Citrate-Mg\(^{2+}\) Transport in Bacillus subtilis

STUDIES WITH 2-FUORO-L-ERYTHRO-CITRATE AS A SUBSTRATE*

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SUMMARY

2-Fuoro-L-erythro-[3,4,5,6-\(^{14}\)C\(_4\)]citrate is actively transported in Bacillus subtilis cells by the citrate-Mg\(^{2+}\) transport system. The kinetic data for uptake of the analogue are very similar to those previously determined for citrate transport. Citrate and 2-fuoro-L-erythro-citrate mutually inhibit their uptake in a competitive manner. Only insignificant metabolism of the analogue was detected after uptake into cells induced for citrate-Mg\(^{2+}\) transport. 2-Fuoro-L-erythro-citrate induces the citrate-Mg\(^{2+}\) transport system with a delay of about one generation time relative to induction caused by citrate. The fuoro analogue probably causes indirect induction of citrate-Mg\(^{2+}\) transport by inhibition of aconitase (EC 4.2.1.3) and subsequent accumulation of endogenous citrate. 2-Fuoro-L-erythro-citrate strongly inhibits aconitase in extracts of B. subtilis cells.

B. subtilis mutants resistant toward growth inhibition by 2-fuoro-L-erythro-citrate in ribose minimal medium were isolated and found to be defective in citrate-Mg\(^{2+}\) transport. These mutants can still grow, albeit very slowly, on plates containing citrate minimal medium. Preliminary results suggest that a second system exists in B. subtilis cells which transports citrate slowly and with lower affinity than the previously described citrate-Mg\(^{2+}\) transport system.

When citrate is taken up by a bacterial cell it is quickly metabolized via the tricarboxylic acid cycle or the citrate fermentation pathway. This fast metabolism of citrate has previously hindered investigations of bacterial citrate transport. For accurate kinetic investigation of this transport system, experimental conditions were needed under which metabolism of the transported substrate is prevented. We knew from our previous inhibitor studies that the hydroxy group in the 3 position and the carboxy group in the 6 position of the citrate molecule were structural prerequisites for a substrate of citrate-Mg\(^{2+}\) transport in B. subtilis (2). A single substitution in any other position of the citrate molecule, however, would give rise to structural isomers, thereby posing the problem of purifying transport studies a single analogue from a synthetic mixture of four optical isomers. This problem could be overcome by making use of the unique properties of the citrate synthase reaction which converted 2-fuoroacetyl-CoA and oxalacetate to 2-fuoro-L-erythro-citrate (3). This isomer was expected to be a strong inhibitor of aconitase in B. subtilis (4). We enzymatically synthesized 2-fuoro-L-erythro-[3,4,5,6-\(^{14}\)C\(_4\)]citrate. In addition, the unlabeled compound was prepared according to Dummel and Kun (4) and used as reference and carrier material. In this publication we describe the properties of this citrate analogue in kinetic studies of the citrate-Mg\(^{2+}\) transport system. Moreover we report on the isolation of B. subtilis mutants resistant toward L-erythro-fluorocitrate and on a characterization of some of them as defective in citrate-Mg\(^{2+}\) transport activity.

EXPERIMENTAL PROCEDURE

Chemicals—Ethyl fluorooacetate, diethyoxalate, cyclohexylamine and sodium fluorooacetate were bought from Fluka (Neu-Ulm). L-Deoxyphedrine and d-deoxyphedrine were products of K & K Laboratories (Plainview, N. Y.). Dowex 50W-X4, Norit A, and sodium L-malate were obtained from Serva (Heidelberg). Enzymes and cofactors were purchased from Boehringer Mannheim. All other chemicals were from Merck (Darmstadt). Cellulose and silica gel thin layer plates were bought from Macherey and Nagel (Düren). L-[U-\(^{14}\)C]malic acid and [1,5-\(^{14}\)C]-citric acid were from Amersham, and [6-\(^{14}\)C]-citric acid was obtained from NEN Chemicals.

