1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6

1 of 1

ANL/CMB/PP--81754

12/23/93

Characterization of the glycolytic enzyme enolase which is abundant in the hyperthermophilic archaeon, *Pyrococcus furiosus*

M.J. Peak,[a] J.G. Peak,[a] F.J. Stevens,[a]

J. Blamey,[b] X. Mai,[b] Z.H. Zhou,[b]

and M.W.W. Adams[b]

[a]Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL (U.S.A.)[d]

and

[b]Department of Biochemistry and Center for Metalloenzyme Studies, University of Georgia, Athens GA (U.S.A.)

Key words: Enolase; Hyperthermophile; Enzyme characteristics; *Pyrococcus furiosus*

[d]Correspondence: M.J. Peak, Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Il (U.S.A.)
Tel. #(708)252-3850; Fax #(708)252-3387; E-mail peak@anlbem.bim.anl.gov

High enolase activity, as measured by the conversion of 2-phosphoglycerate to phosphoenolphyruvate, was found in the cytoplasm of *Pyrococcus furiosus* (an anaerobic, hyperthermophilic archaeon that grows optimally at 100 °C). In this organism, the enzyme probably functions in a sugar fermentation pathway. The enzyme was purified to homogeneity. It had a temperature optimum of >90 °C, and a pH optimum of 8.1. The enzyme was extremely thermostable with a half time for inactivation at 100 °C of 40 min. In contrast, an enolase from yeast was inactivated in 1 min at 88 °C. Both the *P. furiosus* and yeast enzymes required a metal ion for activity, but whereas the yeast enzyme has an absolute requirement for Mg^{++}, the *P. furiosus* enolase was equally active in the presence of Mn^{++}. Both enzymes were competitively inhibited by citrate. *P. furiosus* enolase, as for mesophilic enolases, probably has a homodimeric structure with subunit M_r greater than 45,000. A highly conserved sequence of eight amino acids in the N-terminal region was found in enolases from *P. furiosus* and a wide range of

other organisms including bacteria, yeast, birds, and mammals.

Introduction

Enolase (2-phospho-D-glycerate hydrolyase; E.C. 4.2.11) catalyses the conversion of 2-phosphoglycerate (PGA) to phosphoenol pyruvate (PEP) and is a key enzyme in the glycolytic pathway. Enolases from a wide range of sources have been studied and characterized [1]. Enolase was recently shown to be present in crude cell-free extracts of the archaeon *Pyrococcus furiosus* [2], an archeaon (formerly archaebacterium) which grows at temperatures up to 105 °C by the fermentation of carbohydrates [3]. In this organism, enolase is thought to catalyse a step in a unique, partially non-phosphorylated Entner-Doudoroff pathway [2,4-6]. This enables the conversion of a carbon source such as maltose, to acetate, carbon dioxide, and hydrogen without the participation of nicotinamide nucleotides [5].

Our current knowledge of proteins, including enzymes, from hyperthermophilic archaea was recently reviewed [7]. Several enzymes have been

characterized from these organisms, and so far most have the property of extreme thermostability. For example, a unique hydrogenase that is adapted for H_2 evolution and a ferredoxin, both of which are stable for up to 1 h at 100 °C, have been isolated from *P. furiosus* [8,9]. An exceptionally thermostable glutamate dehydrogenase from the same organism has also been described [10-11]. This has a half life of from 3.5 to 10 h at 100 °C, depending on the concentration of the enzyme. The most thermostable enolase previously reported [12,13] was from the extreme thermophile *Thermus aquaticus*, which grows optimally at 72 °C. This enzyme was found to have an optimum temperature for catalysis close to 90 °C, but above that temperature the activity fell sharply [12]. The aim of our study was to purify the enolase from *P. furiosus* and compare its temperature stability and other properties with enolases from thermophilic and mesophilic organisms. This was of considerable interest because enolases have not previously been purified from the Archaea, which represent the third domain of known life forms [14].

Materials and Methods

Materials

All reagents were of the highest purity available commercially. The substrate, PGA, and the *Saccharomyces cerevisiae* enolase were obtained from Sigma Chemical Co., St. Louis, MO.

Purification of enolase

P. furiosus was grown anaerobically in a 400-l fermenter at 88 °C as described previously [5,8]. Preparation of the cell extract, and enzyme purification by column chromatography were performed anaerobically as described previously [9]. Columns were controlled by an FPLC system from Pharmacia LKB, Piscataway, NJ. Active fractions collected from columns were pooled and concentrated when appropriate using a filtration unit from Amicon, Beverly, MA, fitted with a PM10 membrane.

The crude extract (1400 ml) was directly applied to a (8 cm × 21 cm) DEAE Sepharose column (Pharmacia) previously equilibrated with 50 mM

Tris-HCl buffer, pH 8, containing 10% glycerol, 2 mM dithionite, and 2 mM dithiothreitol. A linear gradient of 0.1–0.5 M NaCl in the same buffer was applied to the column at a flow rate of 8.5 ml/min, and 100–150 ml fractions were collected. Enolase activity started to elute at a concentration of 0.3 M NaCl. Active fractions (total volume 300 ml) were pooled, diluted to 1 l in 50 mM Tris-HCl buffer, pH 8, containing glycerol, dithionite, and dithiothreitol as above, and applied to a (8 × 17.7 cm) Q Sepharose High Performance column (Pharmacia) previously equilibrated with the same buffer. A linear gradient of 0.1–0.5 M NaCl in the same buffer was applied to the column at a flow rate of 8.5 ml/min. In this and all subsequent separations only fractions that contained protein, as indicated by the absorbance at 280 nm (A_{280}), were collected for assay. Fractions ranged in size from 15–100 ml. After the gradient was completed, the column was washed with 250 ml of 1 M NaCl in buffer and the wash was collected for assay. Enolase activity started to elute at 0.4 M NaCl and was detected in all subsequent fractions including

the NaCl wash. Fractions containing enolase activity were applied to a (3.5 × 35 cm) hydroxyapatite column (High Resolution, Behring Diagnostics) that had been washed with 50 mM Tris-HCl, pH 8.4. The sample (350 ml) was diluted to 1200 ml in the same buffer before loading. Fractions were eluted with a linear gradient of 0–0.2 M K^+PO_4 at a flow rate of 3.5 ml/min. Enolase activity started to elute at 0.03 M phosphate. Further purification was obtained by using a (5 × 30 cm) Q-Sepharose (Pharmacia) column with a combination linear/step gradient. A linear gradient of 0.2–0.6 M NaCl in 50 mM Tris-HCl, pH 8.4, was applied to the column at a flow rate of 3.5 ml/min. A major protein peak containing enolase activity started to elute at 0.49 M NaCl so the gradient was held at this concentration until the entire peak had been collected. The linear gradient was then restarted and continued up to 0.6 M NaCl and followed with a 1 M salt wash. Finally, an aliquot (35 mg) of active protein that had been concentrated to 5 mg/ml was applied to a (2 × 15 cm) Phenyl Sepharose column (Pharmacia) after being diluted

50:50 into Tris-HCl buffer, pH 8, containing 5.8 M $(NH_4)_2SO_4$. The column was eluted with a linear gradient of 2.9–0 M $(NH_4)_2SO_4$ at a flow rate of 2 ml/min. The active fraction was desalted using a Sephadex G-50 column to remove possible inhibitory effects of $(NH_4)_2SO_4$. Purified enolase samples were stored at 4 °C in Tris buffer, pH 8, containing 1 mM $MgCl_2$.

