Tritium exchange reactions catalyzed by aconitase have shown the involvement of a basic proton-abstracting group on the enzyme (*11*). Coordination of the fluorine of fluorocitrate to the enzyme-bound metal could facilitate the substitution of fluoride by the proton-abstracting group of the enzyme and lead to irreversible inhibition. The spatial arrangements of the fluorine and the proton-abstracting group are illustrated in Fig. 2. Thus, the crystallographic model explains both the competitive inhibition and the slower inactivation of aconitase by fluorocitrate.

H. L. CARRELL, JENNY P. GLUSKER
J. J. VILLAFRANCA, A. S. MILDVAN
The Institute for Cancer Research, Philadelphia, Pennsylvania 19111
R. J. DUMMEL, E. KUN
Department of Pharmacology and *Cardiovascular Research Institute, University of California, School of Medicine, San Francisco*

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Threonine Deaminase: A Novel Activity Stain on Polyacrylamide Gels

Abstract. *With phenazine methosulfate and nitro blue tetrazolium, an activity stain for threonine deaminase has been developed. Because the deamination reaction does not involve any overall oxidation or reduction, it is proposed that one of the intermediates of the reaction, α-aminocrotonate or α-iminobutyrate, is the reducing agent. Studies with ferricyanide as the artificial electron acceptor indicate that a decarboxylation of the intermediate occurs during the dye reduction.*

The ability to identify a protein band with a specific enzyme activity by an activity stain in situ after electrophoresis on polyacrylamide or starch gels has proven useful in exploring a variety of questions. For example, with such a stain, the existence of isoenzymes can be established, even in relatively crude extracts. Similarly, this simple technique can provide valuable information about changes in protein structure or association-dissociation behavior of enzymes in the presence or absence of ligands. Unfortunately, activity stains in situ have been developed for only a few enzymes. The most familiar activity stain is that for the various dehydrogenases (*1*) where pyridine nucleotide or flavin nucleotide coenzymes participate in the enzyme-catalyzed oxidation of substrates. In addition to the dehydrogenases, activity stains have been developed for polynucleotide phosphorylase (*2*), alkaline phosphatase (*3*), esterases (*4*), and several other enzymes (*4*).

To stain for dehydrogenase activity after electrophoresis, the gel is incubated in a buffered solution containing the oxidized coenzyme and the reduced substrate in the presence of phenazine methosulfate (PMS) and nitro blue tetrazolium (NBT). The oxidation of the substrate in the enzyme-catalyzed reaction reduces the coenzyme which, in turn, reduces the PMS. Finally, the reduced PMS is reoxidized with concomitant reduction of NBT. In the oxidized state, NBT is soluble and colorless, while in the reduced form it is insoluble and has a purple color. Hence, a purple precipitant band is visible on the gel coincident with the site of dehydrogenase activity.

In the search for an activity stain for L-threonine deaminase [L-threonine hydrolyase (deaminating) (E.C. 4.2.1.-16)] from *Rhodospirillum rubrum*, we found that the combination of PMS and NBT in the presence of threonine also generated a purple precipitant band. Threonine deaminase, purified to homogeneity (*5*), was subjected to electrophoresis on polyacrylamide gels (Fig. 1). Gel 2 (Fig. 1A) was stained for protein with amido black, whereas gels 1 and 3 were stained for enzyme activity with L-threonine and L-serine as substrate, respectively (*6*). The two bands in gel 2, corresponding to the native enzyme and a dimer of the native enzyme (*5*), were both active with either substrate. Partially purified enzyme preparations displayed a large number of protein bands, but when stained for enzyme activity showed only two bands in the same relative positions as those seen in Fig. 1A. The control experiments (Fig. 1B) demonstrated that omission of substrate, PMS, or NBT resulted in no purple precipitant band.

To demonstrate that the threonine deaminase was indeed located in those regions showing precipitant bands, puri-

