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Uptake of Ferrienterochelin by *Escherichia coli*: Energy-Dependent Stage of Uptake

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The uptake of the siderophore-iron complex ferrienterochelin was found to be strongly dependent upon an energized membrane state, as demonstrated by its sensitivity to dinitrophenol, azide, and cyanide. Ferrienterochelin uptake may also be dependent upon phosphate bond energy, as indicated by sensitivity to arsenate and iodoacetic acid. Although the adenosine triphosphatase does not appear to be involved in this energy coupling mechanism, ferrienterochelin uptake was shown to be less dependent upon phosphate bond energy than was glutamine uptake. Sensitivity of ferrienterochelin uptake to osmotic shock was shown to be due to the release of a ferrienterochelin binding compound located in the outer membrane of the cells and probably identical to the colicin B receptor protein.

The uptake of nutrients, growth factors, and ions (referred to herein as transport substrates) by *Escherichia coli* involves transfer across two lipid bilayers (7). Transfer across the outer membrane is thought, in most cases, to be an entirely passive or nonspecific process, the transport substrates normally being small enough to diffuse through pores in the outer membrane (27, 29). Transport against a concentration gradient into the cell cytoplasm is an energy-dependent process and requires the presence of one or more specific transport systems (6). Although it is not completely clear how energy is coupled to the uptake process, several workers using different uptake systems have postulated the existence of three distinct mechanisms (15, 38). The group translocation system is the best understood of these three mechanisms but is applicable only to the uptake of a limited number of sugars (15, 38). The remaining two mechanisms can be distinguished by such parameters as sensitivity to uncouplers of oxidative phosphorylation and inhibitors of electron transport or glycolysis, by the effects of loss, through mutation, of cytochrome or adenosine triphosphatase (ATPase) activities, by the direct utilization of fermentable or oxidizable carbon sources to drive active uptake, and by ability to accumulate the transport substrate after anaerobic growth (4, 14, 23, 33). On the basis of results obtained in experiments of this type, several workers postulated that the two energy coupling processes are (i) utilization of an energized membrane state (a proton or charge gradient across the cytoplasmic membrane) generated either by electron transport during respiration or by hydrolysis of adenosine 5'-triphosphate (ATP) by the ATPase under anaerobic conditions, and (ii) direct utilization of a high-energy phosphate bond (probably ATP) (4, 14, 23).

It has been shown that uptake systems that utilize phosphate bond energy are highly sensitive to osmotic shock, and this sensitivity is associated with the loss of specific binding proteins (4, 23, 35). These proteins are located in the periplasmic space, the region between the inner and outer membranes of *E. coli*, and are probably associated with the outer surface of the inner membrane (7). These proteins also appear to be involved in chemotactic responses to certain nutrients (21).

We previously reported the results of a series of experiments on the uptake by *E. coli* K-12 of iron complexed with the siderophore (iron chelator) enterochelin, a cyclic trimer of 2,3-dihydroxybenzoyl serine (30). Enterochelin (also called enterobactin) is secreted by *E. coli* (and by other *Enterobacteriaceae*) under conditions of iron stress and serves to solubilize iron that would otherwise be unavailable to the cells (36). Ferrienterochelin (the ferric ion enterochelin complex) is transported into the cell via specific outer and inner membrane transport systems and is therefore similar to the uptake of vitamin B_{12} and maltose, both of which also have specific outer membrane uptake systems (39, 41). The uptake of ferrienterochelin is inhibited in a number of mutants of *E. coli* that lack outer membrane ferrienterochelin receptor activity (*cbr* mutants) (32; unpublished data) or ferrienterochelin esterase activity (*fes* mu-

tants) (24). The nature of the block in ferrienterochelin uptake in other mutants of *E. coli* is less well understood, but some may involve stages in transfer across the outer or inner membranes (*fep* [8], *feuA* and *feuB* [16, 20], *exbB* and *exbC* [30], and *tonB* [12, 30]). Many of these mutants are also resistant to a number of colicins (20, 30), but the mechanisms through which the colicin action and ferrienterochelin uptake systems interact are not fully understood (31).

To learn more of the stages involved in the uptake of ferrienterochelin we have examined the energy dependence of the active uptake stage by using some of the parameters described above. The results presented in this paper indicate that the uptake of ferrienterochelin is strongly dependent upon an energized membrane state. However, the results also indicate that energy for ferrienterochelin uptake is dependent upon a supply of phosphate bond energy such as ATP. The implications of these results are discussed.

MATERIALS AND METHODS

Bacterial strains and culture media. Strains of *E. coli* K-12 used in this study are listed in Table 1. Mutants defective in ATPase activity (*unc-405* and *uncA*) were cloned at regular intervals and checked for inability to grow with succinate as the sole carbon source. Phosphate minimal salts medium (medium A) was prepared as described previously and was supplemented with amino acids and growth factors (Table 1) and with a trace salts solution at the concentrations used previously (30). Tris(hydroxymethyl)aminomethane minimal salts solution (medium B) contained (per liter): tris(hydroxymethyl)aminomethane base, 12.1 g; NaCl, 4.5 g; KCl, 1.5 g; $Ca(NO_3)_2$, 0.01 g; $MgSO_4 \cdot 5H_2O$, 0.2 g; and $(NH_4)_2SO_4$, 2.0 g (pH 7.3 with HCl). Iron-depleted salts solutions and buffer solutions were prepared by extracting the iron with 8-hydroxyquinoline (30), and sterile glucose was added to the sterilized growth and uptake media at a final concentration of 5 g/liter unless stated otherwise.

