Overexpression and Purification of Ferric Enterobactin Esterase from *Escherichia coli*

DEMONSTRATION OF ENZYMATIC HYDROLYSIS OF ENTEROBACTIN AND ITS IRON COMPLEX*

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Timothy J. Brickman† and Mark A. McIntosh§
From the Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

The *Escherichia coli* ferric enterobactin esterase gene (*fes*) was cloned into the vector pGEM3Z under the control of the T7 gene 10 promoter and overexpressed to approximately 15% of the total cellular protein. The ferric enterobactin esterase (Fes) enzyme was purified as a 43-kDa monomer by gel filtration chromatography. Purified Fes preparations were examined for esterase activity on enterobactin and its metal complexes and for iron reduction from ferric complexes of enterobactin and 1,3,5-tris(N,N',N''-2,3-dihydroxybenzoyl)aminomethylbenzene (MECAM), a structural analog lacking ester linkages. Fes effectively catalyzed the hydrolysis of both enterobactin and its ferric complex, exhibiting a 4-fold greater activity on the free ligand. It also cleaved the aluminum (III) complex at a rate similar to the ferric complex, suggesting that ester hydrolysis of the ligand backbone is independent of any reductive process associated with the bound metal. Ferrrous iron was released from the enterobactin complex at a rate similar to ligand cleavage indicating that hydrolysis and iron reduction are tightly associated. However, no detectable release of ferrous iron from the MECAM complex implies that, with these in vitro preparations, metal reduction depends upon, and is subsequent to, the esterase activity of Fes. These observations are discussed in relation to studies which show that such enterobactin analogs can supply growth-promoting iron concentrations to *E. coli*.

When *Escherichia coli* cells are exposed to limiting growth concentrations of the essential metal nutrient iron, they secrete the powerful catechol siderophore enterobactin (enterochelin) to solubilize and chelate ferric ion for subsequent active assimilation. Transport of the ferrated siderophore requires a specific outer membrane receptor, FepA (Armstrong et al., 1990), and a dedicated multicomponent permease system (Shea and McIntosh, 1991). Once the ferric enterobactin complex enters the cytoplasm of *E. coli*, iron is removed by a mechanism requiring the *fes* gene product. In early investigations into iron metabolism in *E. coli*, ferric enterobactin was shown to be a substrate for an esterase activity which hydrolyzes the ester bonds of the ligand backbone to yield the linear trimer, dimer, and monomer of 2,3-dihydroxybenzoylserine (O'Brien et al., 1971). The level of esterase activity in the cytoplasm was repressed by iron in the growth medium in parallel to the repression of the enterobactin synthetic enzymes, and therefore was proposed to be coregulated.

The necessity for hydrolytic cleavage of the siderophore complex for the release of iron was initially proposed based upon cyclic voltametric studies suggesting that enzymatic hydrolysis of ferric enterobactin to ferric complexes of 2,3-dihydroxybenzoylserine monomers was required to present an opportunity for energetically feasible metal reduction under physiological conditions (O'Brien et al., 1971). Determination of a very low redox potential (~750 mV versus the normal hydrogen electrode, pH 7.0) for ferric enterobactin (Harris et al., 1979) provided additional support for the proposal that hydrolysis may be a necessary prerequisite to the eventual *in vivo* release of iron from the siderophore complex via reduction of the ferric iron center.

Controversy regarding the precise nature and substrate specificity of *Fes* activity has prevailed despite general agreement upon the importance of a hydrolytic mechanism for iron release. Bryce and Brot (1972) reported that chromatographic fractions containing the esterase activity hydrolyze only the free ligand and have no activity on ferric enterobactin, whereas Langman et al. (1972) claimed that Fes prefers the ferric complex. Greenwood and Luke (1978) determined, however, that both the free ligand and the ferric complex are substrates for the esterase, but that free enterobactin is hydrolyzed 2.5-fold faster than ferric enterobactin. The precise reasons for these observed inconsistencies were not established with certainty but were attributed to variations in experimental procedures among groups.

