10	Molybdenum	0.03
Boron	1.54	Cadmium	<0.03
Iron	1.52	Tin	<0.03
Nickel	0.56	Uranium	<0.03
Magnesium	0.38	Zirconium	0.01
Chromium	0.30	Cobalt	<0.005
Barium	0.18	Lead	0.003

Absorption Spectrum. Native *Thermus* X-1 enolase exhibits a maximum at 279 nm in the near-ultraviolet region. A 1% solution of protein has an extinction of 7.94 ± 0.24 cm⁻¹ at 280 nm. The A_{280}: A_{260} of the protein is 2.10.

No absorbance was detected between 320 and 700 nm using a protein concentration of 0.95 mg/ml in a 1-cm light path.

Chemical Composition. Thermus X-1 enolase contains 53.4% carbon, 15.4% nitrogen, and 6.9% hydrogen. An average of 1.9 ± 1.4 mol of phosphorus/mol of enolase was detected in four measurements. The large variability is probably due to leaching of phosphate from glassware during acid hydrolysis of the protein and to trace contaminants in the reagents.

Thermus X-1, rabbit muscle, and yeast enolase each form a colored complex having absorption maxima at 400 and 488 nm upon reaction with phenol–sulfuric acid. Free pentose and hexose also produce a colored complex, but in each case a single maximum is observed occurring at 485 nm for the pentose complex and 489 nm for the hexose complex (Dubois *et al.*, 1956; Montgomery, 1961). The observed $A_{488\ nm}$ for *Thermus* X-1 enolase is equivalent to 1.4 ± 0.7 mol of mannose equivalents/mol of polypeptide chains (six analyses), that of yeast enolase to 0.8 ± 0.5 mol/mol (three analyses), and that of rabbit muscle enolase to 0.06 ± 0.004 mol/mol (three analyses). The nature of the complex having an absorbance maximum at 400 nm is unknown, although it appears common to all three enolases examined.

Results of analysis of a single sample of *Thermus* X-1 enolase for 24 elements by emission spectroscopy is shown in Table II. The relatively high concentrations of silicon, aluminum and potassium are very likely derived from the glassware rather than the protein. An independent measurement of the magnesium content by atomic absorption spectroscopy gave a value of 0.35 g-atom of Mg/mol of enolase in

good agreement with the concentration shown in Table II. Analysis of two other preparations by atomic absorption gave values of 3.8 and 4.3 g-atoms of Mg per mol of protein. However, only 3.5 g-atoms of Zn/mol of protein could be detected colorimetrically using diphenylthiocarbazone to specifically complex Zn^{2+}. Colorimetric Zn analysis of a second enzyme preparation gave 6.6 g-atoms of Zn/mol of protein. The reason for the order of magnitude difference in Zn content measured by emission spectrography and colorimetrically is unclear. All of the Zn should be available for reaction in colorimetric assay since the protein was hydrolyzed in 6 N HCl at 110° for 24 hr prior to complexation with diphenylthiocarbazone. If *Thermus* X-1 enolase were to contain 49 g-atoms of Zn/mol of protein, 60% of the Zn^{2+} added to the growth medium would be required for enolase. This is unlikely in view of the sizeable number of Zn^{2+} metalloenzymes commonly found in microorganisms (Vallee and Wacker, 1970). We are therefore of the opinion that the colorimetric Zn analysis is of the correct order of magnitude and that the high value measured by emission spectrography must represent contamination. No Zn was detected colorimetrically in *Thermus* X-1 enolase after dialysis against 100 mM phosphate buffer–100 mM EDTA (pH 7.2) followed by dialysis against 100 mM phosphate buffer (pH 7.2) to remove the EDTA. This dialyzed protein was used to generate the inactivation curves for *Thermus* X-1 enolase shown in Figure 3.

The results of the amino acid analysis, given in Table III, indicates that amino acids accounted for 101 ± 2% of the composition of the enzyme when recoveries were based on norleucine as an internal standard. Recovery of samples was 97 ± 4% based on dry weight when norleucine was not added. No half-cystine was observed after acid hydrolysis, and no cysteic acid was detected after oxidation with performic acid. Oxidation was complete as indicated by the total conversion of methionine to methionine sulfone. Similarly, no *S*-(2-aminoethyl)cysteine was detected after reaction of *Thermus* X-1 enolase with ethylenimine. Between 0.45 and 0.55 mg of hydrolyzed protein was utilized per analysis in these measurements. No sulfhydryl residues were detected by titration with DTNB. The concentration of enzyme used would have produced a $\Delta A_{412\ nm}$ equal to 0.253 if there were one sulfhydryl residue per subunit. A partial specific volume of 0.738 cm^3/g was calculated from the amino acid composition (Table III) as described by Cohn and Edsall (1943).