Chemicals. [^{3}H]proline (677 mCi/mmol), [^{14}C]-glutamine (57.3 mCi/mmol), [^{3}H]arginine (22 mCi/mmol), and $^{55}FeCl_3$ (11.4 mCi/mmol) were purchased from the Amersham/Searle Corp. and were diluted with appropriate carrier before use. Enterochelin was as used previously (30), and spectinomycin was kindly provided by G. Whitfield of Upjohn Ltd. All other chemicals were obtained commercially and were of the highest purity available.

Protein determinations. Protein concentrations were determined by the method of Schacterle and Pollack (37) by using bovine serum albumin as a standard.

Uptake studies. The method used to study enterochelin-mediated iron uptake under aerobic conditions was as follows unless stated otherwise. Cells were grown overnight in minimal medium A containing an additional 5 μM $FeCl_3$. The cells were harvested by centrifugation and washed twice and suspended in iron-depleted medium A to an initial optical density at 660 nm (OD_{660}) of 0.01. The cells were then grown with good aeration until an OD_{660} of 0.2 was reached, at which stage the cells were chilled to 4°C and harvested by centrifugation. The cells were washed twice and resuspended in fresh iron-depleted medium A containing 100 μM nitrilotriacetic acid (NTA) and 0.5% glucose (uptake medium; final OD_{660}, 0.8). Cells were stored at 4°C until use and were always used within 2 h of harvesting. In studies on the effect of arsenate on uptake, the iron-starved cells were washed and resuspended in iron-depleted medium B containing 100 μM NTA,

TABLE 1. *E. coli K-12 strains*

Strain designation	Relevant genotype[a]	Source (reference)	
AN248	*ilvC7 argH entA*	F. Gibson	(9)
AN249	*ilvC7 argH entA unc-405*	F. Gibson	(9)
7	Prototrophic	R. Kadner	(23)
NR70	*uncA* derivative of strain 7	R. Kadner	(23)
A428	*his proB*	R. Kadner	(23)
N144	*uncA* derivative of A428	R. Kadner	(23)
P1571	*aroE spc*[b] derivative of 7		
P1572	*aroE spc*[b] derivative of NR70		
P1683	*aroE spc*[b] derivative of A428		
P1691	*aroE spc*[b] derivative of N144		
P1552	*thr leu his proA argE supE aroE spc str thi*		(30)
P1554	As P1552 but *exbC51*		(30)
P1555	As P1552 but *exbB71*, *metC*		(30)
AN366	*pabA his arg ilv purE aroE str spc*	G. C. Woodrow	
AT2472	*aroE thi*	B. Stillman	
P1798	*cbr-7* derivative of AT2472		

[a] Terminology as in Bachmann et al. (2) and in quoted references.

[b] *aroE spc* strains derived by cotransduction of these markers from strain AN366 with selection for spectinomycin resistance as described previously (30).

0.5% glucose, and either 10 mM sodium arsenate or 10 mM sodium phosphate buffer, both at pH 7.3. Uptake was initiated by the addition of the ^{55}Fe(III)-labeled ferrienterochelin [final concentrations, 6 μM ^{55}Fe(III) and 12 μM desferrienterochelin] to the cells in plastic vials which were shaken in an oscillating water bath (200 oscillations per min) at 37°C. Samples were removed from the uptake mixture at appropriate time intervals and filtered through a Gelman GA-6 membrane filter (0.45 μm mean pore diameter) that had been presoaked in a solution of ferric-ethylenediaminetetraacetic acid (24). The filters were rinsed twice with 10 ml of saline and dried and placed in scintillation vials. The filters were cleared by the addition of toluene containing 0.6% butyl-1,3,4-phenylbiphenyloxidiazole (Sigma Chemical Co.), and the radioactivity retained by the filter was counted in a Packard model 3003 scintillation counter.

Amino acid uptake was determined under the same conditions as ferrienterochelin uptake (i.e., in iron-depleted medium containing 100 μM NTA) using the same iron-starved cells except where noted in the text. The final concentration of labeled amino acid in the uptake mixture was 500 μM. Chloramphenicol (100 μg/ml) was used to inhibit protein synthesis during amino acid uptake.

Counts of radioactivity retained by the membrane filters were corrected for nonspecific retention of cell-free radioactivity, and the results are expressed as weight of transport substrate per milligram of cell dry weight.

Inhibitors, where used, were added to the washed cells which were then incubated for 20 min at 37°C. Inhibitors were dissolved in uptake medium or in ethanol; control experiments demonstrated that the addition of ethanol to the cell suspensions did not affect uptake. In the case of arsenate-treated cells (see above), cells were again incubated at 37°C for 20 min before uptake was measured.

Treatment with 2,4-dinitrophenol (DNP) to deplete cellular energy was performed by the method of Berger (3) with some modifications. Cells were grown first in iron-depleted medium A containing 1 g of glucose per liter (final OD_{660}, 0.25 to 0.30) and then washed twice and suspended in unsupplemented medium A (with no energy source) plus 200 μM NTA and 2 mM DNP. The cells were incubated with good aeration at 37°C for 10 h (AN248) or 2 h (AN249). After starvation, the cells were harvested and washed twice and resuspended in uptake medium without glucose. Where indicated, energy sources were added to the cells which were then incubated for 10 min at 37°C before uptake was initiated.

