BIOCHEMICAL CHANGES DURING THE DEVELOPMENT OF RESPIRATORY ACTIVITY IN *ESCHERICHIA COLI*

III. PREFERENTIAL DEVELOPMENT OF ACONITASE ACTIVITY BY FLUOROACETATE

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(Received June 23, 1971)

The development in respiratory activity for several substrates during the aeration of anaerobically grown *Escherichia coli* was inhibited by fluoroacetate (FAA), but the inhibition was lessened if formate was further added to the aeration medium. During an aeration with FAA and formate, an enormous increase in the activity of aconitase was observed; under the anaerobic incubation aconitase activity did not show significant increase. The addition of FAA after a certain period of aeration induced a rapid increase in aconitase activity. Aeration before the addition of FAA made subsequent increase of the enzyme activity under anaerobic condition possible.

The increase of aconitase activity during the aeration of anaerobic cells with FAA was repressed by chloramphenicol or by the starvation of cells for amino acids. In pre-aerated cells, however, subsequent increase of enzyme activity by the addition of FAA was not inhibited by chloramphenicol or puromycin, and also not repressed by the starvation of cells for amino acids. During aeration, citrate accumulation in the cell suspension was caused not only by FAA but also by picolinate. However, the latter did not induce the development of aconitase activity.

From these results the role of FAA and formate on the increase of aconitase activity in anaerobically grown cells is discussed.

That low respiratory activity of anaerobically grown cells of *Escherichia coli* K-12 increases to the level of aerobically grown cells by aeration without cell division was shown through the work of HINO and MAEDA (1). In a series of subsequent studies, it has been revealed that this respiratory development is produced mainly by the increase of cellular permeability to some

Abbreviations: FAA, fluoroacetate; TCA cycle, tricarboxylic acid cycle.

respiratory substrates and by the increment in the content of TCA cycle and glyoxylate cycle enzymes (2, 3).

SLONIMSKI (4) showed that the development of respiratory activity of anaerobic cells in yeast by oxygen was not inhibited by such respiratory inhibitors as cyanide and azide. Thence, it was suggested that, in the development of respiration in this organism, oxygen plays the role of an inducer for respiratory enzyme formation rather than to serve as a respiratory electron acceptor.

However, in *E. coli*, these respiratory inhibitors added during aeration repressed the development of respiratory activity (1). Thus in *E. coli*, there is no direct evidence that oxygen plays a role other than respiratory substrate or of an inducer for respiratory enzyme.

As for the mechanism of the synthesis of TCA cycle enzymes by aeration of *E. coli*, an alternative mechanism other than the induced formation by oxygen was considered. As an alternative, a possibility was examined whether intermediates of the TCA cycle may induce the formation of corresponding cycle enzymes. Thus, in the present investigation, examination was made on whether the development of TCA cycle enzyme activities is affected by the addition of FAA, an inhibitor of the cycle. In this case, to preclude a possibility that energy supply from respiration is suppressed as a result of the inhibition of TCA cycle by FAA, formate, which is oxidized independently of TCA cycle, was added during the aeration of anaerobic cells with FAA.

MATERIALS AND METHODS

Organism, culture media, and growth conditions. *Escherichia coli* K-12 (CAVALLI's Hfr strain) was used in this study. The media, culture conditions, and procedures for harvesting and washing the cells essentially followed those described by HINO and MAEDA (1). The cultures were grown aerobically by shaking in a nutrient broth, or grown anaerobically in bottles filled to the neck with a nutrient broth containing 1% glucose. Cultures were maintained for 18 hr at 30°.

Aeration and anaerobic incubation of anaerobically grown cells. Anaerobically grown cells were washed and suspended in an incubation mixture containing, unless stated otherwise, 2% casamino acids (Nissan) in 0.02 M phosphate buffer (pH 7.2). The cell suspension was made sufficiently concentrated (about 5×10^9 cells per ml) to prevent cell multiplication.

Aeration was made by shaking cell suspensions, while anaerobic incubation of the cells was accomplished by repeating four evacuations of the flask containing the cell suspension and flushing with pure nitrogen. Aeration and anaerobic incubation were made at 30°.