Bacteria—B. subtilis SB-26 is a methionine- and tryptophan-requiring derivative of the transformable 168 strain. 60871 is an aconitase-less mutant derived from B. subtilis 60015 which is presumably identical with B. subtilis SB-26 (5). The citrate-Mg\(^{2+}\) transport system has been characterized in SB-26 and 60871 (6). B. subtilis C-11 was derived after mutagenesis from SB-26 by its growth resistance toward L-erythro-fluorocitrate. This isolation and characterization of C-11 are described below. B. subtilis C-11 is active in citrate-Mg\(^{2+}\) transport activity.
subtilis SB-25, auxotrophic for histidine and tryptophan, is also a derivative of the 168 strain and was obtained from Dr. E. W. Nester, University of Washington, Seattle.

**Growth Media**—A minimal salts medium containing 0.3% yeast extract (Difco) (NYE medium) was used for growing *B. subtilis* cells (6). Addition of 5 mM citrate to NYE medium caused maximal induction of the citrate-Mg<sup>2+</sup> transport system. *B. subtilis* cells showed a highest level of citrate uptake when harvested after growth in citrate containing NYE medium to about 100 Klett units (filter No. 54, measured against a water blank). Ribose, citrate, or malate minimal plates contained minimal salts, 1.5% agar and 0.25% ribose, 25 mM potassium citrate or 100 mM potassium L-malate, respectively.

Transformation—DNA was isolated from the donor strain according to the protocol of Marmur (7). It was used to transform a competent culture of *B. subtilis* SB-25, which was prepared as described in Ref. 8.

**Synthesis of L- and D-erythro-fluorocitrate**—The synthesis of these isomers was carried out according to Dummel and Kun (4). The cytochrome c salts of diethylfluorocitrate were prepared for separation by diastereomeric chromatography. Dinitrophenylfluorocitrate was removed by the following treatment: By electron capture electrophoresis at 0°C in 0.1 M sodium phosphate buffer, pH 7.4, and frozen at -20°C. The preparation of a crude extract from these cells and the assays for aconitase activity (EC 4.2.1.3) were carried out as previously described (1). After addition of citrate at 25°C a specific activity of 210 pmol per min per mg of protein was determined for aconitase from the formation of NADPH by coupling the aconitase reaction to isocitrate dehydrogenase.

The abbreviations used are: HEPES, N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid; CCCP, m-chlorophenyl carbonyl cyanide hydrazonide.
genase. NADPH formation was always linearly dependent on citrate concentration (EC 1.1.1.42). We measured the dependency of this reaction on citrate concentration in the presence of two different concentration of L-erythro-fluorocitrate. The activity of NADPH oxidase in the cell extract was also determined and used for correction of aconitate activity as previously reported (14).

Isolation of Mutants Resistant Toward 2-Fluoro-L-erythro-

citrate—A culture (10 ml) of B. subtilis SB-26 growing exponen-
tially in ribose minimal medium was mutagenized with 50 μg of
N'-methyl-N'-nitrosoguanidine per ml for 10 min at 45°. After
filtration and washing the cells were diluted 1:15 in ribose minimal
medium and grown overnight. The cells were then washed four
times with glutamate-free ribose minimal medium and aliquots of
5 × 10⁶ cells were spread on plates containing glutamate-free
ribose minimal medium with 20 mm N-erythro-fluorocitrate.
After 4 to 5 days of incubation some colonies appeared which grew
much faster than the slow growing background cells. Single
colonies were picked and restreaked onto fresh plates contain-
ing N-erythro-fluorocitrate in glutamate-free ribose minimal me-
dium. The mutants were stored on tryptose blood agar (Difco)
before being grown for citrate and malate transport assays.

RESULTS

Metabolism of 2-Fluoro-L-erythro-[3,4,5,6-14C₄]citrate in Vivo—
Dummel and Kun (4) had previously shown that 2-fluoro-L-
erythro-citrate blocked aconitate activity from rat kidney mito-
chondria. As we shall see below this compound also inhibited
aconitate in a crude extract from B. subtilis cells. In order to
rule out extensive degradation of the analogue inside the cells,
we incubated B. subtilis cells induced for citrate transport with
2-fluoro-L-erythro-[3,4,5,6-14C₄]citrate and analyzed the dis-
simulation products (1). Since we had previously found that a
hot water treatment of 2-fluoro-L-erythro-[3,4,5,6-14C₄]citrate
led to extensive destruction of this compound, we chose to take
the cells with a toluene-water mixture (15). This procedure did
not change the chromatographic properties of radioactive L-
erythro-fluorocitrate used as reference material. We analyzed
the toluene water extract by electrophoresis and two different
chromatographic runs on thin layer plates. Details are described
under “Experimental Procedure.” Fig. 1 shows an example of
thin layer chromatography of the toluene-water extract.