In studies on uptake after anaerobic growth, cells were grown in iron-depleted medium A containing 100 μM NTA for 16 h at 37°C in anaerobic jars filled with hydrogen-carbon dioxide gas mixture generated by a GasPak unit (Becton-Dickinson & Co.; final OD_{660}, 0.15 to 0.20.). Cells were washed twice and resuspended as above except that no glucose was added to the medium. The cells were then purged continually with nitrogen gas that had been passed through two towers of pyrogallol (5% [wt/vol] in 14 M KOH), and the glucose was added. Radioactive transport substrates were similarly purged for 10 min before being added to the cells.

The effect of osmotic shock on uptake was studied by using cells grown under the normal conditions of iron deprivation for 16 h and subjected to the osmotic shock procedure of Willis et al. (42). All reagents except the $MgCl_2$ solution were extracted with 8-hydroxyquinoline (see above) and contained 200 μM NTA to prevent iron uptake through the low-affinity uptake system during shock treatment (30). Shocked cells were washed and resuspended in uptake medium and assayed for uptake immediately or after incubation at 37°C for varying lengths of time in the presence or absence of 100 μg of chloramphenicol per ml.

Preparation of periplasmic fluid and membrane fractions. Periplasmic fluid was prepared from 5-liter cultures of cells of strain AT2472 grown under conditions of iron stress (in iron-depleted medium A) or in the presence of 20 μM $FeCl_3$ by the procedure described above. The shock fluid obtained was concentrated by dialysis against Aquacide II (Calbiochem) and then dialyzed extensively against 50 mM sodium phosphate buffer (pH 7.3), both at 4°C. Debris (largely lipopolysaccharide) was removed by centrifugation at 46,000 × *g* for 60 min at 4°C, and the supernatant was stored at −20°C.

Outer and inner membranes were prepared from iron-stressed or iron-supplemented cells (20 μM $FeCl_3$) in the exponential stage of growth. The cells were disrupted in a French pressure cell (Aminco) and the cell envelope was pelleted, after removal of cell debris, by centrifugation at 46,000 × *g* for 60 min at 4°C. The inner membrane was solubilized with 2% Triton X-100 as described previously (30), and the soluble fraction was precipitated in 70% ethanol at −20°C. The ethanol-precipitated material was pelleted by centrifugation at 46,000 × *g* for 60 min at 4°C and washed twice and resuspended in distilled water. Outer membrane fractions (Triton-insoluble walls) were prepared as described previously (30), and both membrane fractions were stored at −20°C.

Binding assays. Ferrienterochelin binding assays were performed using dialysis chambers similar in design to those used by Furlong and Weiner (13). All reagents were dissolved or resuspended in a solution containing 50 mM NaCl, 10 mM sodium phosphate buffer, and 100 μM NTA (pH 7.0). Samples containing putative binding proteins and the ^{55}Fe(III)-labeled ferrienterochelin were separated by a boiled dialysis membrane (Visking) and allowed to dialyze to equilibrium (24 h) at 4°C on a vertical rotating turntable. Samples were removed from both chambers after dialysis and emulsified in a scintillant cocktail containing two parts toluene-butyl-1,3,4-phenylbiphenyloxidiazole (see above) and 1 part Triton X-100 (28) and counted as above. Concentrations of protein and ferrienterochelin were adjusted to give 10 to 30% binding of available radioactivity, and bovine serum albumin was used as control.

PAGE. Polyacrylamide gel electrophoresis (PAGE) membrane fractions and periplasmic fluid were analyzed in the presence of sodium dodecyl sulfate by PAGE by the technique of Lugtenberg

et al. (26) in slab gels. Gels were stained by the method of Fairbanks, Steck, and Wallach (10).

RESULTS

Experimental approach. One of the purposes of this study was to determine the nature of the energy coupling for the active uptake of ferrienterochelin. Uptake of ferrienterochelin was therefore compared with known examples of transport substrates that use either the energized membrane state (proline) (4) or phosphate bond energy (glutamine and arginine (4) for uptake, under the conditions of iron stress which are necessary for detectable iron requirement and for ferrienterochelin uptake. Control experiments demonstrated that iron starvation had no direct effect on uptake of proline or glutamine under aerobic conditions, and results obtained in different experiments may therefore be compared (having regard for minor fluctuations in uptake rates in different experiments). The uptake of ferrienterochelin is, however, strongly dependent upon the level of iron starvation achieved during growth of the bacteria before assay; hence results obtained in different experiments should not be compared. In studies in which the uptake of ferrienterochelin in different strains of *E. coli* was compared, care was taken to ensure that the cells were grown under identical conditions. All experiments were performed at least twice.

Effect of loss of ATPase activity on ferrienterochelin uptake. Previous studies have shown that uptake of a transport substrate may be reduced in some mutants that are defective in ATPase functions (23). The results shown in Fig. 1 indicate that this is not the case with ferrienterochelin uptake by three mutants lacking ATPase activity. The differences noted in Fig. 1 between ferrienterochelin uptake in strain P1683 and in the isogenic *uncA* strain P1691 are probably not significant when possible differences in the level of iron starvation are taken into consideration. Furthermore, treatment of strain AN248 (*unc*$^+$) with *N,N'*-dicyclohexylcarbodiimide, which binds to a specific protein component of the ATPase complex (1) and thereby inhibits ATPase function, did not affect ferrienterochelin uptake.

