Partial Characterization of Fumarase from an Extreme Thermophilic Bacterium Isolated in Indiana

KEITH BITZINGER and ROBERT F. RAMALEY[1]
Department of Microbiology
Indiana University, Bloomington, Indiana 47401

Abstract

Fumarase (L-malate hydro-lyase E.C. 4.2.1.2) was partially purified from a gram negative, non-spore forming, extreme thermophilic bacterium isolated from a steam chamber on the Indiana University Campus at Bloomington, Indiana. This isolate is very similar to the *Thermus* X-1 isolate previously reported by Ramaley and Hixson and is designated as the *Thermus* K-1 isolate.

The K-1 fumarase has an apparent molecular weight of 170,000, a broad bell shaped pH optimum between pH 7.5 and pH 11, with L-malate as a substrate and an apparent Michaelis constant (Km) for L-malate of 3.3 ± 0.4 millimolar. The K-1 fumarase is fully heat stable for 30 minutes at temperatures up to 85° Centigrade. An Arrhenius plot of the enzyme catalyzed reaction showed an inflection (critical point) at 70° Centigrade with an apparent activation energy of 12,000 calories below 70° Centigrade.

Introduction

In 1969 Brock and Freeze (2) reported the isolation of a new extreme thermophilic bacterium, *Thermus aquaticus*, from natural thermal areas (Yellowstone National Park). This gram negative non-spore-forming organism was also found in water samples from thermally polluted areas including areas on the Indiana University Campus (3). In 1970 Ramaley and Hixson (14) reported the isolation of an apparently different extreme thermophilic bacterium which has been designated as *Thermus* X-1 isolate; pending sufficient studies to see if it warrants being assigned a new species name. Since that time, a number of additional isolates have been obtained confirming the widespread distribution of these bacteria.

During the Fall of 1971 one of the authors (K.B.) isolated such a bacterium (Isolate K-1) from a steam chamber on the Indiana University Campus at Bloomington, Indiana. This organism has an optimum growth temperature of 70° C and appears to be similar to the X-1 isolate of Ramaley and Hixson (14).

To gain further information concerning the comparative physiology of these new bacteria, the properties of enzymes from a number of these isolates have been investigated. Fumarase from the K-1 isolate was chosen for the presently reported study because of the extensive studies that have been conducted on the kinetic (15) and physical (6) properties of the enzyme purified from non-thermophilic sources and because in contrast to other hydrolyases no cofactor requirements are needed for the fumarase catalyzed reaction (6).

[1] Present Address: Department of Biochemistry, University of Nebraska Medical Center, Omaha, Nebraska 68105

Methods and Materials

Isolation of a Growth of the K-1 Isolate

K-1 was isolated from a pool of water at the bottom of a cement chamber containing steam pipes between Ballantine and Morrison Hall on the Indiana University campus at Bloomington, Indiana. The method of enrichment and isolation were similar to those used for the isolation of *Thermus* X-1. The K-1 isolate was grown in a 0.1% yeast extract, 0.1% tryptone, Catzenholtz salt medium as previously described (14). Studies on the growth characteristics of the organism were done in 250 ml side arm Bellco flasks containing 50 ml of medium and incubated at 70°C in a Fermentation Design shaking water bath (120 strokes/min). Cells for the purification of fumarase were grown in 10 l of medium in a 14-liter Fermentation Design fermenter.

Partial Purification of the K-1 Fumarase

Approximately 20 g (wet weight) of K-1 cells were suspended in 40 ml of 0.01 M KH_2PO_4 (pH 7.2), lysozyme was added to a final concentration of 0.1 mg/ml and the cells incubated at 37°C for 20 min. The resulting spheroplasts were disrupted by sonic treatment (Bronwill Biosonik sonicator). The cell extract was centrifuged at 76,000 x g for 90 min (Spinco ultracentrifuge) and the supernatant fraction dialized overnight against 4 l of 0.01 M potassium phosphate (pH 7.2).

The dialyzed extract was placed on a 75 x 2.5 cm column of DEAE cellulose (Whatman DE-32, microgranular) previously recycled and equilibrated with the phosphate buffer. One liter of phosphate buffer containing 10^{-4} M 2-mercaptoethanol was placed through the column and the proteins eluted with a linear gradient of potassium chloride from 0 to 0.2 M in the phosphate-mercaptoethanol buffer. The furmarase eluted as a single peak at 0.05 M KCl. The fumarase was concentrated by Lypogel (Gelmann) and stored at —20° C.