Table 1. Production of CO_2 during ferricyanide reduction. Reaction mixtures were placed in Thunberg tubes, with the side arm containing 0.4 ml of 0.1*N* KOH. The first reaction mixture contained 100 μmole of potassium phosphate buffer (*p*H 8), 20 μmole of L-threonine, 0.5 μc uniformly labeled [^{14}C]threonine (130 mc/mmole), and enzyme in a volume of 1.0 ml. In addition, the second reaction mixture contained 2.5 μmole of potassium ferricyanide. The reaction was initiated by adding 40 μg of enzyme to each mixture. After incubation for 5 minutes at 25°C, the reactions were stopped by adding 0.5 ml of a 1 : 1 mixture of 30 percent trichloroacetic acid and 0.2 percent dinitrophenylhydrazine in 2.0*N* HCl, thus trapping α-ketobutyrate formed as the hydrazone derivative to prevent any acid-catalyzed decarboxylation of the keto acid. After further incubation for 30 minutes at 25°C, 0.2 ml of KOH from the side arm was pipetted onto an etched glass disk along with 0.2 ml of 0.1*M* $BaCl_2$ in 50 percent ethanol. The disks were dried and counted on a gas-flow planchet counter. In calculating the actual CO_2 generated, an efficiency of 25 percent was used to convert counts per minute to disintegrations per minute (dpm). Under identical assay conditions with nonradioactive threonine as substrate, 0.47 μmole of ferrocyanide was produced in 5 minutes, as measured spectrophotometrically.

Reaction mixture	$^{14}CO_2$ in 0.4 ml of KOH (dpm)	Total $^{14}CO_2$ (μmole)	μmole ferricyanide reduced/ μmole $^{14}CO_2$ produced
No ferricyanide	240	0.02	
Ferricyanide	6592	0.49	0.96

fied enzyme was subject to electrophoresis (Fig. 1). One gel was stained for threonine deaminase activity, while a duplicate gel was sliced into small sections. Each section was broken up in 1.0 ml of 50 m*M* potassium phosphate buffer, *p*H 6.8, and when 0.10 ml aliquots of these suspensions were assayed for enzyme activity by the colorimetric method of Friedemann and Haugen (*7*), threonine deaminase activity was found only in those sections corresponding to the location of precipitant bands on the first gel.

Since the purified threonine deaminase did not show reduction of nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate in the presence of threonine (*8*), and since the spectrum of the pure enzyme did not reveal the presence of any cofactor other than pyridoxal phosphate (*5*), the reduction of PMS and NBT by threonine dehydrogenase was ruled out. Furthermore, experiments with partially purified preparations of the *Pseudomonas fluorescens* threonine deaminase, which is strongly inhibited by L-isoleucine, revealed that the appearance of the activity band was delayed in incubation mixtures supplemented with L-isoleucine. These results indicate that the activity stain is indeed specific for threonine deaminase.

To explore the chemical basis for this activity stain, we studied the reaction spectrophotometrically with ferricyanide as an artificial electron acceptor. In 100 m*M* potassium phosphate buffer, *p*H 7.8, containing 50 m*M* L-threonine and 2.5 m*M* potassium ferricyanide, the initial rate of ferricyanide reduction, as measured by the decrease in absorbance at 410 nm at 25°C, was proportional to the enzyme concentration. The rate of reduction was linear for approximately 1 minute then decreased rapidly to a near-zero rate after the reduction of 0.47 m*M* ferricyanide.

Although the initial rate was proportional to the enzyme concentration, the extent of the reaction was not, and in all cases approximately 0.47 m*M* ferricyanide was reduced. Dilution of the assay mixture into fresh assay solutions after the reaction had stopped regenerated enzyme activity both with respect to dye reduction and, in the absence of any dye, with respect to threonine deaminase activity. In addition, neither the products of the deamination reaction, α-ketobutyrate and ammonia, nor the combinations of products plus enzyme, or products plus substrate in the absence of enzyme, resulted in dye reduction. These data suggest that some active intermediate of the deamination reaction was responsible for the reduction of ferricyanide. Furthermore, since addition of ferrocyanide (0.3 m*M*), α-ketobutyrate, and ammonia in various combinations did not affect the total amount of ferricyanide reduced, it would appear that an accumulation of the oxidized product of the active intermediate was the cause for the cessation of the ferricyanide reduction.