None of the systems showed significant catabolism of 2-fluoro-L-
erythro-[3,4,5,6-14C₄]citrate. More than 96% of the radio-
activity applied on the plates was concentrated in the position of
L-erythro-fluorocitrate. To check the possibility that 14CO₂ was
released from 2-fluoro-L-erythro-[3,4,5,6-14C₄]citrate during our
transport assays, we incubated citrate-induced cells of B. subtilis
SB-26 under the same conditions as during our transport assays
together with the radioactive substrate in closed Warburg vessels.
After 5 min we stopped the incubation by addition of perchloric
acid from the side arm of the vessel. The released 14CO₂ was
trapped on a strip of filter paper (Whatman No. 1) moistened
with hyamine hydroxide (Packard). After the end of the reac-
tion the paper was dried and counted in scintillation fluid (16).

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acid from the side arm of the vessel. The released 14CO₂ was
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The transport of N-erythro-fluorocitrate showed the same de-
pendency on Mg²⁺ ions as reported for citrate uptake (6). Using
the indicated times as described under “Experimental Pro-
cedure.” Fig. 2 shows the time dependency of transport of 2-fluoro-L-erythro-citrate in B. subtilis SB-26. When [1,5-14C₄]citrate was used as substrate for uptake studies in this strain we found for incubations of up to 1 min similar transport rates as for 2-fluoro-L-erythro-[3,4,5,6-14C₄]citrate. The accumulated label from metabolized [1,5-14C₄]citrate is mainly trapped in the glutamate pool of the cells (1).

The data in Table I show that CCCP, an uncoupler of oxi-
dative phosphorylation, blocked uptake of radioactivity of both
2-fluoro-L-erythro-citrate and citrate. Moreover, fully induced
cells transport both substrates at similar rates. Usually slightly
more fluorocitrate than citrate was taken up by noninduced cells.
The transport of L-erythro-fluorocitrate showed the same de-
pendency on Mg²⁺ ions as reported for citrate uptake (6). Using
cells of B. subtilis SB-26 we determined a Kₘ of 0.4 mM for the
dependency of L-erythro-fluorocitrate transport on Mg²⁺ conen-
**Induction of Citrate-Mg**⁺ Transport by L-erythro-Fluorocitrate**—**Fig. 5 illustrates an experiment to check whether L-erythro-fluorocitrate was able to induce the citrate-Mg⁺ transport system in B. subtilis SB-26 cells growing in NYE medium. Induction caused by L-erythro-fluorocitrate took place only after a 50-min delay relative to induction by citrate itself. The maximal activity induced by L-erythro-fluorocitrate was only two-thirds of the maximum transport activity which is induced by citrate. We conclude that L-erythro-fluorocitrate probably did not directly induce citrate-Mg⁺ transport. It seems more likely that it caused inhibition of aconitase activity, which led to an internal accumulation of citrate. This might have acted as an endogenous inducer similar to the situation found in the aconitase-deficient mutant 60871 (1). Dummel and Kun (4) had reported that 2-fluoro-L-erythro-citrate blocked aconitase from rat liver mitochondria. We confirmed this inhibitory effect of the fluoro analogue on aconitase activity in a crude extract from B. subtilis SB-26. After incubation of the extract (final protein concentration, 0.2 mg per ml) with 2 μM 2-fluoro-L-erythro citrate for 5 min at 25°C prior to addition of citrate we did not observe any NADPH formation.

