Induction and Stabilization of Yeast Aconitase Activity by Fluorocitrate[†]

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Both fluorocitrate and fluoroacetate acted on yeast aconitase as an inducer and a stabilizer of the enzyme. Fluoroacetate appeared to function after conversion to fluorocitrate in the cells. Inhibitors of protein synthesis and terminal respiratory system showed a strong inhibitory effect on the inductive formation of aconitase. In addition, these fluorocompounds were also found to induce various microbial aconitases.

Aconitase(citrate(isocitrate)hydro-lyase, EC 4.2.1.3) catalyses the interconversion of citric, *cis*-aconitic and isocitric acids and is a representative of a class of non-heme iron enzymes.

In spite of much effort, however, on the purification of aconitase, isolation of the enzyme in crystalline form from any biological sources has been unsuccessful, because of the extreme instability of the enzyme.¹⁾ Therefore, relatively little is known on the characteristics of aconitase, notably the role of iron and sulfur atoms in its function.

On the other hand, biochemical information concerning the enzyme formation has been accumulated. Hirsch²⁾ found that aconitase activity in aerobically grown cells of *Saccharomyces cerevisiae* was about ten times that in anaerobically grown cells. Hino *et al.*³⁾ and Takahashi *et al.*⁴⁾ reported that the aconitase in *E. coli* was synthesized under the aerobic conditions. Furthermore, Takahashi⁵⁾ described that in *E. coli*, the aconitase was induced by the addition of fluoroacetate, which is well known to act as an inhibitor of the enzyme after conversion to fluorocitrate.⁶⁾

In the course of studies on the formation of citric acid from *n*-paraffins by yeast,⁷⁾ it

was found that fluorocitrate had abilities not only of induction but also of stabilization of the yeast aconitase activity.

MATERIALS AND METHODS

Microorganisms. *Candida lipolytica* ATCC 20114 was mainly used. *Brevibacterium thiogenitalis* D-248,⁸⁾ *Xanthomonas campestris* IFO 13303, *Trametes sanguinea* IFO 4923 and *Streptomyces aureus* IFO 12371 were used in the experiments on inductive formation of aconitase.

Cultivation. Seed culture medium contained, per liter: glucose, 20.0 g; KH₂PO₄, 1.0 g; K₂HPO₄, 0.5 g; (NH₄)₂SO₄, 4.0 g; MgSO₄·7H₂O, 0.5 g; yeast extract, 2.0 g and CaCO₃, 5.0 g.

Main culture medium contained, per liter: sodium acetate, 5.0 g; KH₂PO₄, 1.0 g; NH₄Cl, 1.0 g; NH₄NO₃, 1.0 g; MgSO₄·7H₂O, 0.5 g; thiamine hydrochloride, 300 µg and sodium monofluoroacetate which was added to the medium, if necessary, in a concentration ranging from 1.0 to 5.0 g.

One part of the seed culture broth, cultivated at 28°C for 24 hr, was transferred to 20 parts of the main culture medium. The cultivation was carried out at 28°C for 72 hr. Acetic acid was fed to maintain the broth pH at 6~8 throughout the run.

Process for the induction of aconitase. The cells were grown in nutrient broth without shaking for 5 days at 28°C. Then the cells were harvested and washed with the induction medium which contained, g per liter; KH₂PO₄ 1.0, NH₄Cl 1.0, NH₄NO₃ 1.0, MgSO₄·7H₂O 0.05 and thiamine hydrochloride 300 µg/liter (pH 6.0). After the cells were suspended in the in-

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duction medium, each test compound was added to the cell suspension, and incubated at 28°C for 150 min with shaking.

Preparation of crude enzyme. The cells were harvested by centrifugation and washed twice with 0.1 M Tris-HCl buffer solution (pH 7.4) and subjected to disruption in a French pressure cell (500 kg/cm²). The extract was centrifuged at 5000×*g* for 10 min to remove debris and the supernatant fluid was used as a crude enzyme preparation.