The absolute requirement for ligand hydrolysis by an esterase for iron removal from the ferric enterobactin complex was questioned by Hollisfield and Neilands (1978) who demonstrated, in growth promotion studies, that a carbocyclic analog of enterobactin (cis-1,5,9-tris-(2,3-dihydroxybenzamido)cyclododecane), devoid of ester linkages and hence not susceptible to esterase activity, was as efficient as the native siderophore in supplying iron to *fes* cells, yet neither compound would support the growth of *fes* or *fep* mutants. The

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† Recipient of a Department of Health and Human Services Predoctoral Traineeship T32 AI07276. Present address: Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840.

‡ To whom correspondence should be addressed: M613 Medical Sciences Bldg, School of Medicine, University of Missouri-Columbia, Columbia, MO 65212. Tel.: 914-882-4193; Fax: 314-882-4287.

The abbreviations used are: Fes, ferric enterobactin esterase; MECAM, 1,3,5-tris(N,N',N''-2,3-dihydroxybenzoyl)aminomethylbenzene; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobase(s).

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authors suggested that fes encodes a component of the iron release mechanism (a reductase) and that hydrolysis of the labile ester bonds is only coincidentally related to the iron delivery step. A report of a ferric siderophore reductase system in cell-free extracts of the Gram-positive bacterium *Bacillus subtilis* which catalyzed the reductive, nonhydrolytic release of iron from ferric enterobactin analogs lacking ester bonds (Lodge et al., 1980) supported the proposal for existence of a widespread reductive mechanism of iron release from bacterial siderophores. In addition, a similar reductase system which reduces iron from the catechol siderophore arobutin was identified in *Agrobacterium tumefaciens* (Lodge et al., 1982).

Furthermore, Heidinger et al. (1983) reported positive growth promotion for the ferric complex of the enterobactin analogs 1,3,5-\(N,N',N''\)-Tris-(2,3-dihydroxybenzoyl)triaminomethylbenzene, 1,3,5-Tris-(2,3-dihydroxybenzoylcarbamido)benzene, and 1,5,10-\(N,N',N''\)-Tris-(5-sulfobutyrylo)triazadecane, all without ester linkages, but again cellular response was dependent upon functional fes and fep permease genes, although in this case it still occurred in mutants lacking the ferric enterobactin outer membrane receptor FepA. Presumably, these analogs are capable of supplying growth-promoting iron concentrations to *E. coli* by an unknown pathway independent of at least some component(s) of the enterobactin uptake system.

Consistent with the proposal of an alternate mechanism of iron delivery by synthetic analogs, Mössbauer spectroscopic analysis of *E. coli* cells grown in the presence of the synthetic enterobactin analog complex Fe(III) MECAM indicated that little iron is actually delivered to the cytoplasmic compartment of *E. coli* by this carrier, whereas soon after uptake the major fraction of iron delivered to the cell as ferric enterobactin appears as Fe(II) in the cytoplasm (Matzanken et al., 1986). The results implied to these authors that specific features of the uptake process for these analogs vary from those of the native siderophore, and led to the suggestion that highly sensitive growth promotion studies may not provide an accurate means of assessing substrate specificity of the enzymatic components involved in iron release from enterobactin and its analogs lacking ester linkages. Taken together, these observations supplied the impetus for the preliminary investiga-
tion of the role of Fes in the release of iron from ferric enterobactin using purified Fes in vitro.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Plasmids—*E. coli strain JM101 (supE44 thi \( \Delta \)lac-proAB) (PhraD6 supAB lacZV22ΔM13) (Norwood et al., 1983) was used as the host for all plasmid construction procedures. The recombinant fes plasmid pITS311 was described previously (Petits and McIntosh, 1987). *E. coli* strain PAM163 (pGPI-1, pITS313) was grown in T broth containing ampicillin and kanamycin at 50 \( \mu \)g/ml, shaken at 30 °C until the A \(_{650}\) reached 1.5, at which time the temperature was rapidly shifted to 42 °C and held for 30 min. Rifampicin was then added to 100 \( \mu \)g/ml final concentration and the cells maintained at 37 °C for 2 h. Cells were harvested by centrifugation, washed with cold 0.9% NaCl, resuspended in 3 ml/wet g cells in cold 100 mM Tris-CHCl, pH 8.0, containing 5 mM dithiothreitol, and lysed by sonication at 0 °C using a Sonicator Cell Disrupter (Heat Systems-Ultronics, Inc., model W225R, Plainview, Long Island, NY) fitted with the microtip for 5 min at 50% output. Unbroken cells were removed by centrifugation at 10,000 \( \times \) \( g \), 4 °C, for 15 min. The sucrose fraction of the sonicate lysate was removed by further centrifugation (100,000 \( \times \) \( g \), 4 °C, 1 h), and nucleic acids were precipitated by the dropwise addition, with gentle stirring, of one-fourth volume of cold 1% protamine sulfate in 100 mM Tris-CHCl, pH 8.0, 5 mM dithiothreitol. Nucleic acid precipitate was removed by centrifugation at 10,000 \( \times \) \( g \), 4 °C, for 15 min to yield crude extract.