**Characterization of B. subtilis Mutants Resistant Toward DL-erythro-Fluorocitrate**—In pilot experiments we had observed that addition of 5 mM DL-erythro-fluorocitrate to glutamate-free ribose minimal medium extended the doubling time of B. subtilis SB-26 cells from 3 hours to 13 hours, although growth never stopped completely. This growth inhibition could be relieved by addition of 100 μg of L-glutamate per ml of medium. This recalled the growth properties of the aconitase-less mutant B. subtilis 60871 which could grow in glucose minimal medium only if glutamate was also present (1). Thus, it seemed likely that DL-erythro-fluorocitrate retarded growth of B. subtilis SB-26 by inhibiting the aconitase activity.

We made use of this observation in a search for mutants resistant toward 2-fluoro-DL-erythro-citrate in ribose minimal medium. Table II lists transport properties of two isolated mutants. Both B. subtilis C-1 and C-11 are derivatives of B. subtilis 60871 is competitively inhibited by 2-fluoro-DL-erythro-citrate with a $K_i$ of 0.3 ± 0.1 mM. For the competitive inhibition of citrate transport by 2-fluoro-DL-erythro-citrate we found in the same mutant a $K_i$ of 0.6 mM. The lack of specificity toward the L and D isomer of fluorocitrate recalls our earlier observation that both L- and D-malate inhibit citrate transport competitively (2). Transport of L-erythro-fluorocitrate in B. subtilis 60871 is competitively inhibited by citrate ($K_i = 0.35 ± 0.1$ mM) as illustrated in Fig. 4B and D-erythro-fluorocitrate ($K_i = 0.35$ mM). These results support the idea that L-erythro-fluorocitrate can be used as citrate analogue in kinetic investigations of the citrate-Mg⁺ transport system in B. subtilis. The measurements of accumulation of label from [1,5-¹⁴C]citrate during short time incubations also yielded results sufficiently accurate for estimation of citrate-Mg⁺ transport activity.

We found for citrate transport in noninduced B. subtilis SB-26 grown in NYE medium an apparent $K_m$ of 1.5 mM and a $V_{max}$ of 18 μmoles per min per g dry weight. The corresponding data for transport of 2-fluoro-L-erythro-citrate were an apparent $K_m$ of 8.3 mM and a $V_{max}$ of 18 μmoles per min per g dry weight (data not shown). The results of our transport measurements under noninduced conditions should be considered as rough estimations. Our measurements of the low maximal velocities showed a considerable variability in the presence of high substrate concentrations which was probably due to variable diffusion and binding of the substrate to the cells.

**Table I**

Comparison of transport data obtained by using L-erythro-fluorocitrate and citrate as substrates

| Strain                  | Transported substrate | Transport | No CCCP treatment | CCCP treatment |
|-------------------------|-----------------------|-----------|-------------------|---------------|
| SB-26 noninduced        | Fluorocitrate         | 9         | 2                 |               |
| SB-26 induced           | Citrate               | 6         | 1                 |               |
| SB-26 induced, no Mg⁺   | Fluorocitrate         | 115       | 3                 |               |
|                         | Citrate               | 110       | 1                 |               |
|                         | Fluorocitrate         | 8         | 3                 |               |
|                         | Citrate               | 10        | 3                 |               |

**Fig. 3.** Uptake of 2-fluoro-L-erythro-citrate as a function of the substrate concentration in the medium. A, transport studies were carried out as usual with cells of Bacillus subtilis SB-26 induced for citrate-Mg⁺ transport. B, data plotted according to Lineweaver and Burk (18) to obtain the apparent $K_m$ and $V_{max}$.

**Fig. 4A** shows that citrate transport in B. subtilis 60781 (6).
induced cells were harvested and processed for transport studies in Bacillus subtilis 60871. Citrate was added simultaneously with citrate to the reaction mixture. Uptake studies were carried out as usual. Final concentrations of inhibitor (2-fluoro-L-erythro-citrate) were 0.02 mM (○), 0.8 mM (●), and no inhibitor (△). B (center), competitive inhibition of 2-fluoro-L-erythro-citrate uptake by citrate in Bacillus subtilis 60871. The experimental conditions were the same as those given above.