Effects of inhibitors of electron transport and uncouplers of oxidative phosphorylation. Inhibitors of electron transport (potassium cyanide) block the production of the energized membrane state by interfering with respiration. Uncouplers of oxidative phosphorylation (DNP and carbonyl cyanide *m*-chlorophenylhydrazone [CCCP]) produce a "leaky" membrane which cannot maintain an energized state. As shown in Fig. 2, these reagents preferentially inhibit proline uptake, which is dependent upon the energized membrane state, and their effect was more pronounced in strain AN249 (*unc-405*) in which the defective ATPase could not be used to energize the membrane by ATP hydrolysis. The effects of DNP and cyanide on glutamine and proline uptake were clearly dependent on the dose of inhibitor used (Table 2), but we were unable to demonstrate preferential inhibition of proline uptake by the uncoupler CCP (Table 2). This effect has been noted previously (14) and is thought to be due to the ability of CCP to form thiol groups (22). The uptake of proline was also strongly inhibited by 10 mM sodium azide, which inhibits oxidative phosphorylation and cytochrome oxidase, whereas glutamine uptake was largely unaffected (Fig. 2). The results in Fig. 2 and in Table 2 indicate that the active uptake of ferrienterochelin was also highly sensitive to DNP, cyanide, and azide, which suggests that this uptake system is strongly dependent upon the presence of an energized membrane.

Effects of inhibitors of ATP production. Inhibitors of glycolysis (iodoacetic acid [IAA] and sodium fluoride) block the production of ATP and thus reduce the level of cellular ATP available to drive active uptake. Results in Fig. 3 show that uptake of glutamine was strongly inhibited by 200 μM IAA, whereas proline and

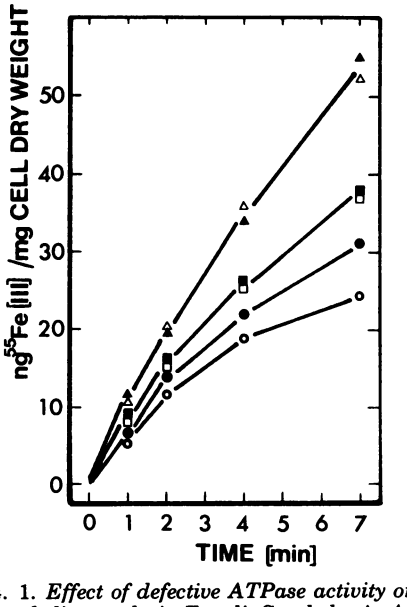

FIG. 1. *Effect of defective ATPase activity on ferrienterochelin uptake in E. coli. Symbols:* ▲, *AN248 (unc*$^+$*);* △, *AN249 (unc-405);* ■, *p1571 (unc*$^+$*);* □, *P1572 (uncA);* ●, *P1683 (unc*$^+$*);* ○, *P1691 (uncA).*

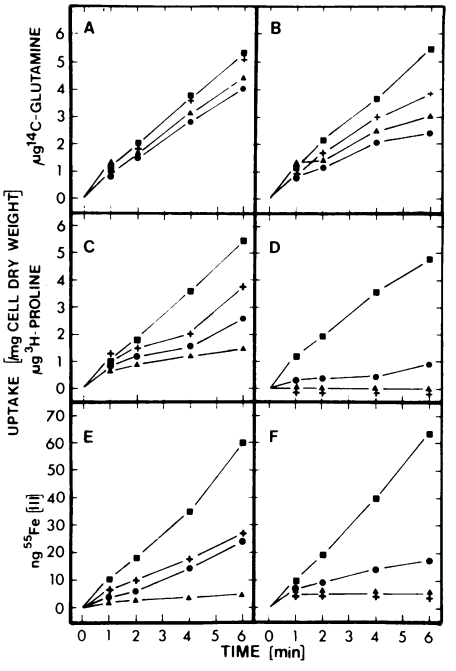

FIG. 2. *Effect of uncouplers of oxidative phosphorylation and inhibitors of electron transport on uptake of glutamine, proline, and ferrienterochelin in strains AN248 (unc$^+$; A, C, and E) and AN249 (unc-405, B, D, and F). Symbols:* ■, *control;* ●, *500 μM DNP;* +, *4 mM KCN;* ▲, *10 mM* NaN_3.

ferrienterochelin uptakes were less affected. However, sensitivity of ferrienterochelin uptake to 200 μM IAA was strongly increased in the absence of a functional ATPase (strain AN249), which suggests that ATP generated by the ATPase and by glycolysis may play a role in the energy-dependent uptake of this compound (Fig. 3F). We were unable to differentiate between the energy coupling involved in the uptake of proline and glutamine with 50 mM sodium fluoride (Fig. 3), but we were able to selectively inhibit glutamine uptake in cells treated with 10 mM sodium arsenate (Fig. 4), which blocks ATP production by competing with phosphate (4). Ferrienterochelin uptake was also inhibited in cells treated with sodium arsenate in the absence of phosphate (Fig. 4). Ferrienterochelin uptake was completely restored by the addition of 100 mM phosphate buffer to the arsenate-treated cells, thus ruling out the possibility that iron had entered the cells in association with the arsenate during preincubation. The results therefore suggest that ferrienterochelin uptake is dependent upon the presence of cellular ATP or an analogous source of phosphate bond energy.

Combined effects of inhibitors of glycolysis and inhibitors of electron transport or uncouplers of oxidative phosphorylation. To determine whether the effects of DNP and IAA were additive, we pretreated cells of strain AN249 (*unc-405*) with a 100 μM concentration of each or both reagents. As shown in Fig. 5, these doses of IAA or DNP individually have only a limited effect on uptake of ferrienterochelin, but the combined effect of the two inhibitors was to strongly inhibit ferrienterochelin uptake. Similar results were obtained with combinations of 10 mM sodium arsenate and 100 μM DNP or 1 mM potassium cyanide.