Enolase Activity

Enolase activity was measured spectrophotometrically by following the conversion of PGA to PEP by the increase in A_{240}. The buffer was 50 mM imidazole, pH 8, supplemented with 1 mM magnesium acetate, unless otherwise stated. The molar extinction (ε_{240}) of PEP at 70 °C in this buffer was found to be 1.28×10^3 M^{-1} cm^{-1}. The reaction was initiated by the addition of PGA and unless otherwise stated assays were performed aerobically at 70 °C.

Gel electrophoresis

Polyacrylamide gels were run using a Mighty Small 11 SE250 electrophoresis system from Hoeffer Scientific Instruments, San Francisco, CA. Denaturing polyacrylamide gel electrophoresis (PAGE) was by the method of Laemmli [15]. Protein samples were denatured at 100 °C for 15 min in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol prior to loading on the gel. The method for nondenaturing gel electrophoresis was described [16]. Protein samples were concentrated to 4 mg/ml for electrophoresis. High and low-molecular weight protein markers were obtained from BIORAD, Richmond, CA.

Protein determination

Protein was determined using the Lowry colorimetric method [17] with bovine serum albumin as standard.

Amino acid composition

Amino acid analysis was performed on an AB1 420A amino acid analyzer (Applied Biosystems, Foster

City, CA) using manual hydrolysis, and precolumn derivatization with phenyl isothiocyanate followed by reverse-phase high performance liquid chromatography [18]. This method yields a 15% loss of serine for which a correction was applied. Tryptophan and cysteine are not measured by this method.

Amino terminal sequencing

Protein from an SDS-PAGE gel was electroblotted onto polyvinylidine difluoride membranes using a Mini Trans-Blot Cell, fiber pads and membranes from BIORAD, Richmond, CA. The N-terminal sequence was determined using repeated automatic cycles of the Edman degradation reaction as described by Matsudaira [19].

Results

Purification and electrophoresis

In the crude cell-free extract of *P. furiosus*, enclase activity could only be detected in aliquots smaller than about 5 µl in the final volume of 1 ml. It therefore appeared than an inhibitor of enolase activity was present in cell extracts, so that activity was measurable only when the inhibitor was sufficiently dilute to be ineffective. This inhibition precluded accurate measurements of enolase activity in cell-free extracts and thus calculations of the degree of purification of the purified enzyme preparation. However, from a starting specific activity of about 1.3 U/mg protein, a specific activity of 13.8 U/mg was obtained using the protocol summarized in Table 1. An aliquot (25 mg protein) of this preparation was passed through a Phenyl Sepharose minicolumn as described in Methods. The active protein fraction that eluted from this column appeared homogenous on an SDS-PAGE gel (Fig. 1). However there was no further increase in measured specific activity, presumably due

to loss or inhibition of activity in the Phenyl Sepharose protocol.

The purified protein was electrophoresed on a denaturing (SDS) gel, alongside ladders of high- and low-molecular weight markers. As shown by Fig. 1, in a 12.5% denaturing gel enolase migrated almost as far as ovalbumin, indicating a subunit molecular weight close to 46,000 (Table 2) which is in agreement with that measured in other organisms [20,21]. The most frequently observed enolase structure is a homodimer [20]. *Pyrococcus* enolase may also have this structure but we cannot rule out the possibility that *Pyrococcus* enolase has a homooctomeric subunit structure as is found in *Zymomonas mobilis* and *Thermus aquaticus* [12,21].

Stability and catalytic activity

All samples of *P. furiosus* enolase were stored at 4 °C and remained completely stable under these conditions for many months. As shown in Fig. 2, the enzyme was extremely thermostable, especially when compared with the enolase from *Saccharomyces*

cerevisiae. The mesophilic enzyme was completely inactivated after 1 min at 88 °C, whereas the half life for the *P. furiosus* enzyme was 300 min at 88 °C and 40 min at 100 °C. The *P. furiosus* enolase is also considerably more thermostable than the enolase from the extreme thermophile, *Thermus aquaticus*, whose activity decreased rapidly upon exposure to 90 °C and was completely destroyed after 5 min at 100 °C [12]. Although several enzymes from *P. woesii*, which is closely related to *P. furiosus*, are stabilized by KCl (22), the half life of the enolase at 100 °C declined by a factor of about three in the presence of 700 mM KCl. Although no enolase activity was detected with *P. furiosus* enolase at 25 °C, it was readily measured at 40 °C, and increased with temperature up to 90 °C, the highest assay temperature used (Fig 3).

Some kinetic properties of the *P. furiosus* enolase are shown in Table 2. The K_m for PGA was 4×10^{-4} M, about four times larger than in the case of *S. cerevisiae* [23], indicating a lower affinity for the substrate. Its pH optimum is 8.1 [compared with 7.8 for *S. cerevisiae*: 24] and it is inhibited by citrate and

to a lesser extent by *iso*citrate. Figure 4 shows the Michaelis-Menton kinetics (double reciprocal plots) and demonstrates that citrate inhibition of the activity of *P. furiosus* enolase is strictly competitive. One possibility for this inhibition is that citrate chelates Mg^{++} ions. This divalent cation is essential for the activity of *P. furiosus* enolase (Fig. 5). This is also true for the *S. cerevisiae* enzyme which has been shown to contain one mole of tightly bound Mg^{++} per subunit [25]. In contrast, any bound Mg^{++} ions appear to readily dissociate from the *P. furiosus* enzyme with a K_m value of approximately 0.5 mM (Fig. 5). After the enzyme was passed through a Sephadex G-50 column equilibrated with 0.1 M Tris, pH 8, in the absence of Mg^{++}, it was no longer active. Addition of exogenous Mg^{++} to the protein restored activity, with complete restoration in the assay at 1 mM Mg^{++} (Fig. 5). Fig. 6 demonstrates that addition of 1 mM Mg^{++} to *P. furiosus* enolase that had been inhibited by 1.25 mM citrate almost completely reversed the citrate inhibition, which supports the Mg^{++} chelation hypothesis of citrate inhibition. This is further supported by the

observation that 1 mM EDTA added to the assay mixture also inhibited the enolase completely, and readdition of 1 mM Mg^{++} reversed this inhibition (data not shown). Fig. 6 also demonstrates that enolase from *P. furiosus* is more sensitive to citrate inhibition than the *S. cerevisiae* enzyme, consistent with the lower affinity of the hyperthermophilic protein for Mg^{++} ions.