Thermus X-1 enolase was cleaved with cyanogen bromide and the reaction mixture subjected to polyacrylamide gel electrophoresis. As shown in Figure 1D, between 10 and 12 bands are stained by Coomassie Blue. If the eight polypeptide chains have identical sequences, the amino acid analysis indicates that each chain should contain nine methionyl residues thereby producing ten peptides after complete cyanogen bromide cleavage. Thus the sequences of the eight polypeptide chains are apparently chemically equivalent by this criterion, although it is possible that aggregation of cyanogen bromide peptides occurred and that the total number of peptides was not detected electrophoretically.

TABLE III: Amino Acid Analyses of *Thermus* Enolase.[a]

Amino Acid	Residues/3.82 × 10^5 g of Protein			
	24 hr[b]	48 hr[b]	72 hr[b]	Av
Alanine	369 ± 7	362 ± 15	371 ± 24	367
Arginine	186 ± 12	176 ± 13	192 ± 10	185
Aspartic acid	370 ± 1	388 ± 8	387 ± 26	382
$^1/_2$-Cystine	nd[c]	nd	nd	0
Glutamic acid	449 ± 9	494 ± 6	447 ± 7	463
Glycine	358 ± 3	353 ± 12	362 ± 19	358
Histidine	52 ± 7	41 ± 2	47 ± 5	47
Isoleucine	198 ± 12	224 ± 1	228 ± 7	228[d]
Leucine	307 ± 16	328 ± 12	322 ± 17	319
Lysine	247 ± 6	214 ± 4	230 ± 17	230
Methionine	72 ± 1	66 ± 1	68 ± 1	68
Phenylalanine	73 ± 1	69 ± 1	72 ± 2	71
Proline	96 ± 2	100 ± 4	95 ± 4	97
Serine	147 ± 2	153 ± 9	118 ± 11	161[e]
Threonine	157 ± 6	165 ± 12	138 ± 19	167[e]
Tryptophan	16 ± 2[f]	na[c]	na	16
Tryosine	122 ± 3	116 ± 1	121 ± 4	120
Valine	265 ± 8	270 ± 20	273 ± 21	273[d]

[a] The variation is that observed for duplicate analyses. [b] Hydrolysis times in 6 N HCl. [c] nd, none detected; na, not analyzed. [d] Values obtained after 72-hr hydrolysis. [e] Values obtained by extrapolation of the content after 24- and 72-hr hydrolysis to zero hydrolysis time. [f] Alkaline hydrolysis.

Discussion

The properties of *Thermus* X-1 enolase are very similar to those reported for the enzyme obtained from the extreme thermophile, *Thermus aquaticus* YT-1 (Stellwagen *et al.*, 1973). Both enzymes are globular proteins containing eight polypeptide chains of equal size. Cyanogen bromide digests suggest that the polypeptide chains within each protein are chemically equivalent. The apparent K_M for the substrate, the specific activity and the energy of activation for the reaction catalyzed by each enzyme are comparable. However, the optimum temperature for catalysis and the $A_{1/2}$ temperatures for *Thermus* X-1 enolase are both about 16° lower than the values measured for *T. aquaticus* enolase. The thermostability of *Thermus* X-1 enolase is thus intermediate between that of mesophilic enolases, such as rabbit muscle and yeast, and that of the extreme thermophile *T. aquaticus* YT-1.

With two exceptions, the structural and catalytic properties of *Thermus* X-1 and *T. aquaticus* enolase are in common with those of rabbit muscle, salmon, trout, *Escherichia coli*, and yeast enolase (Wold, 1971). All enolases contain multiple copies of a polypeptide whose molecular weight spans a narrow range from 4.1 to 5.0 × 10^4 g. The catalytic site of each enolase exhibits comparable values for the K_M of the substrate, the specific activity at 25°, and the energy of activation at 25°. The thermostability of each protein is increased in the presence of 1 mM Mg^{2+}. To the extent that comparisons have been made, these enolases may be considered to constitute a homologous series. Within this series, *Thermus* X-1 and *T. aquaticus* enolase have two unique features: their octameric rather than dimeric quaternary structure and their enhanced thermostability. While it is tempting to consider these properties to be causally related, we are of the opinion that they are not. Firstly, the degree of polymerization cannot account for the 16° difference in the $A_{1/2}$ for *Thermus* X-1 and *T. aquaticus* enolase. Secondly, enzymes with enhanced thermostability obtained from moderate thermophiles generally have a molecular weight characteristic of their thermolabile counterparts (*e.g.*, ATPase, Hachimori *et al.*, 1970; glyceraldehyde-3-phosphate dehydrogenase, Singleton *et al.*,