The crude extract, at 6 °C, was brought to 50% saturation with ammonium sulfate by the slow addition, with gentle stirring, of cold saturated ammonium sulfate solution. The precipitate containing Fes was collected by centrifugation at 10,000 \( \times \) \( g \), 4 °C, for 10 min, and dissolved in 4 ml/wet g precipitate in 100 mM Tris-CHCl, pH 8.0, 5 mM dithiothreitol.

The protein fraction obtained after ammonium sulfate fractionation was applied to a 9.5-2.0 cm column of Sephacryl S-200 HCl, pH 8.0, 5 mM dithiothreitol, and eluted with the same buffer at a flow rate of 20 ml/h, collecting 5-ml fractions. Elution was monitored at 290-nm wavelength using an ISCO absorbance/fluorescence detector model UA-5 (ISCO, Inc., Lincoln, NE).

A single peak emerging at an elution volume corresponding to a species of estimated molecular mass of 42-45 kDa (based on calibration with protein standards: bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa; and RNase, 13.7 kDa) was found to catalyze the hydrolysis of ferric enterobactin.

**Analysis of Proteins—**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% polyacrylamide slab gels as described (Laemmli, 1970). Molecular weight standards were from Bio-Rad. Protein concentrations of Fes samples were determined using the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard.

**Ferric Enterobactin Esterase Activity**—Ferric enterobactin and its ferric complex was assayed by a modification of a previously described method (Greenwood and Luke, 1978). The assay mixture contained 0.75 mM enterobactin or the iron (III) or aluminum (III) complex, 100 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, and 50 \( \mu \)l of S-200 column peak fraction in 100 \( \mu \)l of total reaction volume. After incubation at 37 °C for 15 min in the case of ferric enterobactin substrate, the mixture was acidified by the addition of 33 \( \mu \)l of 1 M HC1, and extracted into 750 \( \mu \)l of ethyl acetate. The resulting emulsion was allowed to equilibrate
for 20 min at room temperature, then the mixture was spun briefly in a microcentrifuge, 600 µl of the organic phase was removed, and counterextracted into 600 µl of 0.1 M sodium phosphate buffer, pH 7.2, to quantitatively extract enterobactin hydrolysis products. Levels of total hydrolysis products were measured spectrophotometrically at 316-nm wavelength and quantitated based upon a millimolar extinction coefficient of enterobactin of 9.39 at 316 nm (O'Brien and Gibson, 1970), and esterase activity was expressed in units of nanomoles of enterobactin hydrolyzed h⁻¹ mg⁻¹. The degree of ligand hydrolysis (that is, the relative quantities of hydrolyzed linear trimers, dimers, and monomers of 2,3-dihydroxybenzoylserylserine produced) by Fes was not determined in esterase assays. Under similar conditions, Langman et al. (1972) reported a yield of an approximately 4:1 mixture of the monomeric and dimeric forms of 2,3-dihydroxybenzoylserylserine.