Effect of divalent cations on activity

Table 3 lists activity of enolases with different divalent cations as cofactor. For *P. furiosus* enolase, the activity is the same for Mn^{++} as for Mg^{++}; Zn^{++} and Ni^{++} do not substitute for Mg^{++} or Mn^{++}. The enzyme from *S. cerevisiae* is different from *P. furiosus* enolase, since it is only 20% as active in the presence of Mn^{++}, compared with Mg^{++}.

Amino acid composition

The amino acid composition of *P. furiosus* enolase is given in Table 4, together with those of the enolases from the thermophiles *Thermus* X-1 and

Thermus aquaticus, yeast, rabbit muscle [13] and *Candida albicans* [26]. The table shows that the *P. furiosus* enzyme has higher percentage levels of glycine, alanine, proline, and valine compared with the other enolases, and lower levels of lysine, serine, and glutamic acid. The content of strongly hydrophilic residues is strikingly reduced. The composition with respect to all other amino acids was closely similar in the six enolases.

N-terminal amino acid sequence

The N-terminal sequence [27 residues] of *P. furiosus* enolase is shown in Table 5, and aligned with those of enolases from *Escherichia coli*, *Zymomonas*, *Candida*, *Xenopus*, and a variety of isoenzymes from rat, mouse, human, rabbit, and duck [21,26–38]. There is significant sequence homology. Isoleucine was common to all of the enzymes at position seven, together with a consensus sequence of six amino acids extending from position 16 to 21. The eucaryotic enolase from *Xenopus* had the strongest

homology with the *P. furiosus* enzyme, extending from position 13 to position 27.

Discussion

We have isolated and purified an enolase from the hyperthermophilic archaeon, *Pyrococcus furiosus*. Enolase is a major activity in this organism, consistent with its probable function as a key enzyme in the partially non-phosphorylated Entner-Doudoroff pathway, thought to be a an important route for sugar degradation in hyperthermophiles [2,5]. Although *P. furiosus*, like many archaea, is a strict anaerobe, the enolase activity that we isolated is not itself sensitive to oxygen.

In a comparison of the kinetic properties of *P. furiosus* with those of enolase from *S. cerevisiae*, the enzymes were found to be qualitatively similar in that both have an absolute requirement for a metal ion catalyst such as Mg^{++}, and both are inhibited by citrate. However, there are quantitative differences. For instance, the pH optimum for the *S. cerevisiae* enolase is slightly lower (pH 7.8 compared with 8.1)

and the yeast enzyme appears to have a stronger affinity for the substrate, PGA, as evidenced by its lower K_m value. The significance of these differences is not clear.

Citrate inhibition of *P. furiosus* enolase activity appeared to be strictly competitive and could be a classic physiological negative feedback (product inhibition) of an earlier step in a biosynthetic pathway. In this case the product (citrate) could be produced by carboxylation of PEP to oxaloacetate which condenses with acetyl-CoA to form citrate. This latter step is catalyzed by the enzyme citrate synthase, which we have demonstrated to be active in cell-free extracts of *P. furiosus* (unpublished data). An alternative possibility is that citrate inhibits enolase by chelating the Mg^{++} essential for its activity. Our finding that the citrate inhibition can be reversed by addition of more Mg^{++} (Fig. 6) supports this hypothesis, as does the enolase inhibition by EDTA that we observed. In this scenario the smaller citrate inhibition seen with *S. cerevisiae* enolase might be explained by the very tight binding of Mg^{++} by the yeast enzyme [25], and

this might also explain its low activity when other divalent cations are substituted. Whereas 1 mM Mn^{++} was able to substitute 100% for Mg^{++} in the assay of *P. furiosus* enolase assay, the activity of the *S. cerevisiae* enzyme in the presence of Mn^{++} was less than 20% the level when Mg^{++} was the catalyst. However, it is difficult to rationalize the data shown in Fig. 4 with a mechanism of inhibition that is purely or predominantly related to chelation of the cation. As shown in Fig. 5, enolase activity was essentially linearly proportional to Mg^{++} concentrations below 1 mM Mg^{++} routinely used in our enzyme assays. Thus, any decrease in free Mg^{++} concentration due to complexation by citrate should directly reduce the measured enzyme activity as a result of decreased concentration of active enzyme. This should reveal itself as a decrease in the apparent V_{max} with increasing chelator concentration, but without an increase in the substrate concentration required to saturate the remaining enzyme complexed with Mg^{++}. Nevertheless, Fig. 4 demonstrates that 0.25 mM citrate increases the K_m for PGA from 0.4 mM to 0.6

mM; a citrate concentration equal to the concentration of Mg^{++} ion increased the K_m by approximately one order of magnitude to 5 mM.

Therefore, we interpret these results to suggest that citrate inhibits enzyme activity primarily through competition with substrate for binding to the catalytic site of the *P. furiosus* enolase. The observation that excess Mg^{++} decreases the effect might indicate that a citrate:Mg complex has less affinity for the catalytic site. When comparing the effects of citrate on activities of the *P. furiosus* and *S. cerevisiae* enzymes, one potential confounding factor is the effect of temperature on the ability of citrate, in fact, to effectively chelate. Because chelation is the result of ionic interactions between the cationic Mg^{++} and the tricarboxylic acid, it is possible that the elevated temperature used to assay the *P. furiosus* enolase substantially diminishes the sequestering of Mg^{++}.

If metal chelation is less effective at the elevated temperatures for which the metabolic pathways of *P. furiosus* and other hyperthermophiles are adapted, then it is possible that comparisons of related enzymes

from hyperthermophiles with those from thermophiles and mesophiles will reveal differential tendencies in the mechanisms used for regulation. Although the fourfold difference between the *P. furiosus* and *S. cerevisiae* enzymes in their K_m values for the substrate PGA might be consistent with some variation in the structure of the catalytic site that could be associated with differential susceptibility to (end) product inhibition, more detailed analyses of enzyme kinetics and a comparison of three-dimensional structures will be required to establish the significance and structural basis of citrate inhibition.

A comparison of the primary stucture of the N-terminal region of the *P. furiosus* enolase with that from several other organisms (Table 5) revealed a near identity, most notably in a segment roughly centered by an arginine residue at position 18. This arginine corresponds to the arginine at position 12 in the enzyme from *S. cerevisiae*. The three-dimensional structure of the *S. cerevisiae* enzyme-substrate complex has been described [39–41]. Although the arginine residue has not been implicated in the

catalytic mechanism of enolase dehydration, it represents a significant contributor to the catalytic environment. The conserved sequence containing this arginine must be of importance to the functioning of the enzyme, since it has been conserved from one of the earliest known forms of life through to extant *P. furiosus* and humans, over a period of perhaps 4 billion years [14].

In contrast to the rather strict conservation of the primary structure of the N-terminus, significant variation of the overall amino acid composition of the *P. furiosus* enzyme is found when compared to enolases from other organisms (Table 4). Presumably, at least some of these variations correlate with the diminished thermal stability of the non-archaean enolases.