Oxygen uptake. Oxygen uptake was measured by the conventional manometric technique at 30°. Respiratory activity was expressed as $Q_{O_2}(N)$ after subtracting the $Q_{O_2}(N)$ of endogenous respiration.

Assay of enzyme activity. Preparation of cell-free extracts and the assay methods for enzyme activity were essentially the same as described previously (3).

Citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) was measured by determining citrate synthesized from acetyl phosphate, CoA, and oxaloacetate (5).

Aconitase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) was measured by observing the increase in absorbance at 240 nm in the presence of DL-isocitrate (6).

Isocitrate dehydrogenase (Ls-isocitrate : NADP oxidoreductase (decarboxylating), EC 1.1.1.42) was determined by following NADP reduction at 340 nm in the presence of DL-isocitrate (7).

Isocitrate lyase (Ls-isocitrate glyoxylate-lyase, EC 4.1.3.1) was measured by observing the increase in absorbance at 324 nm in the presence of DL-isocitrate and phenylhydrazine (8).

Succinate dehydrogenase (succinate : (acceptor) oxidoreductase, EC 1.3.99.1) was measured by observing the reduction of 2,6-dichlorophenolindophenol at 600 nm in the presence of phenazine methosulfate and succinate (9).

Fumarase (L-malate hydro-lyase, EC 4.2.1.2) was determined by measuring the increase in absorbance at 240 nm in the presence of L-malate (6).

Malate dehydrogenase (L-malate : NAD oxidoreductase, EC 1.1.1.37) was measured by observing the rate of NADH oxidation at 340 nm by oxaloacetate (10).

Enzyme activities are expressed as μ moles of the substrate consumed or of the product formed per min per mg nitrogen of the extracts.

Determination of nitrogen and citrate. The amount of cell nitrogen was directly determined by the Kjeldahl method. The nitrogen content of the cell-free extracts was determined indirectly using a standard curve drawn between the amount of protein and nitrogen per ml of the extracts, assayed by the biuret and Kjeldahl method, respectively.

The amount of citrate in cell suspension was estimated by the colorimetric method used in an assay of citrate synthase (5). After precipitating cells by centrifugation, supernatant was autoclaved for 20 min at 2 atm to remove interfering substances such as β -keto acids (11).

RESULTS

Effect of FAA on respiration and on the development of respiration

The inhibitory effect of FAA on the respiratory activity of aerobically grown cells is shown in Table 1. It is known that, in animal tissues, FAA competitively inhibits the oxidation of acetate and is converted enzymically into fluorocitrate (12) which inhibits aconitase (13). In accordance with these facts, the oxidative activity of aerobically grown *E. coli* for acetate, succinate, or malate was remarkably inhibited by FAA, but for formate, which

Table 1. Effect of FAA on the respiratory activity of aerobically grown cells.

The warburg vessel contained 0.04 M of phosphate buffer (pH 7.2), 0.03 M of substrate, cells, and 20 mM of FAA in a total volume of 3.0 ml. Cells and inhibitor were incubated for 10 min before addition of substrate. In this table, as in subsequent tables and figures, each value given is the mean of duplicate or triplicate determinations unless stated otherwise.

	QO ₂ (N) Substrate			
	Formate	Acetate	Succinate	Malate
Control	1,730	1,080	1,660	870
+FAA	1,650	15	340	15

Table 2. Effect of FAA on the development of respiratory activity in anaerobically grown cells.

Anaerobically grown cells were aerated for 2.5 hr in a buffer containing 2% casamino acids in the presence or absence of 20 mM FAA and 50 mM formate. After aeration, cells were collected, washed, and respiratory activity measured on each substrate.

Cells	QO ₂ (N) Substrate			
	Formate	Acetate	Succinate	Malate
Un aerated	285	10	40	15
Aerated with casamino acids	450	170	390	235
Aerated with casamino acids +FAA	345	56	175	95
Aerated with casamino acids +FAA +formate	370	156	290	185

is oxidized independently of the TCA cycle, there was no appreciable inhibition.