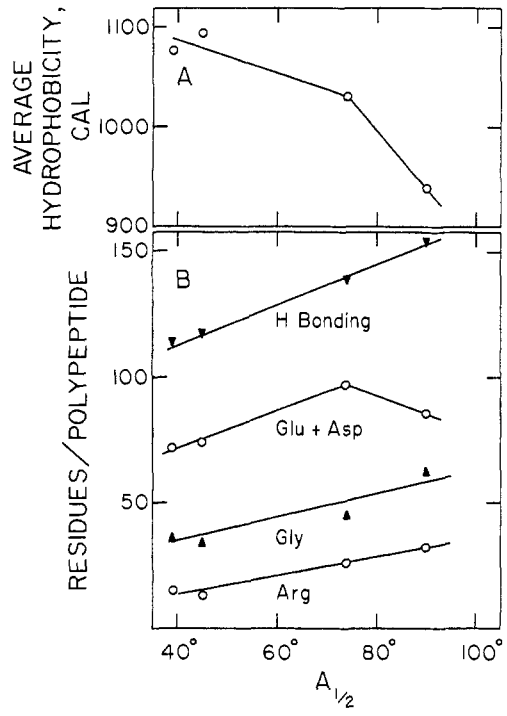

FIGURE 4: Correlation of the composition of various amino acids and amino acid groups with $A_{1/2}$, the temperature at which 50% of the initial catalytic activity is lost in 5 min. (A) The average hydrophobicity of each enolase was calculated according to Bigelow (1967). (B) The sum of the residues capable of forming hydrogen bonds was calculated for each enolase to generate the H-bonding curve. Those residues summed are given in the text. The values of $A_{1/2}$ for rabbit muscle enolase, yeast enolase, *Thermus* X-1 enolase, and *T. aquaticus* enolase are 40, 45, 74, and 90°, respectively.

TABLE IV: Comparison of Amino Acid Composition.

Amino Acid	Residues/Polypeptide			
	Rabbit Muscle	Yeast	*Thermus* X-1	*T. aquaticus* YT-1
Alanine	38	47	46	42
Arginine	15	13	23	26
Aspartic acid	38	43	48	29
$^1/_2$-Cystine	6	1	0	0
Glutamic acid	34	31	58	57
Glycine	36	34	45	63
Histidine	9	11	6	7
Isoleucine	22	20	28	14
Leucine	34	35	40	36
Lysine	34	35	29	25
Methionine	6	5	9	5
Phenylalanine	13	14	9	12
Proline	13	13	12	15
Serine	16	26	20	41
Threonine	16	17	21	18
Tryptophan	5	4	2	4
Tyrosine	9	8	15	7
Valine	28	33	34	27

1969; α-amylase, Pfueller and Elliot, 1969; formyltetrahydrofolate synthetase, Brewer *et al.*, 1970). We are therefore of the opinion that the structural basis for enhanced thermostability resides in one or more chemical components of the protein.

The chemical analysis of *Thermus* X-1 enolase makes it very unlikely that nonamino acid moieties contribute significantly to enhanced thermostability. A minimal concentration of 1 mol of chemical moiety/mol of polypeptide is considered significant. The sensitivity of the absorbance spectral measurements indicates the absence of visible chromophores whose extinction exceeds 3.8×10^3 M^{-1} cm^{-1}, *e.g.*, FMN, NADH, pyridoxal phosphate, vitamin A, and heme. The low phosphorus content, about 0.2 P/polypeptide precludes consideration of nucleotide phosphates and phospholipids. While *Thermus* X-1 enolase contains the equivalent of about 1 mannose/polypeptide, thermolabile yeast enolase contains nearly the same amount. The mannose equivalent may be derived from the chromatographic material, *e.g.*, agarose, employed in the purification procedure. Both thermolabile and thermostable enolase form a complex in phenol–sulfuric acid which has an absorption maximum at 400 nm. Although the nature of the protein moiety generating this complex is unknown it cannot be responsible for enhanced thermostability since its presence is independent of $A_{1/2}$. The elemental analysis of *Thermus* X-1 enolase (Table II) indicates that, except for glassware contaminants, only Zn is present in excess of 1 atom/polypeptide. Since the $A_{1/2}$ temperature for *Thermus* X-1 enolase stripped of Zn^{2+} is 74°, this element cannot be responsible for the enhanced thermostability of the enzyme. It is of interest to note that the low concentration of divalent metal cations in *Thermus* enolase, particularly Ca^{2+}, indicates that ligation of these cation is not responsible for thermostability, in contrast to the situation reported for thermolysin (Feder *et al.*, 1971). Because of the limited amounts of *T. aquaticus* enolase available, these analysis have not been extended to this enzyme. Since the enhanced thermostability of *Thermus* X-1 enolase is two-thirds that of *T. aquaticus* enolase relative to either rabbit muscle or yeast enolase, we have assumed that *T. aquaticus* enolase is also devoid of significant concentrations of these chemical entities.