A high level of glycine is found, which because of its small side chain might contribute to some relatively tighter beta turns and closer internal packing of amino acid side chains. Most striking perhaps, is a comparison of the ratio of hydrophobic to hydrophilic amino acids (Table 6). The ratio ranges

from 0.9 to 1.2 for the five non-archaeal enzymes but is 2.9 for the *P. furiosus* enolase. This threefold higher ratio would suggest that energetically, the difference between the "outside" and "inside" of the enzyme is significantly more distinct for the hyperthermophilic enzyme and may contribute to enhanced resistance to denaturation. However, Barnes and Stellwagen [13] calculated the average hydrophobicity (HQ_{ave}) of enolases from rabbit muscle, yeast, *Thermus* X-1 and *Thermus aquaticus* using data for relative hydrophobicity of individual amino acids tabulated by Bigelow [42] and found, in fact, a reciprocal relationship between the thermostability of these enolases and their HQ_{ave}.

Clearly, as previously pointed out (see 7), detailed structure informatin is required for insights into mechanisms of protein "hyperthermostability."

Acknowledgements

Work supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract No. W-31-109-ENG-38 (to M.J.P.) and

by a grant from the Office of Naval Research (N00014-90-J-1894) to M.W.W.A. We thank E. Smith, S. Mukund, K. Ma, and V. Ramakrishnan for generous assistance in the laboratory, and Dr. J. M. Brewer for useful discussions.

References

1 van der Streeten, D., Rodrigues-Pousada, R.A., Goodman, H.M. and Montagu, M.V. (1991) Plant Cell 3, 719–735.

2 Schäfer, T. and Schönheit, P. (1992) Arch. Microbiol. 158, 188–202.

3 Fiala, G. and Stetter, K.O. (1986) Arch. Microbiol. 145, 56–61.

4 Conway, T. (1992) FEMS Microbiol. Rev. 103, 1–28.

5 Mukund, S. and Adams, M.W.W. (1991) J. Biol. Chem. 266, 14208–14216.

6 Adams, M.W.W. (1993) FEMS Microbiol. Rev. 47, 627–658.

7 Adams, M.W.W. (1993) Ann. Rev. Microbiol. 47, 627-658.

8 Aono, S., Bryant, F.O. and Adams, M.W.W. (1989) J. Bacteriol. 171, 3433–3439.

9 Bryant, F.O. and Adams, M.W.W. (1989) J. Biol. Chem. 264, 5070–5079.

10 Robb, F.T., Park, J.-B. and Adams, M.W.W. (1992) Biochim. Biophys. Acta 1120, 267–272.

11 Consalvi, V., Chiaraluce, R., Politi, L., Vaccaro, R., De Rosa, M. and Scandurra, R. (1991) Eur. J. Biochem. 202, 1189-1196.

12 Stellwagen, E., Cronlund, M.C. and Barnes L.D. (1973) Biochemistry 12, 1552–1559.

13 Barnes, L.D. and Stellwagen, E. (1973) Biochemistry 12, 1559–1565.

14 Woese, C.R., Kandler, O. and Wheelis, M.L. (1990) Proc. Nat. Acad Sci. U.S.A. 87, 4576–4579.

15 Laemmli, U.K. (1970) Nature 227, 680–685.

16 Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) Current Protocols in Molecular Biology, vol. I, Wiley, New York.

17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.

18 Ozols, J. (1990) in Methods in Enzymology (Deutscher, M.D., ed.), Vol. 182, pp. 587–591, Academic Press, San Diego, CA.

19 Matsudaira, P. (1990) in Methods in Enzymology (Deutscher, M.D., ed.), Vol. 182, pp. 602–613, Academic Press, San Diego, CA.

20 Wold, F. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. 5, 3rd. ed., Academic Press, New York, NY.

21 Burnett, M.E., Liu, J. and Conway, T. (1992) J. Bacteriol. 174, 6548–6553.

22 Scholz, S., Sonnenbichler, J., Schäfer, W. and Hensel, R. (1992) FEBS Lett. 306, 239-242.

23 Wold, F. and Ballou, C.E. (1957) J. Biol. Chem. 227, 301–312.

24 Holt, A. and Wold, F. (1961) J. Biol. Chem. 236, 3227–3231.

25 Brewer, J.M. (1985) FEBS Letts. 182, 8–14.

26 Sundstrom, P. and Aliaga, G.A. (1992) J. Bacteriol. 174, 6789–6799.

27 Weng, M., Makaroff, C.A. and Zalkin, H. (1986) J. Biol. Chem. 261, 5568–5574.

28 Segil, N., Shoutkowski, A., Dworkin, M.B. and Dworkin-Rastl, E. (1985) Biochem. J. 251, 31-39.

29 Sakimura, K., Kushiya, E., Obinata, M. and Takahashi, Y. (1985) Nucleic Acids Res. 13, 4365-4378.

30 Sakimura, K., Kushiya, E., Obinata, M., Odani, S. and Takahashi, Y. (1985) Proc. Nat. Acad. Sci. U.S.A. 82, 7453-7457.

31 Giallongo, A., Oliva, D., Cali, L., Barba, G. and Showe, L.C. (1986) Proc. Nat. Acad. Sci. U.S.A. 83, 6741-6745.

32 Kaghad, M., Dumont, X., Chalon, P., Lelias, J.M., Lamande, N., Lucas, N., Lazar, M. and Caput, D. (1990) Nucleic Acids Res. 18, 3638-3638.

33 Ohshima, Y., Mitsui, H., Takayama, Y., Kushiya, E., Sakimura, K. and Takahashi, Y. (1989) FEBS Letts. 242, 425–430.

34 Peshavaria, M. and Day, I.N.M (1991) Biochem. J. 275, 427–433.

35 Chin, C.C.Q. (1990) J. Protein Chem. 9, 427–432.

36 Lamande, N., Mazo, A.M., Lucas, M., Montarras, D., Pinset, C., Gros, F., Legault-Demare, L. and Lazar, M. (1989) Proc. Nat. Acad. Sci. U.S.A. 86, 4445–4449.

37 Russell, G.A., Dunbar, B. and Fothergill-Gilmore, L.A. (1987) Biochem. J. 236, 115–126.

38 Wistow, G.J., Lietman, T., Williams, L.A., Stapel, S.O., de Jong, W.W., Hortwitz, J. and Piatigorsky, J. (1988) J. Cell. Biol. 107, 2729–2736.