As has been noted (1), the respiratory activity of anaerobically grown cells increased during the aeration with 2% casamino acids (Table 2). This increment of the respiratory activity, measured on acetate, succinate, or malate as a substrate, was repressed to 30%, 40%, and 35% of the control, respectively, by the addition of FAA to the aeration mixture. The repressive effect of FAA was partially removed by the supplementary addition of formate in an aeration medium.

Changes in the level of TCA cycle enzymes by FAA during aeration

To elucidate the cause of the action of FAA and formate on the develop-

Table 3. Effect of the addition of FAA and carbon sources during aeration on the levels of enzyme activity of anaerobically grown cells.

Casamino acids, FAA, and carbon sources were added to cell suspension at a concentration of 2%, 20 mM, and 100 mM, respectively. Cells were aerated for 4 hr.

Enzyme	Enzyme activity (μ mole/min/mg N) in extracts from anaerobically grown cells					
	Unaerated	Aerated with casamino acids plus				
		None	FAA	FAA and formate	FAA and succinate	FAA and glycerol
Citrate synthase	0.180	1.150	0.560	0.715	—	—
Aconitase	0.040	0.176	0.326	0.861	0.304	0.688
Isocitrate lyase	0.008	0.072	0.008	0.041	0.005	0.004
Isocitrate dehydrogenase	0.142	0.470	0.255	0.475	0.255	0.615
Succinate dehydrogenase	0.009	0.061	0.017	0.029	0.021	0.019
Fumarase	0.044	0.244	0.115	0.147	0.075	0.171
Malate dehydrogenase	0.412	2.080	0.860	1.540	0.675	1.280

Table 4. Effect of FAA concentration on the development of aconitase activity.

Washed suspensions of anaerobically grown cells were added to a buffer containing 2% casamino acids, 100 mM formate, and various concentrations of FAA, then shaken in air for 4 hr.

	Extracts from anaerobically grown cells							
	Unaerated	Aerated with FAA at concentrations of						
		0	2	5	10	20	40	100 mM
Enzyme activity (μ mole/min/mg N)	0.059	0.209	0.729	0.866	0.710	0.800	0.607	0.449

ment of respiratory activity in anaerobically grown cells, the levels of TCA cycle and glyoxylate cycle enzymes in cell-free extracts before and after aeration were measured (Table 3). Test was also made to see whether succinate or glycerol, a carbon source other than formate, affects the levels of these enzymes during aeration with FAA.

By aeration in the absence of FAA, the activity of these enzymes increased 3- to 9-fold as compared with the original cells. When formate was absent from the aeration mixture, FAA repressed the development of the

Table 5. Effect of aerobic and anaerobic incubation on the development of aconitase activity in the presence of FAA.

Washed suspension of anaerobically grown cells was added to a buffer containing 2% casamino acids, 5 mM FAA, and/or 50 mM carbon source. Aerobic incubation was done by shaking in air and anaerobic incubation was accomplished in pure nitrogen. Incubation was maintained for 4 hr.

Conditions of incubation of anaerobically grown cells			Enzyme activity (μ mole/min/mg N)
FAA	Carbon source	Oxygen	
—	None	+	0.268
+	Formate	+	0.780
+	Formate	—	0.147
+	Glycerol	—	0.163
Anaerobically grown cells before incubation			0.075

activity of all the enzymes tested except aconitase, whose activity was rather enhanced. Among the enzymes that were repressed, the levels of isocitrate lyase and succinate dehydrogenase were extremely reduced.

As to the effect of carbon sources added to aeration medium, formate was the most effective for the release from FAA repression on the increase of these enzyme activities. Formate also remarkably stimulated the rise in the level of aconitase by FAA.

During the aeration of anaerobically grown cells without FAA, the level of aconitase increased 3- to 4-fold of the original level even in the absence of FAA, but various concentrations of FAA further promoted the increase of aconitase activity (Table 4). The pronounced stimulation was observed between 2 and 20 mM of FAA and the highest level was about 15-times greater than the level in anaerobically grown cells. Higher concentration of FAA repressed the development of the enzyme activity.