Respiration-dependent uptake. Treatment of cells with high doses of DNP over long periods of time in the absence of energy sources completely reduces cellular ATP levels (3). Uptake of transport substrates can thus be coupled directly with the utilization of exogenously added energy sources. As shown in Fig. 6, uptakes of glutamine and proline can both be driven quite effectively by exogenous glucose added to iron-starved, DNP-treated cells of strain AN249 (*unc-405*). Similar results were obtained with strain AN248 (*unc*$^+$). Glucose-driven uptake of ferrienterochelin could, however, only be demonstrated in cells of strain AN249 (*unc-405*) after DNP treatment. Furthermore, glucose-driven proline uptake was somewhat reduced in iron-starved, DNP-treated cells of both strains tested (cf. Fig. 6 with Fig. 2). We also noted that proline uptake could not be effectively coupled with lactate respiration in iron-starved cells of either strain tested (Fig. 6), even though proline uptake is respiration dependent and is normally highly efficient. However, higher rates of proline uptake were obtained with DNP-treated cells of strain AN249 (*unc-405*) which were not subjected to the iron-starvation regimen.

Effect of anaerobiosis on uptake. Growth of *E. coli* under anaerobic conditions in the absence of an electron acceptor eliminates electron transport and inhibits respiration-dependent uptake of transport substrates (14). Control experiments demonstrated that glutamine uptake in strain AN248 (*unc*$^+$) was unaffected by anaerobic growth under iron stress, whereas proline uptake was severely inhibited (20 to 30% of control aerobic uptake). We were unable to demonstrate any active uptake of ferrienterochelin after anaerobic growth.

Effects of sodium arsenate and potassium cyanide on mutants partially defective in ferrienterochelin uptake. We previously described two classes of mutants (*exbB* and *exbC*) that are partially defective in ferrienterochelin uptake (30). We reexamined ferrienterochelin uptake in these mutants following treatment

TABLE 2. *Effect of DNP, CCCP, and KCN on active uptake of glutamine, proline and ferrienterochelin in strains AN248 (unc$^+$) and AN249 (unc-405)*[a]

Inhibitor and concentration	Inhibition of active uptake (%)					
	[^{14}C]glutamine		[^{3}H]proline		^{55}Fe(III)-labeled ferrienterochelin	
	AN248	AN249	AN248	AN249	AN248	AN249
DNP						
100 μM	6	0	25	12	7	31
200 μM	15	40	52	57	33	77
500 μM	33	62	76	90	66	80
1,000 μM	64	74	89	97	88	85
1,500 μM	86	86	96	100	96	98
2,000 μM	100	100	100	100	100	100
CCCP						
10 μM	11	14	13	7	4	11
20 μM	51	56	62	72	72	68
30 μM	85	93	95	96	93	93
50 μM	90	92	100	100	98	95
KCN						
2 mM	0	26	38	94	42	92
4 mM	3	51	40	97	44	89
6 mM	23	62	55	98	66	93
8 mM	62	75	53	97	69	95
10 mM	74	78	68	98	74	93
20 mM		72		100		98

[a] Amino acid uptake rates were determined from three samples taken within the first 3 min of uptake. Ferrienterochelin uptake rates [^{55}Fe(III)] were determined from three samples taken between min 1 and 4 of uptake to avoid problems caused by initial binding of the ferrienterochelin to the outer membrane receptor protein. KCN, Potassium cyanide.

with 10 mM sodium arsenate or 4 mM potassium cyanide. The results in Fig. 7 indicate that uptake of ferrienterochelin in the parent strain (P1552) was inhibited after treatment with arsenate or cyanide. Uptake of ferrienterochelin was also inhibited in strains P1554 (*exbC*) and P1555 (*exbB*) after treatment with arsenate or cyanide. We conclude that these mutants are not defective in ferrienterochelin uptake through inability to couple either phosphate bond energy or the energized membrane state with the active uptake process.

Effect of osmotic shock on ferrienterochelin uptake. Sensitivity to osmotic shock is thought to indicate dependence upon a periplasmic binding protein and is usually correlated with utilization of phosphate bond energy for active uptake (4). Results in Fig. 8 show that arginine, proline, and ferrienterochelin uptake are all strongly inhibited when cells are assayed immediately after osmotic shock. Proline uptake, which involves no periplasmic binding protein (4), was almost completely restored after 30 min of incubation in the presence of chloramphenicol (Fig. 8B), whereas arginine and ferrienterochelin uptake were not. Uptake of arginine could, however, be fully restored in shocked cells incubated for 2 h in the absence of chloramphenicol, under which conditions fresh periplasmic binding protein(s) could be synthesized. Uptake of ferrienterochelin could be partially restored by incubation in the absence of chloramphenicol after osmotic shock. This restoration was largely achieved through an increase in apparent uptake during the first minute (approaching normal after 2 h of incubation) rather than an increase in the rate of active uptake of ferrienterochelin (no better than 50% recovery after 2 h of incubation compared with over 80% in the case of arginine uptake).