39 Lebioda, L., Stec, B. and Brewer, J.M. (1987) J. Biol. Chem. 264, 3685–3693.

40 Leboida, L. and Stec, B. (1991) Biochemistry 30, 2817–2822.

41 Leboida, L. and Stec, B. (1991) Biochemistry 30, 2823–2827.

42 Bigelow, C.C. (1967) J. Theoret. Biol. 16, 187–211.

Table 1. Purification of enolase from *P. furiosus*

Step	Protein (mg)	Activity (U[1])	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Crude	56000	72000	1.3	1	100
DEAE Sepharose	8500	46900	5.5	4.2	65
Q-Sepharose HP[2]	5000	31300	6.2	4.8	43
HAP[3]	2200	26600	11.8	8.9	37
Q-Sepharose	800	11100	13.8	10.6	15

[1]U=1 μmol PEP produced from PGA in 1 min

[2]High performance

[3]Hydroxyapatite

Table 2. Properties of *P. furiosus* enolase

Parameter	Substrate	Value
K_m	2-PGA	0.40 mM
K_i	Citrate	0.13 mM
pH optimum	2-PGA	8.1
Subunit molecular weight		46,000
50% activity	Citrate	0.30 mM
	*Iso*citrate	0.20 mM
	(K-PO_4)	20.0 mM

Table 3. Effect of divalent cations on enolase activity

Enzyme	Cation, 1 mM	% Activity
P. furiosus	Mg^{++}	100
	Mn^{++}	100
	Zn^{++}	<1
	Ni^{++}	<1
S. cerevisiae	Mg^{++}	100
	Mn^{++}	20

Table 4. Amino acid composition[1] of six enolases

Amino acid	*Pyrococcus furiosus*[2]	*Thermus* X-1[3]	*Thermus aquaticus*[3]	*Candida albicans*[4]	Yeast[3]	Rabbit muscle[3]
Ala	13	10	10	10	12	10
Arg	3	3	6	3	3	4
Asn	0			4		
Asp	7	11	7	7	11	10
Cys	ND[5]	0	0	0	0	2
Glu	3	13	13	6	8	10
Gly	19	10	15	9	9	10
His	0	1	2	2	3	2
Ile	8	6	3	7	5	6
Leu	11	9	8	9	9	9
Lys	4	6	9	8	9	9
Met	0.3	2	1	1	1	2
Phe	4	2	3	3	4	3
Pro	7	3	3	4	3	3
Ser	2[6]	4	10	7	7	4
Thr	4	5	4	4	4	4
Trp	ND	0	1	1	1	1
Tyr	2	3	2	3	2	2
Val	14	8	6	6	8	8

[1]Percentage

[2]This study

[3]Barnes and Stellwagen, [13]

[4]Sundstrom and Aliaga, [26]

[5]Not determined

[6]A correction for 15% loss of serine was applied

Table 5. Sequence homology between enolases

Organism	N-Terminal Amino Acid Sequence	Reference
	1 5 10 15 20	
P. furiosus	MENPYEIVGVVAREIL**DXRGNP**TVEVD	[this study]
E. coli	......I.....REI.**DSRGNP**.....	[27]
Zymomonas; candida	......I.....**DSRGNP**TVEVD	[21,26]
Xenopus, γ	......I.....REIL**DSRGNP**TVEVD	[28]
Rat, α; rat, β; human, α; mouse, α	......I.....REI.**DSRGNP**.....	[29-32]
Rat, β; human, β; rabbit, β; mouse, β, γ; chick, β; duck, α	......I....AREIL**DSRGNP**.....	[33-38]

Table 6. Ratio of hydrophobic to hydrophilic amino acids in six enolases

Organism	Ratio[1]
P. furiosus	2.94
Thermus X-1	1.06
T. aquaticus	0.86
c. albicans	1.25
Saccharomyces	1.22
Rabbit muscle	1.03

[1]Ala + Phe + Ile + Leu + Val / Arg + Asp + Glu + Lys

Legends to Figures

Fig. 1. SDS-PAGE of the purified enolase from *Pyrococcus furiosus*. Enolase samples (3 mg/ml) were incubated in the presence of SDS (1% w/v) for 15 min at 100 °C prior to electrophoresis on a 10% acrylamide gel containing SDS (0.1% w/v). Approximately 4 and 2 μg protein were applied to lanes 2 and 3 respectively, numbered from left to right. Lanes 1 and 4 are protein standard mixtures. Lane 1 contained (top to bottom) myosin (M), β-galactosidase (G), phosphorylase b (P), bovine serum albumin (A) and ovalbumin (O). Lane 4 contained (top to bottom) P, A, O, and carbonic anhydrase (C).

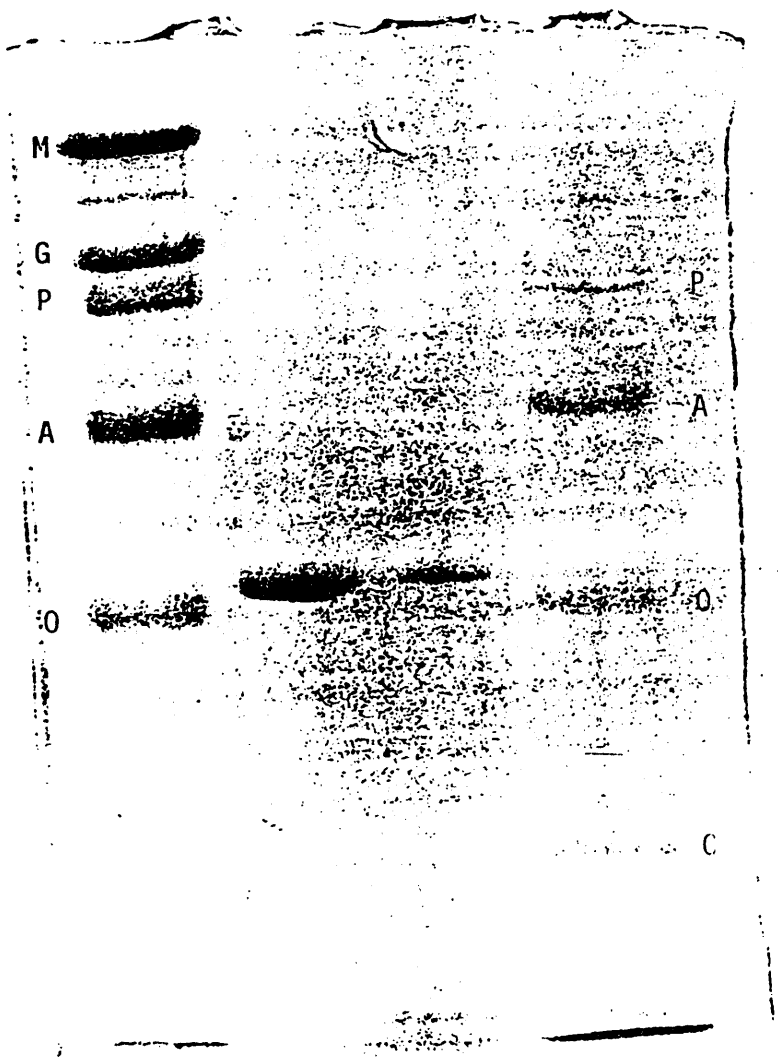
Fig. 2. Stability of enolase from *P. furiosus* at 88 °C (circles) and 100 °C (squares) and at 100 °C in the presence of 1 M KCl (triangles). After incubation for different time periods at the elevated temperatures, activity was measured at 70 °C.