Assay methods

i) **Aconitase activity.** Assay solutions contained; Tris-HCl buffer (pH 7.4) 100 mM, sodium *d*-isocitrate 10 mM and enzyme preparation in a final volume of 3.0 ml. Increase in absorbance at 240 nm was measured at 25°C with a Hitachi Model 124 spectrophotometer with 1.0 cm lightpath length cells. One unit of enzyme activity was defined as the amount of enzyme to form 1 μ mole of *cis*-aconitate ($\epsilon_{240\text{nm}} = 2.4 \times 10^3$) per min under the assay conditions and the specific activity of the enzyme was expressed as units per mg protein.

ii) **Citrate synthase activity.** The activity was measured by the method of Stere *et al.*⁹⁾

iii) **DNA and protein.** DNA and protein were determined by the method of Dische¹⁰⁾ and of Lowry *et al.*,¹¹⁾ respectively.

iv) **Radioactivity.** Radioactivity was measured by a liquid scintillation counter, Aloka Model LSC 502 (Nihon-Musen K.K., Tokyo).

Chemicals. DL-Fluorocitrate (barium salt) was purchased from Calbiochem, Los Angeles and *d*-isocitrate (monopotassium salt) was purified from the culture broth of *Candida lipolytica* ATCC 20114 by the method of Vickery *et al.*¹²⁾ with slight modifications. Others were obtained from Wako Pure Chemical Industries, Ltd., Osaka.

RESULTS AND DISCUSSION

Inductive formation of aconitase

Candida lipolytica ATCC 20114 produced citric and isocitric acids in the ratio of about 60:40 from *n*-paraffins. In the presence of fluoroacetate which is a precursor of fluorocitrate, a well-known aconitase inhibitor, the production ratio of the two acids changed to about 80:20.⁷⁾

Based on the above facts, aconitase activity in the yeast cells, grown in the presence of sodium monofluoroacetate, was determined.

As shown in Table I, the aconitase activity in the yeast cells grown in the presence of the compound was unexpectedly several times

TABLE I. EFFECT OF FLUOROACETATE CONCENTRATION ON FORMATION OF ACONITASE BY GROWING YEAST CELLS

Cultivations were carried out under the conditions described in *Cultivation*.

Sodium monofluoroacetate (mM)	Aconitase (Specific activity)	Citrate synthase (Specific activity)
0	0.84	0.517
10	6.35	0.534
20	6.70	0.526
30	7.40	0.501
40	7.74	0.575

higher than that grown in the absence of the compound. However there was no significant difference between citrate synthase activities of the cells grown in the presence and absence of fluoroacetate.

The formation of citrate synthase and aconitase has been well known to be regulated simultaneously in aerobic bacterial cells.¹³⁾ However, Takahashi⁵⁾ reported recently that aconitase activity in *E. coli* was developed in the presence of fluoroacetate, while the level of citrate synthase activity was not affected by the addition of the compound. The present finding with yeast cells was in accord with the data reported by Takahashi.⁵⁾

Figure 1 shows the time course of the inductive formation of the yeast aconitase by the growing cells in the presence of 10 mM fluoroacetate. The aconitase activity in the cells increased approximately two times even after the yeast growth ceased.

In order to confirm the inductive action of fluoroacetate, the effect of fluoroacetate and fluorocitrate on the inductive formation of aconitase by resting cells was examined. As illustrated in Table II, both fluoroacetate and fluorocitrate had a strong effect on the inductive formation of aconitase. At a lower concentration the latter exhibited a stronger effect than the former. No cell growth occurred during the induction period; this was

FIG. 1. Time Course of Inductive Formation of Aconitase by Growing Yeast Cells in the Presence of Fluoroacetate.

○—○, Specific activity of aconitase; ●—●, O.D.₆₆₀. Five liters of the seed broth were transferred to a 200 liter fermenter containing 100 liters of the main culture medium. The main culture conditions were as follows: temperature, 28°C; air flow rate, 0.3 VVM; agitation, 200 rpm; culture time indicated; inner pressure, 1 kg/cm²G.