Effect of oxygen on the development of aconitase activity

As it was shown that oxygen is indispensable for the development of respiratory activity in anaerobically grown *E. coli* (1), examination was made to see whether oxygen is necessary for the increase of the aconitase activity (Table 5). When oxygen was eliminated, no great increase in the activity was seen. Clearly, molecular oxygen is indispensable in the development of this enzyme activity.

When FAA was added at various time intervals during aeration of anaerobically grown cells with formate, a marked change in the pattern of the increase of aconitase activity occurred (Fig. 1). When the aeration was initiated in the presence of FAA, the enzyme level increased with time of aeration (Fig. 1B), but the addition of FAA after the lapse of a period of

Fig. 1. Change of the levels of aconitase activity after the addition of FAA during aerobic incubation.

Cells grown anaerobically were aerated in a buffer containing 2% casamino acids and 50 mM formate. In A, FAA was not added in the cell suspension. In B, C, D, E, and F, FAA (10 mM) was added in cell suspensions at the time (in min) indicated by an arrow. Before and after addition of FAA, samples were withdrawn at intervals for assay of the enzyme activity.

aeration with formate accelerated the rate of the increase in enzyme activity. When FAA was added after 2 hr of aeration, the level of aconitase rose rapidly to that reached by the addition of FAA in the beginning of the aeration (Fig. 1F).

Since this fact suggests that aeration of cells with FAA is not a prerequisite for the increase of aconitase activity, further investigation was made by the transition of cells from the aerobic to the anaerobic condition. If anaerobically grown cells had been aerated for a certain period, the increase of aconitase activity by the addition of FAA occurred even under nitrogen (Fig. 2).

Dependency of the development of aconitase activity on protein synthesis

Examination was made on whether the increase of aconitase activity

Fig. 2. Development of aconitase activity by a shift from aerobic to anaerobic incubation with FAA.

Cells grown anaerobically, suspended in a buffer containing 2% casamino acids and 50 mM glycerol, were aerated for 2 hr and then, immediately following the addition of 10 mM FAA to the cell suspension, they were incubated anaerobically (at arrow) by sparging with pure nitrogen. —●— Cells maintained without FAA in air; —x— Cells maintained with FAA in anaerobiosis (N₂ gas); --●-- Cells maintained without FAA in anaerobiosis.

during aeration requires protein synthesis (Table 6). When anaerobically grown cells were aerated in the absence of casamino acids, the activity of aconitase did not increase. In the presence of casamino acids, the increase in enzyme activity was appreciably suppressed by chloramphenicol. These evidences show that protein synthesis is a prerequisite for the rise in the level of aconitase, as for the development of respiratory activity (1).

Results shown in Fig. 1E or 1F and Fig. 2 indicate that there are at least two processes whereby the enhanced level of aconitase is induced in the presence of FAA. Experiments were then designed to examine the participation of protein synthesis in the two processes. During aeration of anaerobically grown cells, cells were transferred from the primary incubation mixture without FAA to the secondary incubation mixture with FAA. As indicated in Table 7, casamino acids were omitted from the primary and secondary incubation mixtures, and antibiotics to inhibit protein synthesis were added

Table 6. Effect of chloramphenicol and casamino acids on the development of aconitase activity.

Anaerobically grown cells were aerated for 4 hr in a buffer or incubation medium containing casamino acids (2%), FAA (5 mM), formate (100 mM), and chloramphenicol (200 μ g/ml in Expt. 1 and 400 μ g/ml in Expt. 2) as indicated.

Extracts from cells	Enzyme activity (μ mole/min/mg N)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Unaerated	0.029	0.048	0.043	0.030
Aerated in buffer	—	—	0.038	0.019
Aerated with casamino acids	0.140	0.226	0.240	0.240
Aerated with casamino acids + chloramphenicol	0.023	0.073	—	—
Aerated with casamino acids + FAA + formate	0.746	1.037	—	—
Aerated with casamino acids + FAA + formate + chloramphenicol	0.268	0.166	—	—

to either incubation mixture. Therefore cells received casamino acids or an antibiotic before or simultaneously with the addition of FAA. Thus nine kinds of cells (A~I in Table 7) were prepared, and the enzyme activity in extracts from each type of the cells was assayed.