We are then left with the amino acid residues themselves as the origin of enhanced thermostability. The magnitude of the intrinsic viscosity of *Thermus* X-1 enolase in concentrated guanidine hydrochloride, 39.6 ml/g, is virtually identical with the value calculated (Tanford *et al.*, 1967) for a randomly coiled polypeptide chain containing 444 residues. This agreement negates the presence of intra-chain covalent cross-links bridging amino acid residues located in distant regions of the sequence. Although the measured and calculated $[\eta]$ for *T. aquaticus* enolase in 6 M guanidine hydrochloride allowed for the occurrance of a modest amount of cross-linking, the low concentrations of protein available for viscosity measurements limited the precision of the measured $[\eta]$. We then must turn to the noncovalent interactions as the source of enhanced thermostability. Since sedimentation measurements indicate that the octameric quaternary structure of *T. aquaticus* enolase persists up to its $A_{1/2}$ of 90° (Stellwagen *et al.*, 1973), both the inter-chain as well as the intrachain noncovalent interactions must be equally thermostable. If the subunits are globular in the octameric protein, as indicated for *T. aquaticus* enolase (Stellwagen *et al.*, 1973), the number of inter-chain noncovalent interactions must be much smaller than the number of intrasubunit interactions. Thus, thermostability can be associated with a relatively low number of noncovalent interactions.

In the absence of detailed conformation information, only the potential for various noncovalent interactions can be calculated. The amino acid compositions of four enolases are compared in Table IV. Changes in the content of various amino acids or functional groups with $A_{1/2}$ are plotted in Figure 4. In generating this plot, the $A_{1/2}$ measured in the absence of Mg^{2+} was used since we considered the thermostability of the protein rather than its Mg^{2+} complex to be more relevant. Similar results were obtained if the residues per 10^5 g of protein rather than residues per polypeptide chain were used. The average hydrophobicity of each protein was calculated using the hydrophobicity factor for the individual amino acids (Bigelow, 1967). As shown in Figure 4A, the average hydrophobicity is inversely related with $A_{1/2}$ indicating that hydrophobic interactions are unlikely to be a significant source of thermostability. The inverse relationship could only generate thermostability if hydrophobic residues were to exist on the surface of rabbit muscle and yeast enolase and if these surface hydrophobic residues were progressively eliminated in *Thermus* X-1 and *T. aquaticus* enolase. While the total number of basic residues (almost exclusively Arg and Lys at pH 7.5) is independent of $A_{1/2}$ the content of acidic residues increases with $A_{1/2}$, although rather irregularly, as shown in Figure 4B. The content of residues whose side chains are capable of participating in hydrogen bonds increases much more regularly with $A_{1/2}$. This is the case whether we consider only the sum of Arg, $^1/_2$-Cys, His, Met, Ser, Thr, Trp, and Tyr or also include an estimate of the Gln and Asn content (45% of the Glu + Asp) in the total. The estimated Gln and Asn contents are included in the total numbers of residues capable of hydrogen bonding plotted in Figure 4B. An increase in the number of internal side-chain hydrogen bonds would be expected to increase the $A_{1/2}$. The concommitant increase in the Gly content with increasing $A_{1/2}$ may be necessary to facilitate proper orientation of the proposed internally hydrogen bonded residues. The increasing content of Arg and Asp and Glu residues with increasing $A_{1/2}$ could also contribute to thermal stability by forming hydrogen-bonded ion pairs.

Since the amino acid composition of only four proteins with different $A_{1/2}$ values is available, the statistical merit of the trends noted above may be questioned. If $A_{1/2}$ temperatures for the four enolases measured in the presence of 1 mM Mg^{2+} are compared, the amino acid composition of a fifth protein, chum salmon enolase having an $A_{1/2}$ of 64° measured for a 3-min preincubation in 1 mM Mg^{2+} (Wold, 1971), supports the trends noted above.

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