Material released by osmotic shock from iron-starved or iron-supplemented cells of strain AT2472 or P1798 (*cbr*) was examined for ferrienterochelin binding activity. As shown in Table 3, the level of ferrienterochelin binding activity in the periplasmic material from iron-starved cells of strain AT2472 was higher than that released from the corresponding iron-supplement cells. An increase in ferrienterochelin binding activity was also detected in the outer membranes prepared from iron-starved cells of this strain. However, material released by osmotic shock from iron-starved cells of strain

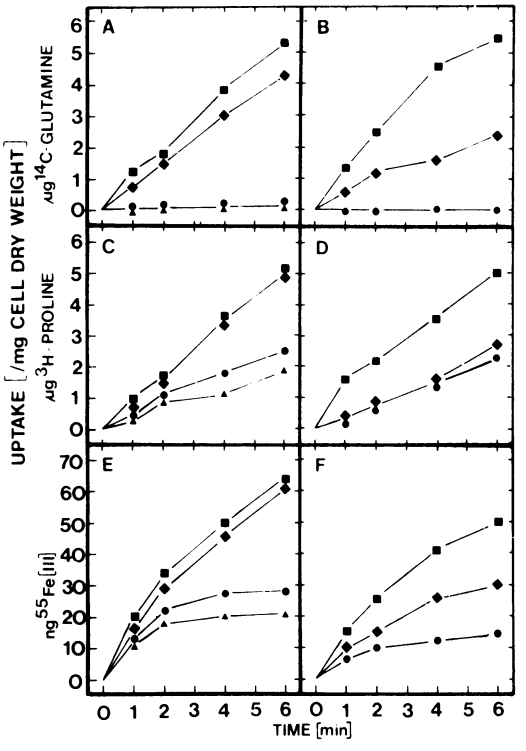

FIG. 3. *Effect of inhibitors of glycolysis and ATPase function on uptake of glutamine, proline, and ferrienterochelin in strains AN248 (unc⁺; A, C, and E) and AN249 (unc-405; B, D, and F). Symbols: ■, control; ◆, 50 mM NaF; ●, 200 μM IAA; ▲, 200 μM IAA plus 100 μM N,N'-dicyclohexylcarbodiimide.*

P1798, which lacks outer membrane receptor activity for ferrienterochelin (Table 3) and for colicins B and D (32), did not contain ferrienterochelin binding activity (Table 3).

Samples of the two periplasmic protein preparations and the membrane preparations from strain AT2472 were examined by PAGE in the presence of sodium dodecyl sulfate by the method of Lugtenberg et al. (26). Outer membranes prepared from iron-starved cells and examined by this method showed strong derepression of three outer membrane proteins with molecular weights ranging from 76,000 to 85,000, as judged by their positions in the cells compared with proteins of known molecular weights (16, 32; A. P. Pugsley and P. Reeves, Biochem. Biophys. Res. Commun., in press). No similar derepression of any proteins was detected in either the periplasmic or inner membrane preparations.

DISCUSSION

The uptake of iron complexed with the siderophore enterochelin by *E. coli* is clearly more complex than the systems involved in the uptake of other transport substrates such as amino acids. Apart from the involvement of specific systems for transporting the complex across the outer membrane (19, 20, 30, 40), there also appear to be several stages involved in transfer across the inner membrane. The data presented in this paper indicate that the energized membrane state is necessary for this latter, active uptake stage, as demonstrated by the effects of azide, and particularly of cyanide and DNP in an *unc*⁻ mutant (Fig. 2; Table 2).

Arsenate and IAA were also shown to have some effect on the uptake of ferrienterochelin (Fig. 3 and 4) although the effects were not as pronounced as those of the same reagents upon glutamine uptake. The incomplete effects of IAA and arsenate on ferrienterochelin uptake compared with glutamine uptake may be due to the fact that residual levels of cellular ATP, which remain after treatment with these reagents, are sufficient for the relatively low level (in molar terms) of ferrienterochelin up-

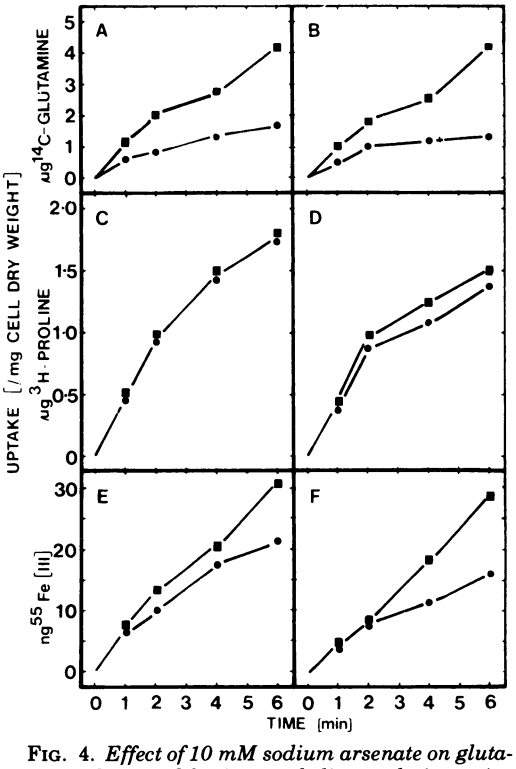

FIG. 4. *Effect of 10 mM sodium arsenate on glutamine, proline, and ferrienterochelin uptake in strains AN248 (unc⁺; A, C, and E) and AN249 (unc-405; B, D, and F). Symbols: ■, cells incubated in medium B containing 10 mM sodium phosphates; ●, cells incubated in medium B containing 10 mM sodium arsenate.*

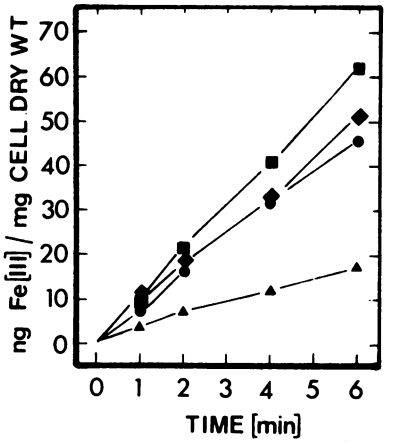

FIG. 5. *Combined effects of IAA and DNP on uptake of ferrienterochelin in strain AN249 (unc-405). Symbols* ■, *control;* ◆, *100 μM IAA;* ●, *100 μM DNP;* ▲, *100 μM IAA plus 100 μM DNP.*

take. An absolute or partial requirement for cellular ATP or a similar source of phosphate bond energy for ferrienterochelin uptake may therefore be indicated. In support of this, we have consistently shown that ferrienterochelin uptake is more sensitive to treatment with arsenate than is proline uptake, which is dependent solely upon an energized membrane state, irrespective of the strain used. On the other hand, it is also possible that the effects of IAA and arsenate on glucose uptake may also result in a reduction of energy available to derive other uptake systems.