Fumarase Assay

Fumarase was assayed by a modification of the method of Racker (13). The standard reaction mixture contained 0.15 M L-malate in 0.05 M (KH_2PO_4) at pH 7.6 and the formation of fumarate determined by the increase in absorbancy at 240 nm with the use of a Gilford 2000 recording spectrophotometer. The temperature of the reaction mixture was maintained at 60° C unless otherwise specified. One unit of K-1 fumarase in the present report is defined as that amount of enzyme causing an increase in absorbancy at 240 nm of one absorbancy unit in 1 min at 60° C. Protein was determined by the method of Lowry *et al.* (8) with bovine serum albumin as a standard and the specific activity is defined in terms of units per milligram of protein.

Other methods such as the isolation of the DNA (9), determination of per cent guanosine plus cytosine content of the DNA (16), determination of the K-1 fumarase molecular weight by Sephadex G-200 gel filtrations (17) and use of Wang computer for Km determination (17) have been previously described.

Results

Properties of the K-1 Isolate

The K-1 isolate has the same general properties as previously reported for the *Thermus* X-1 isolate including optimum growth at 70° C (48 min doubling time), lack of a pronounced yellow carotenoid pigment, morphology, negative gram stain, Actinomycin D sensitive, guanosine plus cytosine base ratio of the DNA (65%) and lack of growth on nutrient agar (14).

Properties of the K-1 Fumarase

DEAE cellulose chromatography of K-1 cell free extracts gave a 10-fold purification of the fumarase (to 0.22 units/mg protein) used for the present studies. Some studies have also been conducted with enzyme purified more extensively but no significant differences are observed over those reported here (R. Ramaley, unpublished observations).

Figure 1 shows the thermostability and effect of temperature on the rate of snzymatic reaction catalyzed by the K-1 fumarase. The enzyme was fully stable for 30 min at temperatures up to 85° C. Studies conducted on the rate of loss of enzymatic activity at 90° C and 95° C showed a simple first order loss of activity with no apparent effect by the presence or absence of malate and fumarate.

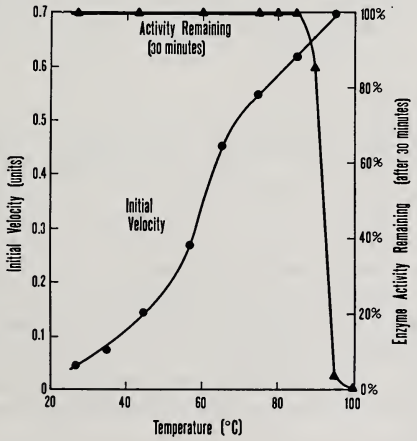

FIGURE 1. *Thermostability and effect of temperature on the initial velocity of the K-1 fumarase.*

Figure 1 also shows the effect of temperature on the initial enzymatic reaction rate of the K-1 fumarase. There was an increasing rate at temperatures up to 95° C (the highest temperature tested). However, there is a discontinuity in the rate as can be seen in an Arrhenius plot of the data given in Figure 1. There is an inflection point "critical point" near 70° C and calculation of the apparent activation

energy gave a value of 12,000 calories below 70° C and 3,000 calories above 70° C.

The K-1 fumarase has a broad symmetrical bell shaped pH optimum between pH 7.5 and pH 11 and an apparent Michaelis constant (Km) for L-malate of 3.3 ± 0.4 millimolar (calculated by the use of a Wang 700 computer). The apparent molecular weight of the enzyme as determined by Sephadex gel filtration (12) was 170,000 and there was no evidence for any additional molecular forms of the enzyme.

Discussion

This report confirms and provides an additional example of an enzyme from a thermophilic microorganism that is more thermostable and catalyzes its enzymatic reaction at higher temperatures than the same enzyme from mesophilic sources (1). Fumarase, especially the fumarase from swine heart, has been the subject of some classical kinetic studies by Massay (10), Alberty and their co-workers (6). Recently, fumarase has been the subject of "a definitive isotope exchange study" (15) by Hansen, Dinova and Boyer (5) and the physical properties of the enzyme have received considerable study in Hill's laboratory (6).

The K-1 fumarase showed a discontinuity at 70° C in the Arrhenius plot at pH 7.3 when L-malate was used as the substrate. Similar discontinuities have been observed with fumarate as a substrate by Massey (10). However, the explanation of the thermal discontinuity is still subject to interpretation (4). The fact that the discontinuity occurred at 70° C (the optimum growth temperature of the organism) may be of some interest.