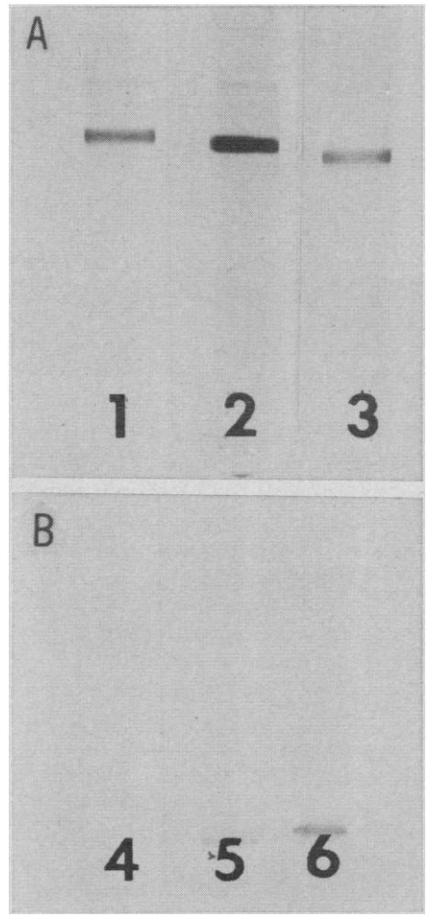

Fig. 1. Polyacrylamide-gel electrophoresis of threonine deaminase. Electrophoresis of 45 μg of pure threonine deaminase was carried out in 7.5 percent polyacrylamide gels at *p*H 9.3 as described by Davis (*11*) until the tracking dye had just run off the bottom of the gel. (A) Gel 2 was stained for protein using 1 percent amido black in 7 percent acetic acid. Gels 1 and 3 were stained for threonine deaminase activity by incubation in a solution containing 100 μmole of L-threonine (gel 1) or 100 μmole of L-serine (gel 3), 500 μmole of potassium phosphate buffer (*p*H 8.0), 5 mg of nitro blue tetrazolium, ad 5 μg of phenazine methosulfate in a total volume of 5.0 ml. The reaction was allowed to proceed in the dark for 10 to 15 minutes before transferring to 7 percent acetic acid. (B) The gels were stained for activity as described except that with gel 4, no NBT was included, with gel 5, PMS was omitted, and with gel 6, substrate was omitted. The tracking dye is visible at the bottom of gels 5 and 6.

The proposed mechanism of threonine deamination (*9*) involves the pyridoxal phosphate-dependent dehydration of L-threonine to generate α-aminocrotonate. This compound is believed to tautomerize on the enzyme to α-iminobutyrate, which, when released from the enzyme, spontaneously hydrolyzes to α-ketobutyrate and ammonia. Because of the reactivity of these intermediates, it was considered likely that one or the other was acting as the reducing agent in the reaction. Using uniformly labeled [^{14}C]threonine, we found that for every mole of ferricyanide reduced, one mole of $^{14}CO_2$ was generated (Table 1). This suggested that a decarboxylation of one of the intermediates was associated with the reducing equivalent.

A relevant reaction has been described by Flavin and Slaughter (*10*) using the enzyme γ-cystathionase, which catalyzes the formation of α-ketobutyrate from cystathionine. In the presence of *N*-ethylmaleimide, a new compound was formed, α-keto-3-[3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl)]butyric acid (KEDB). Flavin and Slaughter have proposed that KEDB is formed by the addition of α-aminocrotonate across the double bond of the maleimide due to the carbanion character at C-3 of the α-aminocrotonate. It is possible that in the threonine deaminase-catalyzed dye reduction, the electron is also provided by the C-3 carbon of α-aminocrotonate, and that this oxidation is followed by a decarboxylation to yield a three carbon radical. Because of the limited extent of the reaction, it has not been possible as yet to identify the product of the reduction reaction.

In addition to the intrinsic interest in the mechanism of this novel oxidation-reduction reaction, the use of PMS and NBT for an activity stain for threonine deaminase will be a powerful tool in studying the various properties of this enzyme. Furthermore, this activity stain may also be of practical value in studying other aminocrotonate or aminoacrylate-generating enzymes, such as γ-cystathionase, tryptophanase, and cystathionine γ-synthetase.

ROSS S. FELDBERG*
PRASANTA DATTA
Department of Biological Chemistry, University of Michigan, Ann Arbor 48104

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* Present address: Department of Biochemistry, University of Aberdeen, Aberdeen AB9, 1AS, Scotland.

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A Thermophilic, Acidophilic Mycoplasma Isolated from a Coal Refuse Pile

Abstract. *A thermophilic, acidophilic procaryote lacking a cell wall has been isolated from a coal refuse pile which had undergone self-heating. Electron micrographs, chemical assays for hexosamine, and the inability of vancomycin to inhibit growth confirm the lack of a cell wall. The apparent ability of the organism to reproduce by budding and the low guanine plus cytosine content of its DNA indicate a relation to the mycoplasmas. The temperature optimum of the organism is 59°C, and growth occurs over a range of 45° to 62°C. No growth occurs at 37°C or at 65°C. The optimum* pH *for growth is between 1 and 2, and growth occurs between* pH *0.96 and 3.5 but does not occur at* pH *0.35 and only poorly at* pH *4.0. We propose to call this organism* Thermoplasma acidophila. *The existence of this organism extends considerably the range of habitats in which mycoplasma may occur.*

Free-living procaryotes which lack cell walls and grow as spheres or pleomorphic filaments are generally called mycoplasmas. In recent years, interest in these organisms has increased greatly due to the fact that they have been shown to be associated with a wide variety of disease syndromes in animals and humans (*1*). We report the isolation and characterization of a very unusual microorganism, clearly related to the mycoplasmas, which grows only at low *p*H and moderately high temperatures. The existence of this unusual organism forces a considerable enlargement of the idea of a mycoplasma (*1*).