If two distinct energy coupling mechanisms are indeed involved in the uptake of ferrienterochelin, they do not appear to interact in the way demonstrated previously for phosphate uptake, for which there are two parallel uptake systems that are genetically and functionally independent (33). In the case of ferrienterochelin, the evidence suggests that both coupling mechanisms are required for a single uptake system. The coupling of energy with the uptake system may be further complicated by the involvement of a requirement for reduction of the metal ion from the ferric to the ferrous form during uptake. This possibility arises (i) by analogy with the postulate of Leong and Neilands (25) that iron in complex with the siderophore ferrichrome must be reduced to the ferrous form before it is transported across the cytoplasmic membrane of *E. coli*, and (ii) by analogy with the uptake of ferric sucrose by rat liver mitochondria where ferric irons are reduced by reducing equivalents supplied by the mitochondrial cytochrome system during uptake (11).

No uptake of ferrienterochelin was observed in cells grown anaerobically. One might predict that a lower level of iron would be required by anaerobically grown *E. coli* since one of the major sites of incorporation of iron is the cytochrome system, which would not be operating

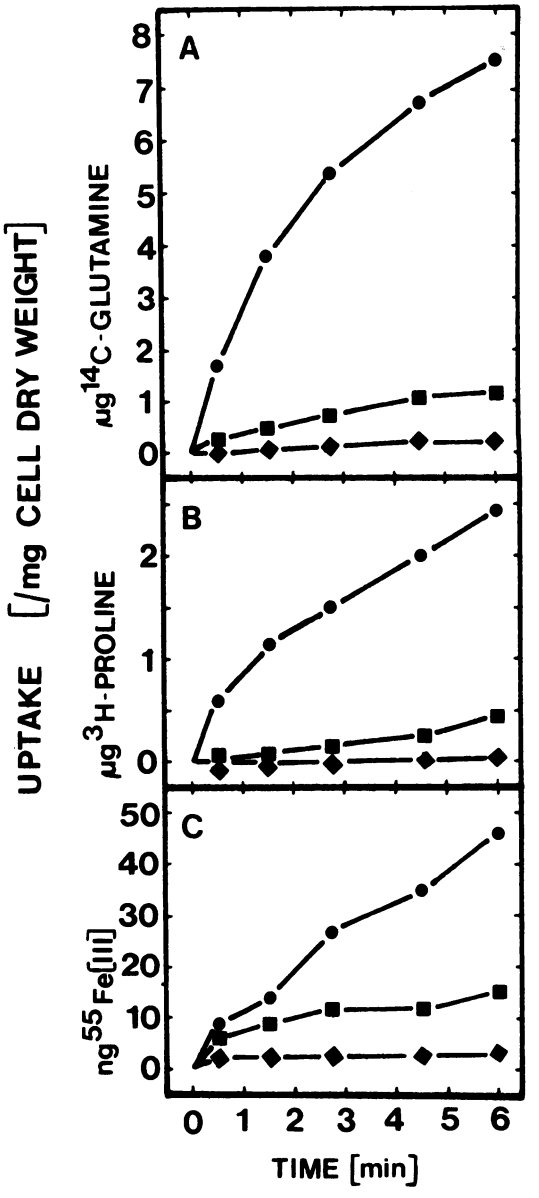

FIG. 6. *Uptake of glutamine, proline, and ferrienterochelin in iron-starved cells of strain AN249 (unc-405) following treatment with 2 mM DNP for 2 h in the absence of energy source. Symbols:* ◆, *uptake in the absence of exogenous energy;* ■, *uptake after 10 min of incubation with 20 mM* DL*-lactate;* ●, *uptake after 10 min of incubation with 20 mM glucose.*

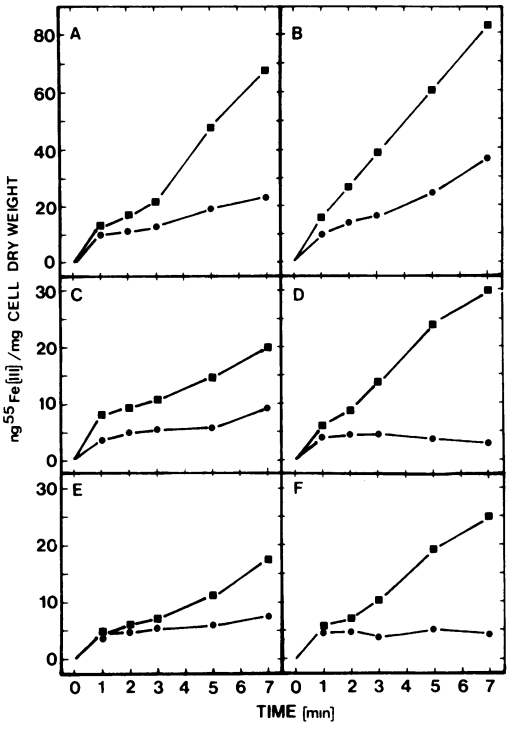

FIG. 7. *Uptake of ferrienterochelin in strains P1552, P1554 (exbC51), and P1555 (exbB71) incubated for 20 min in the presence of 10 mM sodium arsenate (A, C, and E) or 4 mM KCN (B, D, and F). Symbols: ■, controls; ●, cells treated with inhibitors.*

under these conditions. Furthermore, since iron is soluble in the ferrous form under anaerobic conditions, siderophore-dependent systems for the uptake of iron would not normally be necessary, and siderophore uptake systems may be repressed by anaerobiosis. It has been shown previously that enterochelin is not hypersecreted by mutants defective in enterochelin uptake when the cells are grown anaerobically (31). Cells incubated aerobically in the presence of DNP may also have a reduced requirement for iron, particularly after long periods of incubation.