![Graph](https://via.placeholder.com/150)

**FIG. 4.** A (left), competitive inhibition of citrate uptake by 2-fluoro-L-erythro-citrate in Bacillus subtilis 60871. Citrate-induced cells were harvested and processed for transport studies as described under “Experimental Procedure.” The inhibitor was added simultaneously with citrate to the reaction mixture. Uptake studies were carried out as usual. Final concentrations of inhibitor (2-fluoro-L-erythro-citrate) were 0.02 mM (○), 0.8 mM (●), and no inhibitor (△). B (center), competitive inhibition of 2-fluoro-L-erythro-citrate uptake by citrate in Bacillus subtilis 60871. The experimental conditions were the same as those given above.

**TABLE II**

| Strain | Transport | Citrate | Malate |
|--------|-----------|---------|--------|
|        | Fluorocitrate |        |        |
| SB-26  | 112 (9)    | 121 (6) | 244 (96) |
| CI     | 19 (8)     | 8 (2)   | 182 (52) |
| CI1    | 17 (14)    | 12 (9)  | 176 (111) |
| SB-25  | 122 (7)    |         |        |
| SB-25-9-7 | 7 (7)    |         |        |
| SB-25-4-42 | 7 (7)  |         |        |
| SB-25-8-28 | 9 (8)  |         |        |

**DISCUSSION**

The effects of fluoro analogues on di- or tricarboxylate transport systems in B. subtilis and in mitochondria are completely different. Kun and associates had reported that 3-fluoro-L-erythro-malate and 2-fluoro-L-erythro-citrate did not penetrate the inner mitochondrial membrane (19, 20). 2-Fluoro-L-erythro-malate, however, competitively inhibited the mitochondrial malate carrier and 2-fluoro-L-erythro-citrate reversibly inactivated the tricarboxylate carrier in mitochondria at a concentration of 10^{-16} M (20). The citrate transport system in Aerobacter aerogenes was also competitively inhibited by fluoro-citrate (probably a mixture of all isomers) but according to Stein and Sachan the analogue was not taken up by these bacteria (21).

In B. subtilis on the other hand, 3-fluoro-L-erythro-malate is a substrate for C-4-dicarboxylate transport (22), and 2-fluoro-L-erythro-citrate is a substrate for the citrate-Mg^{2+} transport system. Although the fluoro analogues were accumulated in these bacteria 10- to 30-fold over the external concentration, no extensive metabolism of these compounds was detected. Thus, the fluoro analogues may replace malate and citrate in transport studies under conditions where fast metabolism of the transported substrate would influence interpretation of the results.

Lawford and Williams (23) had blocked aconitase activity with...
dl-fluorocitrate in two species of *Pseudomonas* in order to study transport of citrate without concomitant metabolism. They pointed out that dl-fluorocitrate also inhibited the uptake of citrate under their experimental conditions. This complicated the interpretation of their transport kinetics. In *B. subtilis* we observed a strong inhibition by dl-erythro-fluorocitrate of citrate catabolism via aconitase and isocitrate dehydrogenase. We were able to isolate mutants resistant to this inhibition which proved to be defective in citrate transport activity. At present we cannot say, however, whether these mutants are blocked in induction of citrate-Mg²⁺ transport or in a structural gene of a corresponding transport protein. The fluorocitrate-resistant *B. subtilis* mutants could still grow on plates containing citrate minimal medium, albeit very slowly when compared to the parent strain. This suggested that *B. subtilis* cells can transport citrate under those conditions via another transport system. Probably this second system is expressed in cells grown in yeast extract medium (NYE medium) without addition of citrate. We tried to determine the apparent $K_m$ for uptake of citrate and of l-erythro-fluorocitrate under these conditions. Our results were somewhat variable due to the relatively high substrate concentrations necessary to saturate the second transport system. We estimated that both $K_m$ values were about 3- and 15-fold higher, respectively, than the corresponding data determined for citrate-Mg²⁺ transport in citrate-induced cells. Thus, it seems likely that this low affinity system which transported citrate relatively slowly is normally used by *B. subtilis* cells for transport of other tricarboxylates or possibly C-4 dicarboxylates. We had earlier reported that L-malate competitively inhibited citrate uptake in *B. subtilis* (2). In this context it may be added that according to a recent report three different transport systems for tricarboxylic acids could be induced in *Salmonella typhimurium* (24).

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