To monitor the appearance of free ferrous iron associated with Fes activity, a modification of the method of Lodge et al. (1982) used to examine iron reduction in the A. tumefaciens siderophore agaractin, was employed. Assays were not performed anaerobically. Reaction mixtures contained 0.16 mM ferric enterobactin or ferric MECAM, 0.16 mM NADH, 0.015 mM FMN, 1 mM MgCl₂, 100 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol and 0.80 mM ferrozine and 50 µl of S-200 column peak fractions in 4.50 mM of the organic phase was removed, and counterextracted into BOO dimers, and monomers of 2,3-dihydroxybenzoylserylserine produced) by Fes was not determined in esterase assays. Under similar conditions, Langman et al. (1972) reported a yield of an approximately 4:1 mixture of the monomeric and dimeric forms of 2,3-dihydroxybenzoylserylserine.

RESULTS

Construction and Analysis of fes Plasmids—The recombinant plasmid pITS311 (Pettis and McIntosh, 1987), which carries a 1.8-kilobase (kb) HpaI chromosomal DNA fragment including the fes gene in the Smal cloning site of the lac reporter plasmid pMC1403 (Casadaban et al., 1980), was digested with EcoRI and BamHI, which flank the Smal-cloning site in the pMC1403 polyclinker, and the insert fragment was purified from low melting temperature agarose. The purified fragment was further digested with SspI (which cuts at a site within the -10 region of the fes promoter), and the 1.4-kb SspI-BamHI DNA fragment was recovered from low melting temperature agarose and ligated with Smal-BamHI-digested pGEM3Z plasmid vector DNA to produce pITS513 (Fig. 1). This plasmid carries the entire fes-coding region and the 5'-coding region of entF, oriented in pGEM3Z to allow transcription of fes from the vector-supplied T7 bacteriophage promoter in the host strain PAM163 (pGP1-2). In this context, translation of the bacteriophage T7 promoter-directed fes transcript is controlled by the native fes translational signals (Pettis et al., 1989).

Genetic Complementation of a fes Mutation—When the fes mutant strain MM272-60 was transformed to ampicillin resistance with pITS513 growth on nutrient agar plates containing 100 µM dipyridyl was fully restored. The MM272-60 fes lesion (in the promoter-proximal fes cistron of the polycistronic fes-entF transcript) exerts no detectable polar effect upon expression of the downstream entF gene, thus fes complementation by the recombinant plasmid pITS513 results in wild-type growth on this low iron medium. Obviously, low level transcription of fes in this multicopy plasmid construct (without induction of transcription from the vector-supplied T7 promoter) is sufficient for full complementation despite the absence of much of the native fes control sequences which map upstream of the SspI restriction site used to construct pITS513 (Pettis et al., 1989). The pink-colored colony phenotype characteristic of ferric enterobactin accumulation in fes mutants was also eliminated by the introduction of pITS513 into MM272-60.

In Vivo Labeling of pITS513-encoded Fes Polypeptide—In vivo Fes labeling experiments using the procedure of Tabor and Richardson (1985) to exclusively radiolabel pITS513-encoded polypeptides revealed a product (Fig. 2) with an apparent size of approximately 43 kDa, corresponding to the known size of the Fes polypeptide (Fleming et al., 1985; Pettis et al., 1989). This product was not expressed from vector control cultures (data not shown) and was observed under inducing growth conditions (lane B) and when rifampicin-resistant T7 RNA polymerase-directed transcription of the fes-coding sequence from the vector T7 bacteriophage promoter (lane C) resulted in exclusive expression of Fes.

Overexpression and Purification of Fes—With the overproducing strain PAM163 (pGP1-2, pITS513), the fes gene product constitutes approximately 15% of total cellular protein as judged by SDS-PAGE and Coomassie Blue staining (data not shown). The purification of Fes was accomplished by a simple three-step procedure involving production of a crude extract from induced cultures of PAM163 (pGP1-2, pITS513), followed by ammonium sulfate fractionation and gel filtration chromatography. When the Fes-containing protein fraction resulting from ammonium sulfate fractionation was separated by gel filtration chromatography on Sephacryl S-200, a single peak of ferric enterobactin esterase activity (assayed as described under "Experimental Procedures") was detected (Fig. 3A), emerging at an elution volume corresponding to a native tronic fes-entF transcript was constructed as described in the text and includes the entire fes coding region on a 1.4-kb SspI-BamHI DNA fragment from the recombinant plasmid pITS311 (Pettis and McIntosh, 1987), oriented to allow high level inducible transcription of fes from the plasmid vector T7 gene 10 bacteriophage promoter in the E. coli strain PAM163 (pGP1-2, pITS513).