Some of the data presented in this paper may also be useful in determining the role of the *tonB* gene product in iron uptake. The *tonB* product is required for the action of the group B colicins (31), for irreversible adsorption of bacteriophages T1 and ϕ80 and for vitamin B_{12} uptake (C. A. Schnaitman, personal communication). It has recently been shown that irreversible adsorption (deoxyribonucleic acid injection) of bacteriophages T1 and ϕ80 requires an energized membrane state (17), and the *tonB* gene product may be involved in coupling this energization with some stage of T1 or ϕ80 infection. If this were so, one might predict that group B colicin action and other chelator-mediated iron uptake systems, as well as ferrienterochelin uptake (this paper) and vitamin B_{12} uptake (5), would also require a functional electron transport system or energized membrane state and would be coupled to it by the *tonB* gene product. At this stage it is still not possible to say whether the *tonB* gene product is located in the inner or outer membranes of *E. coli* (12).

The sensitivity of ferrienterochelin uptake to osmotic shock seems to be due to the release of

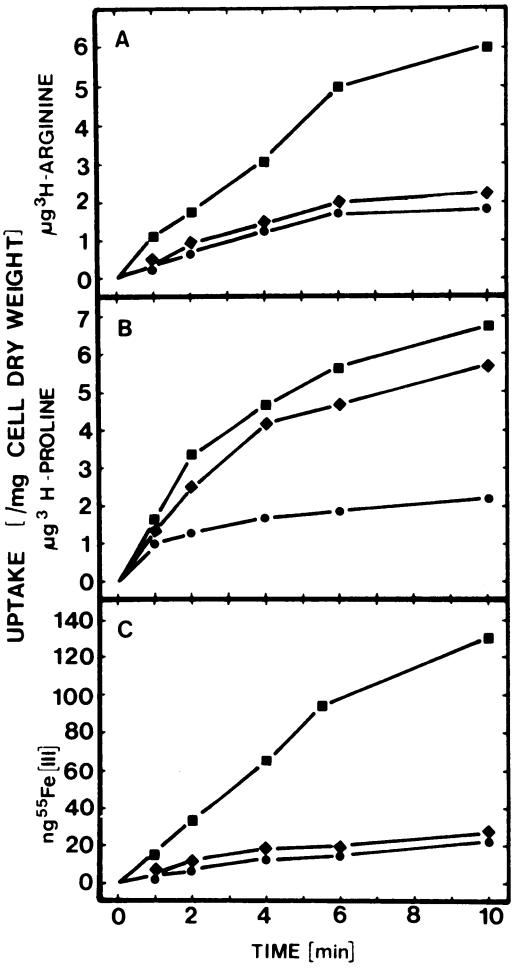

FIG. 8. *Uptake of arginine, proline, and ferrienterochelin in cells of strain AT2472 before and after osmotic shock. Cells were tested immediately before osmotic shock (■), immediately after osmotic shock (●) or after 30 min of incubation in iron-depleted medium A containing 100 µg of chloramphenicol per ml, 0.5 mg of glucose per ml, and 100 µM NTA (◆).*

TABLE 3. *Ferrienterochelin binding activity of outer membranes and periplasmic proteins prepared from iron-starved cells of strains AT2472 and P1798 (cbr)*

Cells	ng of ^{55}Fe(III)-labeled ferrienterochelin/mg of protein[a]			
	Outer membrane		Periplasmic proteins	
	AT2472	P1798	AT2472	P1798
Iron-starved	15.14	0.44	3.32	0.36
Iron-supplemented	0.17	0.18	0.37	0.37

[a] Ferrienterochelin binding to bovine serum albumin occurred at a rate of 0.23 ng of ^{55}Fe(III)-labeled ferrienterochelin/mg of protein. The ferrienterochelin reagent contained 70 nM ^{55}Fe(III) and 140 nM of desferrienterochelin. Protein concentrations ranged from 1.0 to 0.02 mg/ml. Each result is the mean of several independent experiments.

the outer membrane ferrienterochelin receptor by the shock treatment rather than to the loss of a periplasmic binding protein. The evidence for this is that the initial rate of ferrienterochelin uptake, which is considered to be largely binding to the outer membrane receptor (30), is severely reduced by osmotic shock (Fig. 8), and that the ferrienterochelin receptor mutant (P1798, *cbr-7*) did not release ferrienterochelin binding activity after osmotic shock. We could not, however, detect appreciable amounts of any protein with a similar electrophoretic mobility to the outer membrane ferrienterochelin-colicin B receptor protein in any of the osmotic shock fluids examined by PAGE. These observations raise the possibility that other uptake systems, which are thought to have both outer membrane and periplasmic binding proteins, in reality have only the outer membrane receptor. This possibility has already been considered in the case of the uptake of vitamin B_{12} (5, 41).

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