The enzyme activity in extracts from cells B, F, H, and I was nearly the same as that from cells A which were incubated with casamino acids in the absence of the inhibitor. On the contrary, levels of enzyme in cells C, D, E, and G were strongly reduced. Chloramphenicol and puromycin added in the primary incubation mixture inhibited the rise of the enzyme activity to the same level (cells E and G).

These results indicate that, for the development of the enzyme activity by FAA, protein synthesis is necessary during primary incubation without FAA, but not necessary after the addition of FAA.

Effect of FAA and picolinate on aconitase and citrate appearance

KOVÁČ *et al.* (14), in their studies on aerobic growth of *E. coli*, and FORGET and PICHINOTY (15), in their experiments on the respiration of *A. aerogenes*, observed an accumulation of citrate in the presence of FAA. Citrate accumulation when anaerobically grown *E. coli* are aerated with FAA was examined. During the aerobic incubation with FAA, the increased accumulation of citrate occurred, accompanied with the increment of the level in aconitase (Table 8). No appreciable accumulation occurred when cells were aerated without FAA or incubated under nitrogen with FAA.

To examine whether the prominent increase in the level of aconitase by FAA depends on the accumulation of citrate, the effect of picolinate, which is an inhibitor of aconitase (16), on the level of this enzyme was tested

Table 7. Dependence of the development of aconitase activity on protein synthesis before or after the addition of FAA.

Cells ^a	Omission or addition in incubation mixture		Enzyme activity (μ mole/min/mg N)
	Primary ^b	Secondary ^c	
A	None	None	0.890
B	None	—Casamino acids	0.865
C	—Casamino acids	None	0.320
D	—Casamino acids	—Casamino acids	0.235
E	+Chloramphenicol	None	0.210
F	None	+Chloramphenicol	0.640
G	+Puromycin	None	0.172
H	None	+Puromycin	0.710
I	None	—Casamino acids +Puromycin	0.790
Anaerobically grown cells			0.054

^a Nine types of cells (A~I) were obtained by the combination of primary and secondary incubations with or without constituents as indicated. Washed anaerobically grown cells were suspended in the primary incubation mixture and shaken in air for 2 hr, then cells were pelleted by centrifugation, washed two to three times with a buffer, resuspended in the secondary incubation mixture, and aerated for 1 hr. Concentrations of chloramphenicol and puromycin were 600 μ g/ml and 400 μ g/ml, respectively.

^b Primary incubation mixture contained 50 mM of formate and 2% casamino acids.

^c Secondary incubation mixture contained 10 mM of FAA in addition to primary incubation mixture.

Table 8. Effect of FAA and picolinate on the development of aconitase activity and on the accumulation of citrate.

Picolinate was added at the concentration of 5 mM. Aeration time was 3 hr. Other conditions were the same as described in the legend to Fig. 1.

Unaerobically grown cells	Enzyme activity in extracts (μ mole/min/mg N)	Citrate in cell suspension (μ mole/ml)
Anaerated	0.045	0
Aerated without FAA, picolinate	0.205	0.023
Aerated with FAA	0.560	0.334
Aerated with picolinate	0.063	0.336

(Table 8). Although citrate was accumulated in cell suspension by picolinate, there was no stimulative effect on the development of aconitase activity. This fact suggests that citrate accumulated does not induce the development of aconitase activity.

DISCUSSION

In a previous experiment it was shown that inhibitors for respiratory electron transport, such as cyanide, azide, and carbon monoxide, inhibit the development of respiratory activity by aeration of the unaerobically grown *E. coli* (1). In the present experiment, it was shown that FAA, an inhibitor of TCA cycle, also inhibits the development of respiratory activity and the development of some TCA cycle enzyme activities; the inhibitory effect of FAA can partially be removed by the simultaneous presence of formate.

It was shown in the previous experiments (1, 2) that enzyme proteins are synthesized during the development of respiratory activity by aeration. The FAA inhibition of the development of enzyme activities may be accounted for by the restriction of energy supply, caused by the suppression of the TCA cycle, for the synthesis of enzyme proteins. The recovery from FAA inhibition by formate may be explained by the fact that formate is directly oxidized through formate : cytochrome b_1 oxidoreductase (EC 1.2.2.1) and thus can supply energy even when TCA cycle is inhibited by FAA.