TABLE II. EFFECT OF FLUOROCITRATE AND FLUOROACETATE CONCENTRATIONS ON INDUCTION OF ACONITASE BY RESTING YEAST CELLS

The cells were prepared according to the method described in *Process for the induction of aconitase*.

Concentration (mm)	Specific activity	
	Sodium monofluorocitrate	Sodium monofluoroacetate
0	trace	trace
0.002	1.25	0.05
0.005	1.95	0.15
0.02	2.50	0.50
0.05	2.50	1.55
0.2	2.55	2.15
0.5	2.50	2.50

confirmed by measuring DNA concentration in the reaction mixture.

Figure 2 shows the time course of the inductive formation of aconitase by the resting cells in the presence of 0.02 mM fluorocitrate. From the figure, it is apparent that the

FIG. 2. Time Course of Inductive Formation of Aconitase by Resting Yeast Cells in the Presence or Absence of Fluorocitrate.

The cells were prepared according to the method described in *Process for the induction of aconitase*. ○—○, 0.02 mM Fluorocitrate; ●—●, No addition.

aconitase was inductively formed in proportion to the incubation time, at least for 5 hr.

Furthermore, the effect of some intermediates of organic acid metabolism and redox reagents on the inductive formation of aconitase was examined. As is clear from Table III, all

TABLE III. EFFECT OF VARIOUS COMPOUNDS ON INDUCTION OF ACONITASE BY RESTING YEAST CELLS

The cells were prepared according to the method described in *Process for the induction of aconitase*.

Addition (0.02 mm)	Specific activity
Sodium fluorocitrate	2.5
Sodium citrate	trace
Sodium fluoroacetate	0.5
Sodium acetate	trace
Sodium α -ketoglutarate	trace
Sodium fumarate	trace
Sodium pyruvate	trace
Sodium isocitrate	trace
Sodium <i>cis</i> -aconitate	trace
Sodium itaconate	trace
Ferrous sulfate	trace
Cysteine	trace
Sodium ascorbate	trace
Glutathione (reduced)	trace
Sodium citrate (20 mm)	2.0
None	trace

compounds tested showed no effect except for fluoro-compounds in the concentration of 0.02 mM. On the other hand, citrate showed some effect in the excessively high concentration. From the above results, it is suggested that aconitase in yeast cells would be inductively formed by fluorocitrate which might be derived from fluoroacetate added to the medium. In order to substantiate the above conception, yeast cells grown in a medium containing 10 mM sodium monofluoroacetate were removed by centrifugation and the resulting supernatant solution was concentrated to one-twelves of the original volume. The resulting precipitates were filtered off and the filtrate was passed through a cation-exchange resin, IR-120 (H^+ -form). The passed solution was concentrated to one-fifth and the resulting crystals of citric acid were separated from the mother liquor.

The mother liquor was freeze-dried and organic acids in the dried powder were analysed by gas-liquid chromatography after methylation by the following method. Organic acids in the powder, extracted with a solvent mixture of ether and methanol (9:1), were methylated with diazomethane prepared from *p*-tolylsulfonylmethylnitrosamide (0.6 M) in 20% potassium hydroxide solution containing carbitol.

It is obvious from the chromatogram shown in Fig. 3 that fluorocitrate was formed in the fluoroacetate-added culture broth.

Stabilization of aconitase

Stability of aconitases from the cells grown in the presence or absence of fluoroacetate was determined as shown in Table IV. The aconitase from the latter cells was completely inactivated by heating at 45°C or by dialysis against running water, and the aconitase from the former cells was, on the contrary, extremely stable to such treatments. On the basis of the above information, the effect of various compounds on the thermostability of the enzyme was examined.

Table V shows that only fluorocitrate showed a strong activity to stabilize the enzyme

FIG. 3. Detection of Fluorocitrate in Broth by Gas Chromatography.

—, Sample; ----, Authentic samples; Citrate (A) and fluorocitrate (B).

Column: 3% PEG 20 M on Chromosorb W (60~80 mesh) in stainless steel column (3 mm ID $\times$ 1.5 m). Temperature: Programed 170°C to 200°C (4°C/min). Carrier gas: Helium 1.5 kg/cm²G (flow rate: 100 ml/min).