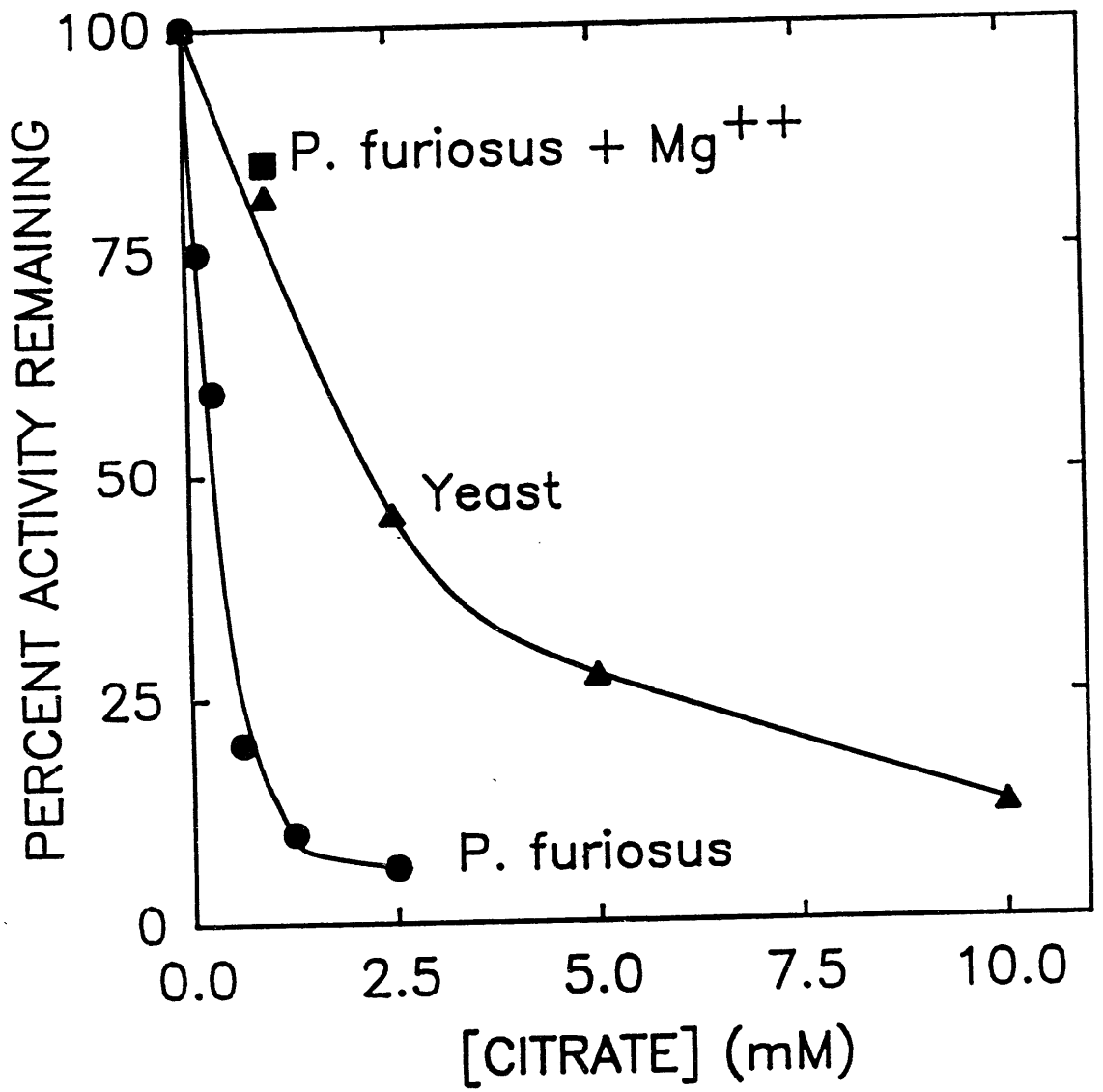
Fig. 3. Effect of temperature upon the activity of enolase from *P. furiosus*. The activity was measured in the spectrophotometer cuvette at a range of different temperatures.

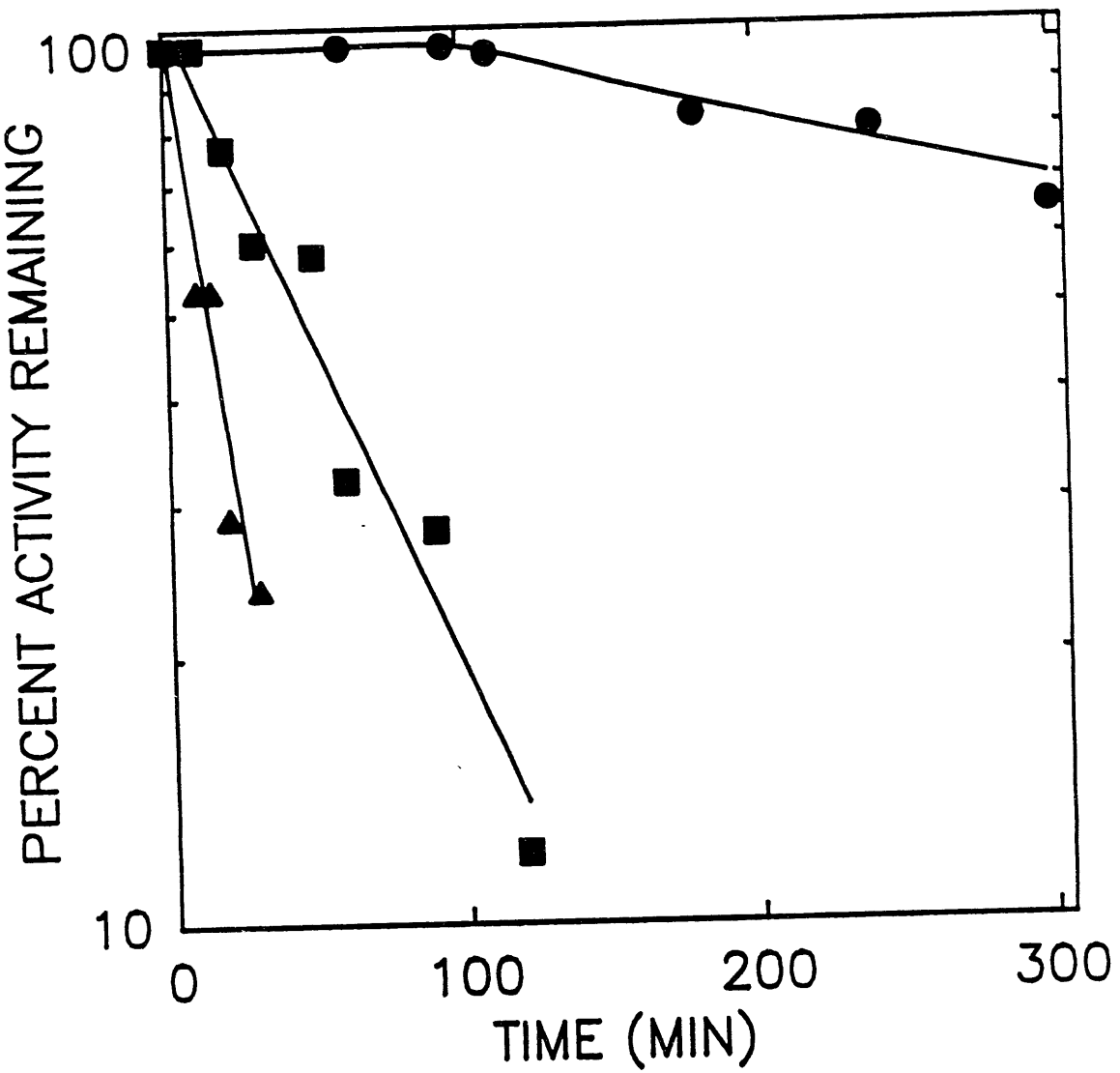
Fig. 4. Reciprocal plots of reaction velocity (v) against substrate concentration (s) for enolase at different concentrations (mM) of citrate demonstrating competitive inhibition of activity by citrate.