When the development of the activities of several TCA cycle and glyoxylate cycle enzymes is inhibited by FAA, only the development of aconitase activity is stimulated. The preferential development of aconitase activity by FAA is more pronounced when energy is supplied by formate oxidation. The preliminary analysis showed that whole process of aconitase development requires the presence of amino acids and is inhibited by inhibitors of protein synthesis. It appears as if aconitase protein is synthesized *de novo*.

In animal tissue, FAA is known to be enzymically converted into fluorocitrate, which in turn acts as an inhibitor for aconitase and prevents the utilization of citrate. Accumulation of citrate during aeration of *E. coli* with FAA strongly suggests that in this case also FAA have converted into fluorocitrate. Thus, the preferential development of aconitase activity by FAA, accompanied by protein synthesis, may most easily be explained by enzyme induction mechanism, *i.e.*, aconitase protein is induced by either citrate or fluorocitrate. In this case citrate may not be an effective inducer, since aconitase was not developed by picolinate, in spite of the appreciable accumulation of citrate.

Although this enzyme induction mechanism for the preferential development of aconitase activity by FAA appeared to be attractive, further analysis revealed that underlying mechanism is more complex. The results of the experiment designed to transfer the cells from an incubation mixture without FAA to that with FAA, and the experiment, in which cells were given FAA

under anaerobic condition after a certain period of pre-aeration, clearly show that at least two processes are involved in the development of aconitase activity. An important indication is that the development of aconitase activity by FAA seems to proceed without direct participation of protein synthesis, provided that cells have been pre-incubated under the condition that allows protein synthesis and formate oxidation to occur.

Thus, the result apparently contradicts the idea that aconitase protein is induced after the contact with FAA or with fluorocitrate. At present alternative mechanism to explain the observed results is difficult to offer. More detailed examination is under progress.

The writer of this paper expresses his deep gratitude to Prof. S. Hino, Hiroshima University, for his encouragement, valuable advice, and discussion.

REFERENCES

- 1) S. HINO and M. MAEDA, *J. Gen. Appl. Microbiol.*, **12**, 247 (1966).
- 2) Y. TAKAHASHI and S. HINO, *J. Gen. Appl. Microbiol.*, **14**, 183 (1968).
- 3) Y. TAKAHASHI and S. HINO, *J. Gen. Appl. Microbiol.*, **14**, 429 (1968).
- 4) P.P. SLONIMSKI, *In Proc. 3rd Intern. Congr. Biochem.*, Brussels 1955, ed. by C. LIÉBECQ, Academic Press, Inc., New York (1956), p. 242.
- 5) S. OCHOA, *Method Enzymol.*, **1**, 685 (1955).
- 6) E. RACKER, *Biochim. Biophys. Acta*, **4**, 211 (1950).
- 7) A. KORNBERG, *Method Enzymol.*, **1**, 705 (1955).
- 8) G.H. DIXON and H.L. KORNBERG, *Biochem. J.*, **72**, 3p (1959).
- 9) O. ARRIGONI and T.P. SINGER, *Nature*, **193**, 1256 (1962).
- 10) S. OCHOA, *Method Enzymol.*, **1**, 735 (1955).
- 11) G.D. NOVELLI and F. LIPMANN, *J. Biol. Chem.*, **182**, 213 (1950).
- 12) R.A. PETERS, R.W. WAKELIN, D.E.A. RIVETT and L.C. THOMAS, *Nature*, **171**, 1111 (1953).
- 13) J.F. MORRISON and R.A. PETER, *Biochem. J.*, **58**, 473 (1954).
- 14) L. KOVÁČ, F. BERTA, M. PŠENÁK and V. SLEZÁRIKOVÁ, *Folia Microbiol.*, **11**, 263 (1966).
- 15) P. FORGET and F. PICHINOTY, *Ann. Inst. Pasteur*, **112**, 261 (1967).
- 16) P. FORTNAGEL and E. FREESE, *J. Biol. Chem.*, **243**, 5289 (1968).