The molecular weight of the K-1 fumarase (170,000) is similar to the molecular weight of the *Pseudomonas putida* fumarase (166,000) which was purified by Lamartiniere *et al.* (7). The Km of the K-1 fumarase for malate (3.3 mM) is similar to the Km for other fumarases (6) including bacterial fumarase (7). However, the kinetic constants for the enzyme are dependent on pH, ionic strength and anions (6). The phosphate buffer employed in the present study is slightly inhibitory to the enzyme reaction and actually shifts the pH optimum to a slightly more alkaline pH (6). However, since the change in pH as a function of temperature for phosphate is much less than that observed with other buffer (*e.g.*, Tris), phosphate was used throughout the present study.

The present study shows some of the utility of these new extreme thermophilic bacteria for general enzymology and enzyme regulation (11) and provides an additional example of the pronounced thermostability of their enzyme.

During these studies one of the authors (K.B.) also isolated an apparently new "pink", non spore-forming, thermophilic bacterium (K-2) with an optimum temperature of 60° C from below a hot water discharge into the Jordan River on the Indiana University Campus

(downstream from Station 30 shown in Figure 1 of Brock and Yoder (3) and this organism is currently being characterized. Thus, it has become apparent that we have only just begun to appreciate the components and biology associated with "man-made thermal environments".

Literature Cited

1. AMELUNXEN, R., and M. LINS. 1968. Comparative thermostability of enzymes from *Bacillus cereus*. Arch. Biochem. Biophys. 125:765-769.

2. BROCK, T. D., and H. FREEZE. 1969. *Thermus aquaticus*, gen. n and sp. n., a nonsporulating extreme thermophile. J. Bacteriol. 98:289-297.

3. BROCK, T. D., and I. YODER. 1971. Thermal pollution of a small river by a large university: bacteriological studies. Proc. Indiana Acad. Sci. 80:183-188.

4. DIXON, M., and E. C. WEBB. 1964. Effect of temperature. p. 145-166. *In* DIXON, M., and E. C. WEBB, Enzymes, 2nd ed., Academic Press, New York, N. Y. 950 p.

5. HANSEN, J. N., E. C. DINOVO, and P. D. BOYER. 1969. Initial and equilibrium ^{18}O, ^{14}C, ^{3}H and ^{2}H exchange rates as probes of the fumarase reaction mechanism. J. Biol. Chem. 244:6270-6279.

6. HILL, R. L., and J. W. TEIPEL. 1971. Fumarase and crotonase. p. 539-571. *In* BOYER, P. D. (ed.), The Enzymes, V, 3rd ed., Academic Press, New York, N. Y. 734 p.

7. LAMARTINIERE, C. A., H. D. BRAYMER, and A. D. LARSON. 1970. Purification and characterization of fumarase from *Pseudomonas putida*. Arch. Biochem. Biophys. 141:293-302.

8. LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

9. MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208-218.

10. MASSEY, V. 1953. Studies on fumarase. 3. The effect of temperature. Biochem. J. 3:72-79.

11. PENNER, P. E., and L. H. COHEN. 1969. Effect of adenosine triphosphate and magnesium ions on the fumarate reaction. J. Biol. Chem. 244:1070-1075.

12. PENNER, P. E., and L. H. COHEN. 1971. Fumarase: demonstration, separation and hybridization of different subunit types. J. Biol. Chem. 246:4261-4265.

13. RACKER, E. 1950. Spectrophotometric measurement of the enzymatic formation of fumaric and cis-aconitic acid. Biochem. Biophys. Acta 4:211-214.

14. RAMALEY, R. F., and J. HIXSON. 1970. Isolation of a nonpigmented thermophilic bacterium similar to *Thermus aquaticus*. J. Bacteriol. 103:527-528.

15. ROSE, I. A. 1970. Enzymology of proton abstraction and transfer reactions. p. 281-320. *In* BOYER, P. D. (ed.) The Enzymes, II, 3rd ed., Academic Press, New York, N. Y. 734 p.

16. SCHILDKRAUT, C. L., J. MARMUR, and P. DOTY. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4:430-443.

17. SEDMAK, J., and R. RAMALEY. 1971. Purification and properties of *Bacillus subtilis* nucleoside diphosphokinase. J. Biol. Chem. 246:5365-5372.