Fig. 1. The Fes expression plasmid pITS513. The expression plasmid pITS513 was constructed as described in the text and includes the entire fes coding region on a 1.4-kb SspI-BamHI DNA fragment from the recombinant plasmid pITS311 (Pettis and McIntosh, 1987), oriented to allow high level inducible transcription of fes from the plasmid vector T7 gene 10 bacteriophage promoter in the E. coli strain PAM163 (pGP1-2, pITS513).

Fig. 2. In vivo labeling of pITS513-encoded Fes polypeptide. Fes was labeled in vivo in E. coli strain PAM163 (pGP1-2, pITS513) using TRAN'S-LABEL (ICN Biomedicals, Inc., Costa Mesa, CA) by the exclusive labeling procedure of Tabor and Richardson (1985). Lanes: A, noninducing growth conditions; B, inducing growth conditions; C, inducing growth conditions in the presence of the E. coli RNA polymerase inhibitor rifampicin. Positions of molecular mass markers are given on the left in kDa.
molecular mass of approximately 43 kDa, indicating a monomeric structure for the enzyme. Examination of S-200 column fractions displaying peak esterase activities by SDS-PAGE (Fig. 3B, fractions 46-50), revealed the corresponding predominant polypeptide (Fes) of approximately 43 kDa. Fractions 48 and 49 (Fig. 3B) were pooled, yielding approximately 16 mg of highly purified Fes from 1 liter of culture. SDS-PAGE of the resulting pool is shown in Fig. 4.

**Examination of Fes Activity**—Purified Fes preparations were examined for esterase activity on enterobactin and metal complexes of enterobactin and for activity resulting in out-transfer of ferrous iron from ferric complexes of enterobactin and a structural analog of enterobactin lacking ester linkages (Fe(III)-MECAM), as described under “Experimental Procedures.” Specific activities on these substrates are summarized, as follows. In esterase assays, purified Fes effectively catalyzed the hydrolysis of ferric enterobactin, hydrolyzing 303 nmol of Fe(III)-enterobactin h⁻¹ mg⁻¹. Consistent with the findings of Greenwood and Luke (1978), Fes was more active on free enterobactin, hydrolyzing 1280 nmol of enterobactin h⁻¹ mg⁻¹, approximately 4-fold greater than with the ferrated ligand, indicating that Fes-mediated hydrolysis of the siderophore-ester bonds is independent of any reductive step associated with iron removal from the ferric complex. In addition, the non-reducible Al(III) complex was cleaved by Fes at a rate similar to that of the ferric complex (approximately 80%), resulting in the hydrolysis of 238 nmol of Al(III)-enterobactin h⁻¹ mg⁻¹. This result suggests that reduction of the metal center of the siderophore complex is not an absolute requirement for ester bond cleavage and supports the assertion that Fes exerts a distinct esterase activity on enterobactin and its metal complexes.

In ferrozine assays monitoring the appearance of ferrous iron associated with Fes activity, 354 nmol of ferrous ferrozine was produced h⁻¹ mg⁻¹ from ferric enterobactin substrate, a value in close agreement with the nearly equimolar amount of ferric enterobactin hydrolyzed by Fes enzyme preparations in esterase assays (303 nmol of Fe(III)-enterobactin h⁻¹ mg⁻¹). However, no detectable release of ferrous iron from the Fe(III)-MECAM complex was observed at this pH, suggesting that the appearance of ferrous iron depends upon, and is subsequent to, the esterase activity of Fes on the iron-siderophore complex.

**DISCUSSION**

Despite considerable past efforts to characterize the role of the fes gene product in iron removal from the ferric enterobactin complex, the precise nature and substrate specificity of Fes activity remained controversial. To analyze the catalytic function of Fes, we achieved high level conditional overexpression of Fes using a T7 promoter-polymerase system and examined Fes activity in vitro using highly purified enzyme preparations.