Instrument: Hitachi K-53 Gas Chromatography.

TABLE IV. STABILITY OF ACONITASE FROM YEAST CELLS CULTIVATED IN THE PRESENCE OR ABSENCE OF FLUOROACETATE

Addition (mM)	Relative activity	
	After heating at 45°C for 20 min	After dialysis against running water at 10°C for 24 hr
None	0	2
10	100	100

against heat treatment. Citrate, a substrate of aconitase, showed a weak activity at a markedly high concentration. Table VI shows the effect of various concentrations of fluorocitrate and citrate on the thermostability of aconitase, and indicates that the thermostabilizing activity of both compounds depends upon the concentration of each in the incubation mixture and that there may be a certain stoichiometric relationship between the aconitase and fluorocitrate.

Figure 4 shows that aconitase activity was completely stable after heat treatment at below 46°C for 30 min in the presence of

FIG. 4. Effect of Fluorocitrate and Citrate on Thermostability of Yeast Aconitase at Various Temperatures.

The conditions were the same as in Table V except for the incubation time and temperature. After incubation for 30 min, aconitase activities were determined. ●—●, 0.05 mM Fluorocitrate; ■—■, 10 mM Citrate; ○—○, No addition.

TABLE V. EFFECT OF VARIOUS COMPOUNDS ON THERMOSTABILITY OF ACONITASE

Crude enzyme was prepared by the method described in the text. Each compound was added to the enzyme preparation at the concentration cited. After heating the enzyme preparation at 45°C for 20 min, residual activity was measured.

Addition (0.05 mM)	Residual activity of aconitase (%)
Sodium fluorocitrate	106
Sodium citrate	0
Sodium fluoroacetate	0
Sodium acetate	0
Sodium succinate	0
Sodium α -ketoglutarate	0
Sodium fumarate	0
Sodium pyruvate	0
Sodium isocitrate	0
Sodium <i>cis</i> -aconitate	0
Sodium itaconate	0
Ferrous sulfate	0
Cysteine	0
Sodium ascorbate	0
Glutathione	0
Sodium citrate (20 mM)	60
None	0

0.05 mM fluorocitrate. Figure 5 shows the time course of the heat inactivation of the activity with and without fluorocitrate at 45°C, indicat-

FIG. 5. Time Courses of Heat Inactivation of Yeast Aconitase at 45°C in the Presence or Absence of Fluorocitrate.

○—○, 0.05 mM Fluorocitrate; ●—●, No addition.

ing that the aconitase was inactivated after almost 10 min without fluorocitrate, but remained fully active in the presence of the compound.

It is evident from the above results that fluorocitrate had dual role on the aconitase except for inhibition; (1) inductive formation of aconitase and (2) stabilization of the enzyme activity. Such actions of fluorocitrate were usefully applied to the purification of aconitase, which will be given elsewhere.

Incorporation of radioactive fluoroacetate into the yeast cells

From the above facts, it was assumed that fluorocitrate, derived from fluoroacetate in the yeast cells, would be combined with the aconitase and stabilized the enzyme activity. To confirm this conception, the absorption of radioactivity derived from radioactive fluoroacetate onto the aconitase was examined.

After yeast cells were grown on the main culture medium supplemented with 10 mM sodium monofluoroacetate-2-¹⁴C (50 μ Ci/ μ mole), crude enzyme preparation was obtained by the procedure described in METHODS. Ethanol was added to the crude enzyme preparation to make a final concentration of

77% (v/v). The resulting precipitate was harvested by centrifugation and dissolved in 0.05 M Tris-HCl buffer solution (pH 7.4). The solution was subjected to Sephadex G-100 gel filtration and the active fractions were applied onto a DEAE-Sephadex column (1 × 15 cm) which was previously equilibrated with the same buffer. The enzyme was eluted stepwise with Tris-HCl buffer of 0.05 M to 0.5 M as shown in Fig. 6.

FIG. 6. Distribution of Protein, Radioactivity and Aconitase after Chromatography on DEAE-Sephadex A-50.