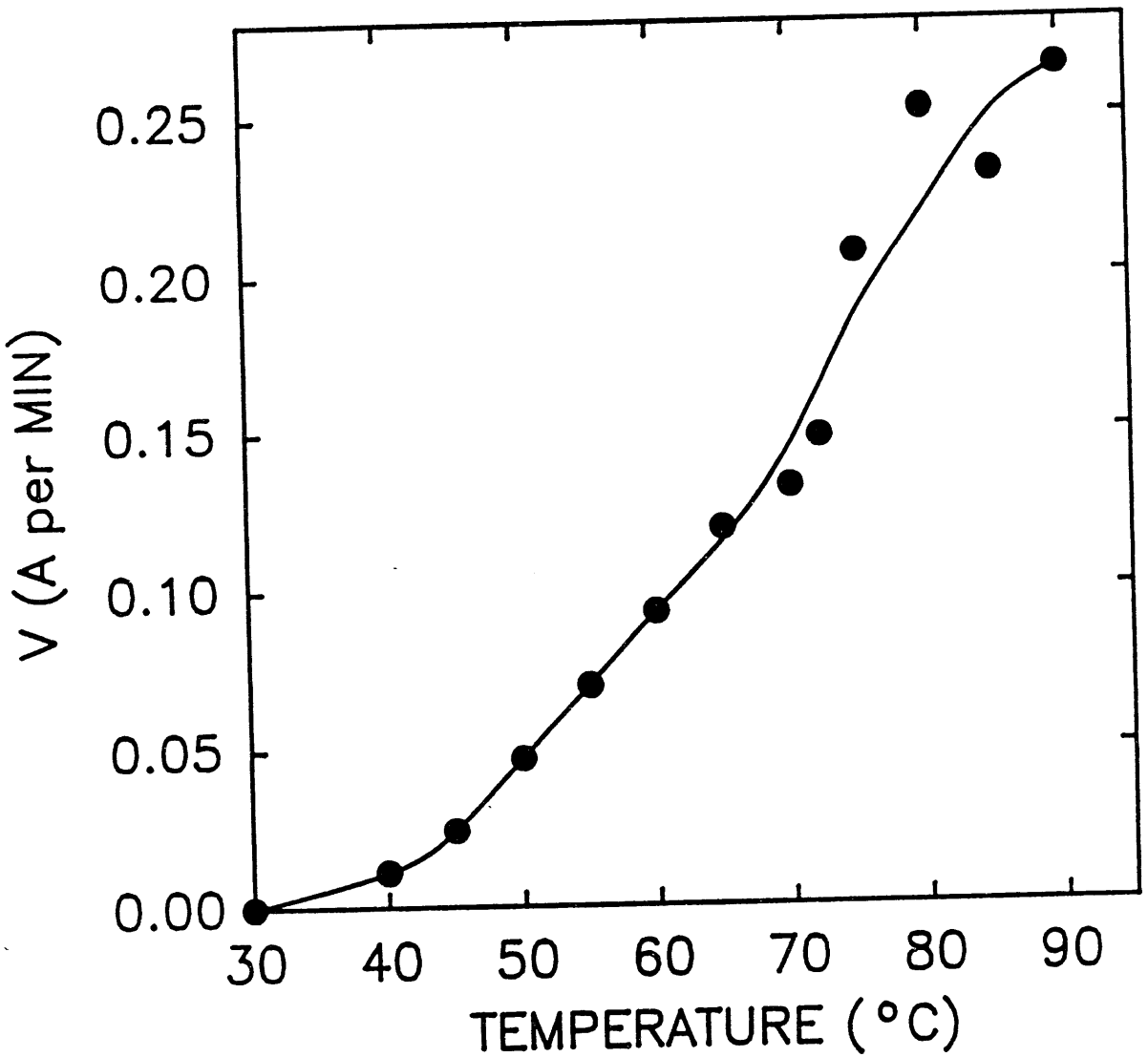
Fig. 5. Effect of Mg^{++} concentrations upon *P. furiosus* enolase activity.