In this study, we have demonstrated that purified Fes catalyzes the hydrolysis of free enterobactin (at a rate 4-fold greater than the ferric complex) indicating that Fes possesses a distinct esterase activity that is independent of any potential reductive mechanism of out-transfer of iron from the ferric enterobactin complex. These data are moderately consistent with the relative rates reported by Greenwood and Luke (1978) for the hydrolysis of enterobactin and its ferric complex by a single component esterase, formerly the so-called B component of Fes (O'Brien et al., 1971). The observed difference in hydrolytic activities of ferric enterobactin esterase on enterobactin and the ferric complex may reflect an inhibitory effect of a metal near the site of ester cleavage, as was suggested by Emery (1976) for esterase activity in the fungal fusarinine system. The biological relevance of Fes activity on the deferrisiderophore is presently obscure since Fes is local-
ized to the cytoplasm of *E. coli* and is presumably compartmentalized from the site of the final stages of siderophore assembly which are suggested to be membrane-associated through EntD-mediated linkage of enterobactin synthetase to the cytoplasmic membrane (Armstrong et al., 1989). However, this Fes activity would clearly be beneficial for hydrolysis of any unwanted intracellular enterobactin.

Fes was also capable of hydrolyzing the non-reducible aluminum (III) complex of enterobactin at a rate similar to that of the ferric complex, demonstrating that reduction of the metal center of the complex is not a necessary prerequisite for enzymatic hydrolysis of the ligand by Fes under the conditions examined.

Furthermore, in assays using the ferrous indicator ferrozine at pH 8.0, purified Fes exhibited activity on the ferric enterobactin complex resulting in production of ferrous iron, yet was incapable of iron removal from the ferric MECAM complex, which is devoid of ester linkages, indicating that Fes-mediated hydrolytic cleavage of the ester backbone is a likely requirement for subsequent removal of iron from the siderophore complex with the associated appearance of the ferrous species. It is not known whether reduction of the ferric center of the enterobactin complex subsequent to hydrolysis is specifically catalyzed by Fes. The requirement for NADH for the appearance of ferrous iron in ferrozine assays was not examined in this study. However, the sensitivity of ferric enterobactin esterase activity to inactivation by N-ethylmaleimide, and its stabilization by dithiothreitol suggests the involvement of an essential thiol group for enzymatic activity (Greenwood and Luke, 1978). This thiol group is likely to participate in a hydrolysis-associated metal reduction step.

The data presented in this report clearly establish that Fes exhibits esterase activity *in vitro* on enterobactin and its metallated derivatives and support the previous suggestion that hydrolysis of the ester backbone precedes the iron reduction step in iron delivery to *E. coli* via enterobactin. The results also indicate that the biological removal of iron from enterobactin analogs like MECAM occurs under different physiological conditions than those required for enterobactin itself although several common functions are incorporated in both processes. One potential variable factor is the pH of *E. coli* cellular compartments (Matzkanke et al., 1986). Binding and competition experiments (Ecker et al., 1986) indicated that ferrated enterobactin and MECAM efficiently utilize the same receptor protein, FepA, to gain access to the cellular periplasmic space, where they encounter a pH near 6.0 expected to protonate the catecholate groups of MECAM but not enterobactin. This difference was suggested to result in discrimination during the inner membrane recognition process allowing only the unprotonated iron catechol complex efficient entry to the cytoplasmic compartment (Matzkanke et al., 1986). As a result, metabolism of enterobactin resulted in the appearance of intracellular Fe(II) species detected by Mössbauer spectroscopy, and the majority of iron delivered via this process could not be released by osmotic shock. MECAM on the other hand was unable to deliver its iron as these intracellular Fe(II) species at detectable levels within the time frame of the experiments. Thus, it was concluded that pH-dependent reduction of the Fe(III)-MECAM complex in the periplasm is not sufficient to enable its delivery of growth-promoting iron concentrations and that nonspecifically metallized MECAM complex provides enough iron for normal growth of *E. coli*.

However, this conclusion is not entirely consistent with previous growth promotion studies that showed a specific requirement for both Fes and the inner membrane Fep per-
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