○—○, O.D.₂₈₀; ●—●, Radioactivity; ●—●, Aconitase activity; —, Concentration of Tris-HCl buffer.

From the results, it is clear that the radioactive compound derived from fluoroacetate-2-¹⁴C was specifically attached to the enzyme and stabilized the aconitase activity.

Activity of the enzyme containing the fluorocompound could be easily assayed under the same conditions as described in MATERIALS AND METHODS. It seems likely that fluorocitrate is known to be the competitive inhibitor of aconitase and the amount of *d*-isocitrate added to the reaction mixture for the assay is significantly larger than that of the fluorocompound attached to the enzyme.

Inhibitory effect of various compounds on the inductive formation of aconitase

The effect of various biochemical inhibitors

TABLE VI. EFFECT OF FLUOROCITRATE AND CITRATE CONCENTRATIONS ON THERMOSTABILITY OF ACONITASE

Experimental conditions; the same as in Table V.

Fluorocitrate		Citrate	
Concentration (mM)	Residual activity (%)	Concentration (mM)	Residual activity (%)
0.002	44	0.2	15
0.005	68	0.5	29
0.02	79	2	44
0.05	106	5	51
		20	61
		50	66

TABLE VII. INHIBITORY EFFECT OF VARIOUS COMPOUNDS ON INDUCTIVE FORMATION OF YEAST ACONITASE

The cells were prepared according to the method described in *Process for the induction of aconitase*.

Addition (10 mM)	Inhibition (%)
Mitomycin	0
5-Bromouracil	26
Cycloheximide	51
<i>p</i> -Fluorophenylalanine	100
Quinacrine	60
Potassium cyanide	100
Sodium azide	100
2,4-Dinitrophenol	28
None	0

on the inductive formation of aconitase in yeast cells was examined. Each inhibitor cited in Table VII was added to the incubation medium at the start of incubation. As shown in the table, it was found that the inhibitors of protein synthesis such as cycloheximide and *p*-fluorophenylalanine and the inhibitors of terminal respiratory enzymes such as quinacrine, potassium cyanide and sodium azide strongly inhibited the inductive formation of aconitase, but inhibitors of DNA synthesis such as mitomycin C and 5-bromouracil did not.

The results suggest that the inductive formation of aconitase by yeast is closely related to protein synthesis as well as respiratory activity as previously shown in *E. coli*.^{4,5)}

TABLE VIII. EFFECT OF FLUOROACETATE ON INDUCTION OF ACONITASE BY VARIOUS MICROORGANISMS

Condition: Incubation at 28°C for 72 hr with shaking.

Microorganism	Specific activity		Carbon source of the medium	
	No addition	Addition		
<i>Candida lipolytica</i> ATCC 20114	0.84	6.70	Sodium acetate	0.5%
<i>Brevibacterium thiogenitalis</i> D-248 ^{a)}	0.75	6.10	Sodium acetate	0.5%
<i>Xanthomonas campestris</i> IFO 13303	0.09	1.53	Glucose	2%
<i>Trametes sanguinea</i> IFO 4923	0.16	2.02	Sucrose	5%
<i>Streptomyces aureus</i> IFO 12371	0.03	0.81	Soluble starch	3%

Medium; CSL 0.2%, NH₄Cl 0.1%, KH₂PO₄ 0.1%, MgSO₄·7H₂O 0.05%, CaCl₂ 0.1%, FeSO₄·7H₂O 10 µg/liter, Thiamine·HCl 100 µg/liter and Biotin 10 µg/liter.

^{a)} Sodium oleate (200 µg/liter) was supplemented.

Induction of aconitase by various microorganisms

In order to confirm the universal validity of the activity of fluorocitrate inducing microbial aconitase, the effect of fluoroacetate was investigated using various microorganisms as shown in Table VIII. When 0.2 M fluoroacetate was added to the medium, the aconitase activity in the cells of each microorganism tested was induced in the range of about 8- to 27-fold increase.

From the results, it is confirmed that microbial aconitase is induced in the presence of fluorocitrate or fluoroacetate.

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