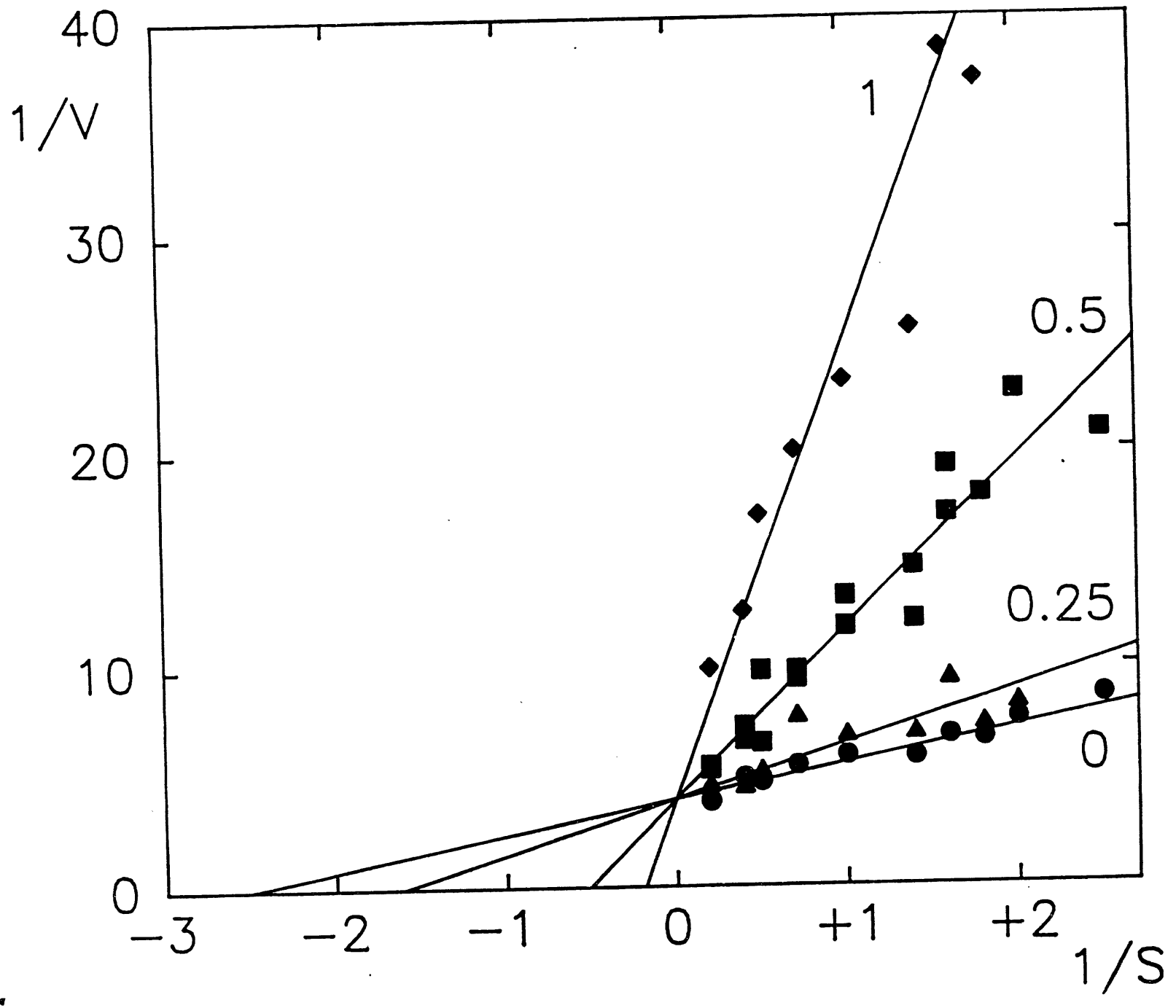
Fig. 6. Comparison of effects of citrate on yeast (triangles) and *P. furiosus* (circles) enolase. The square symbol shows the almost complete restoration by 1 mM Mg^{++} of the activity of *P. furiosus* enzyme that had been totally inhibited by 1 mM citrate.

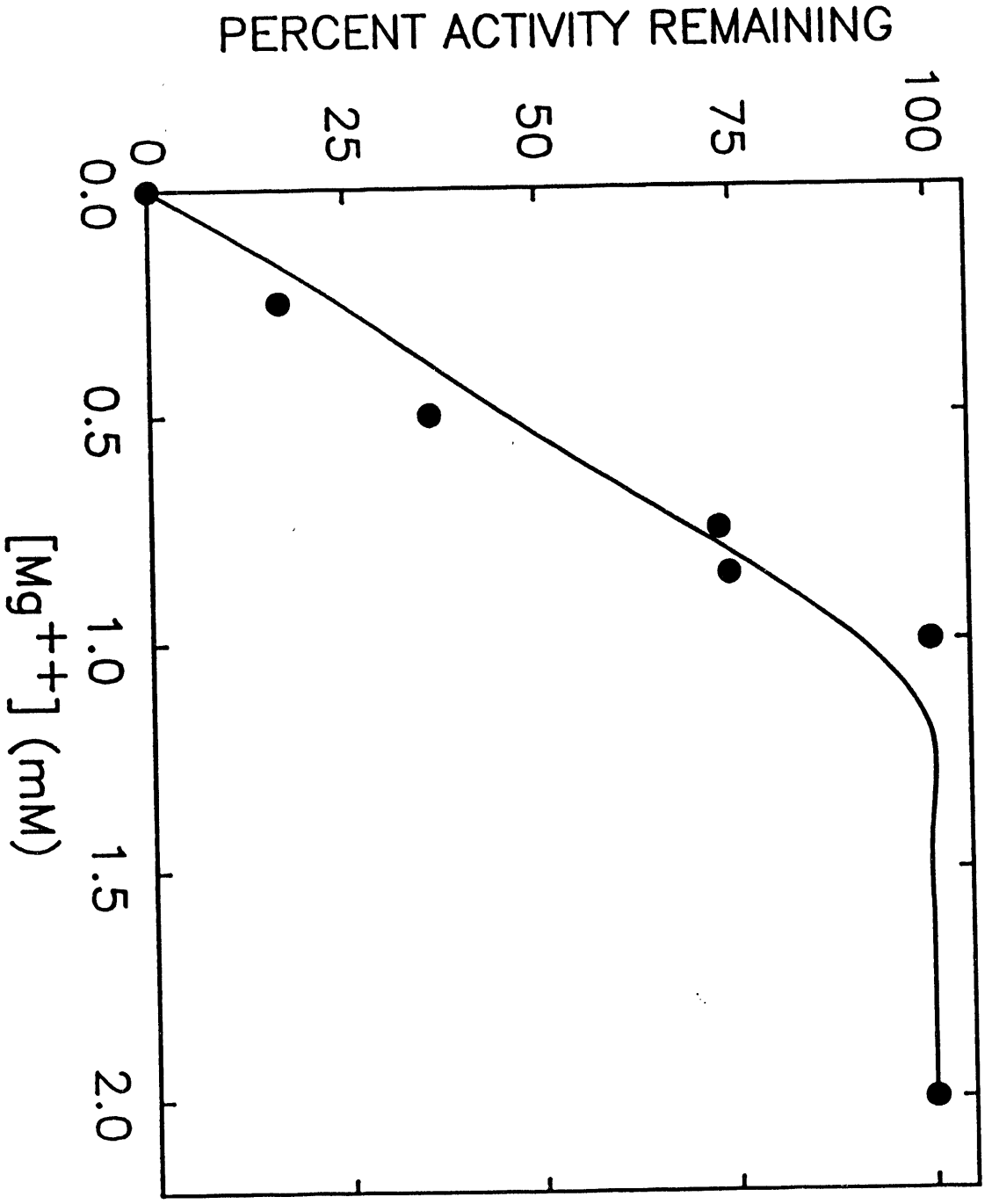

M
G
P
A
O
P
A
O
C


100
75
50
25
0
PERCENT ACTIVITY REMAINING
P. furiosus + Mg++
Yeast
P. furiosus
0.0
2.5
5.0
7.5
10.0
[CITRATE] (mM)


100
10
PERCENT ACTIVITY REMAINING
0
100
200
300
TIME (MIN)


0.25
0.20
0.15
0.10
0.05
0.00
V (A per MIN)
30
40
50
60
70
80
90
TEMPERATURE (°C)


40
1/V
30
20
10
0
−3
−2
−1
0
+1
+2
1/S
1
0.5
0.25
0


PERCENT ACTIVITY REMAINING
0
25
50
75
100
0.0
0.5
1.0
1.5
2.0
[Mg++] (mM)

DATE FILMED

3/22/94

END