The organism was isolated from a burning coal refuse pile at the Friar Tuck mine in southwestern Indiana. The temperature of the refuse pile from which the organism was isolated was 56°C, and the *p*H (1 : 1 mixture in water) was 1.96.

Approximately 0.5 g of black refuse from this site was inoculated into 5.0 ml of a medium containing 0.02 percent $(NH_4)_2SO_4$, 0.05 percent $MgSO_4$, 0.025 percent $CaCl_2 \cdot 2H_2O$, 0.3 percent KH_2PO_4, and 0.1 percent yeast extract (Difco). The *p*H was adjusted to either 2 or 3 with 10*N* H_2SO_4. Glucose was added after autoclaving to yield a final concentration of 1.0 percent. The tubes were incubated at 55°C. After about 2 weeks, the medium was slightly turbid. Dilutions were made into fresh medium and turbidity was detected by 3 days. Two more transfers resulted in microscopically pure cultures. Two isolates were obtained, 122-1B2 and 122-1B3 by enrichment at *p*H 2 and 3, respectively. Another isolate, 3-24, was obtained 6 months later from a different location in the same pile.

Table 1. The effect of antibiotics on the growth of *Thermoplasma acidophila*. The antibiotic was added to the indicated concentration in standard medium [0.02 percent $(NH_4)_2SO_4$, 0.05 percent $MgSO_4$, 0.025 percent $CaCl_2 \cdot 2H_2O$, 0.3 percent KH_2PO_4, 0.1 percent yeast extract, and 1.0 percent glucose] at *p*H 3. The cultures were examined after 5 days at 56°C. Isolate 104-1A is a spore-forming rod which is thermophilic and acidophilic and is used as a control to show that the antibiotics are active under the extreme conditions used.

Isolate	Minimum inhibitory concentration (μg/ml)	
	Novobiocin	Vancomycin
122-1B2	0.1	> 5000
122-1B3	0.1	> 5000
104-1A	10	50

Morphologically the isolates are pleomorphic spheres which, by phase microscopic examination, appear to reproduce by budding. Although a sphere appears to be the basic structural unit, filamentous structures are often seen, particularly in young cultures. The cells vary in diameter from about 0.3 to 2 μm. In keeping with the small size of some of the cells, it was demonstrated that viable units are able to pass through 0.45 μm membrane filters.

Electron micrographs reveal the relatively simple structure of a procaryotic organism. Nuclear material is dispersed throughout the cell with no evidence of a limiting nuclear membrane (Fig. 1A, left). No indication of membraneous organelles within the cytoplasm is seen. Unlike bacterial cells, however, these isolates lack a rigid cell wall and are separated from the surrounding environment by only a double membrane (Fig. 1A, right). A freeze-etch preparation of the organism shows in more detail the structure of the membrane (Fig. 1B). The outer membrane contains particles (arrows) which are about 20 nm in diameter (*2*).

The failure to detect a cell wall with the electron microscope is in keeping with several pieces of indirect evidence. The addition of sodium lauryl sulfate to a culture of the organism results in a very rapid cell lysis, with a decrease in optical density at 540 nm of about 80 percent occurring within 30 seconds. We were unable to demonstrate the existence of hexosamine by the Elson-Morgan assay for amino sugars (*3*). Finally, the isolates are insensitive to the antibiotic vancomycin at concentrations as high as 5 mg/ml (Table 1). Since this antibiotic is a specific inhibitor of cell wall synthesis, blocking the addition of the muramic acid–lipid complex to an acceptor (*4*), the inability of this antibiotic to inhibit growth suggests that a cell wall is not essential for cell growth. However, the isolates were inhibited by novobiocin, an antibiotic that inhibits mycoplasmas, at a concentration of approximately 0.1 μg/ml. Since the antibiotic tests were performed under rather severe conditions, *p*H 3 and 56°C, it was necessary to have a control which showed that the antibiotics were indeed active under these conditions. We used another acidophilic thermophile, isolate 104-1A, a spore-forming rod which grows at *p*H 3 and 56°C. The inhibition of this organism by both antibiotics showed that the antibiotics were active. Because