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Ferrochelatase activity in *Azospirillum brasilense* with reference to the influence of metal cations

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Summary. Ferrochelatase in membrane preparations from *Azospirillum brasilense* displayed an activity of 2.17 μmol protoheme formed·h^{-1}·mg protein^{-1} which is 10-fold greater than previous reports for other bacteria. This ferrochelatase showed an apparent K_m of 20.9 μM for Fe^{2+}, a pH optimum of 6.0–6.5, and stimulation by oleic or stearic acids. Co^{2+}, Cu^{2+} and Zn^{2+} inhibited the incorporation of Fe^{2+} into protoporphyrin IX while Ni^{2+} and Mg^{2+} had no effect on protoheme synthesis. Activity with Fe^{2+} and mesoporphyrin IX was less than with protoporphyrin IX but deuteroporphyrin IX produced the highest rate of protoheme synthesis. The membrane fraction containing ferrochelatase activity was found to insert Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} enzymatically into protoporphyrin IX to produce metalloporphyrins. Cu^{2+} incorporation into protoporphyrin IX proceeded at a rate greater than with Fe^{2+} and the K_m for Cu^{2+} was 21.9 μM.

Key words: Heme synthesis — Iron metabolism — Membranes — Cation inhibition — Metalloporphyrin

Introduction

In the last stage of heme biosynthesis, ferrous iron is inserted into the protoporphyrin ring by an enzyme which has been referred to as heme synthase (Porra and Ross 1965), heme synthetase (Riethmueller and Tuppy 1964), iron-protoporphyrin chelating enzyme (Labbe and Hubbard 1961), ferrochelatase (Porra and Jones 1963), and protoheme ferrolyase, EC 4.99.1.1. From the review of earlier literature by Kassner and Walchak (1973) it was apparent that the acceptance of the role of ferrochelatase in heme synthesis was relatively recent because iron and porphyrin had long been known to combine to produce heme under conditions where no protein was present. Ferrochelatase has been reported in chloroplasts (Goldin and Little 1969), inner mitochondrial membranes (Jones and Jones 1969) and in cytoplasmic membranes of photosynthetic and aerobic bacteria (Dailey and Lascelles 1974). In addition to the recognition of Fe^{2+}, ferrochelatases have been reported to catalyze the insertion of various heavy metal cations into the protoporphyrin molecule (Labbe and Hubbard 1961; Dailey and Lascelles 1974; Dailey 1982; Dailey 1977; Jones and Jones 1970; Taketani and Tokunaga 1981; Wagner and Tephly 1975; Goldin and Little 1969; Porra and Jones 1963; Bugany et al. 1971). Also, attention has been focused on the role of Cu^{2+} in regulation of ferrochelatase activity (Wagner and Tephly 1975). With this diversity of interactions between metal cations and ferrochelatase, it is apparent that additional research is needed to gain a better insight into this important step in heme biosynthesis.

Azospirillum brasilense was selected for this study because, as a microaerophilic, nitrogen-fixing bacterium, it represented a unique physiological group. We report here that ferrochelatase activity in *A. brasilense* was clearly inhibited by Co^{2+}, Cu^{2+}, and Zn^{2+}. Additionally, the catalytic insertion of Cu^{2+} into protoporphyrin with a membrane preparation from *A. brasilense* occurred at a rate comparable to synthesis of heme.

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Materials and methods

Cultures. Cultures of *A. brasilense* ATCC 29145 were maintained as previously described (Shagam et al. 1988) in a medium containing: 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.001% $FeCl_3 \cdot 6H_2O$, 0.0002% $NaMoO_4 \cdot 2H_2O$, 0.5% sodium malate, and 0.005% yeast extract (Difco Laboratories, Detroit, MI) with the pH adjusted to 7.4. The cultures were incubated in 12-l bottles at 25° C for 5 days. The bacterial cells were harvested by centrifugation and were stored for up to two months at −20° C without loss of activity.

Membrane preparation. Cells were suspended in 0.01 M Tris/HCl, pH 7.5 (1 g cells/10 ml buffer) and were disrupted by a single passage through the French pressure cell at 10000 *g* for 10 min to remove whole cells and debris. Membranes were separated from the above soluble extract by ultracentrifugation at 35000 *g* for 90 min and were resuspended in 0.01 M Tris/HCl, pH 7.5. Protein was determined by the Biuret method (Gornall et al. 1949) using bovine serum albumin fraction V as the protein standard.

Ferrochelatase assay. Aqueous solutions of protoporphyrin IX, mesoporphyrin IX, and deuteroporphyrin IX (Porphyrin Products, Logan, UT) were stored at −20° C in the dark until used. Solutions of $FeSO_4$ and other heavy metal salts were freshly prepared for each experiment. The standard assay mixture for ferrochelatase contained 66.7 μM protoporphyrin IX, 1 μM dithiothreitol, 0.1 μM $FeSO_4$ and 5 mg membrane protein in a final volume of 1.5 ml. Changes in the ferrochelatase assay are discussed in specific experiments. In experiments employing ^{59}Fe, this radioactive iron was purchased from ICN Radiochemicals (Irvine, CA) as ferrous sulfate (11.2 Ci/g Fe) in 0.05 M H_2SO_4. Measurements of ^{59}Fe were made with a Nuclear of Chicago gamma counter. The reaction was incubated under N_2 in a 37° C water bath in the dark for 1 h and terminated by addition of cold acidified acetone (1% concentrated HCl in acetone). Ferrochelatase was assayed using a technique described by Jacobs and Jacobs (1976). The reactions were stirred on ice for 1 h in the dark to extract quantitatively any protoheme bound to the membrane. Following centrifugation at 6000 *g* for 10 min, the supernatant was removed and to it was added 3 ml cold 1% HCl in ether. The protoheme present was extracted into the ether layer. This ether layer was extracted four times with 3 vol. cold 1.5 M HCl. The amount of protoheme present was determined by evaporating the ether layer to dryness under a stream of N_2 gas, followed by addition of 0.78 ml cold 1% NaOH and 0.22 ml pyridine. The resulting pyridine hemochromagen was reduced with 0.1 mg sodium hydrosulfite and scanned continuously over 600–450 nm on Varion Cary 219 spectrophotometer. The formation of protoheme, deuteroheme and mesoheme was measured by using the absorption coefficients of 34.4, 26.4 and 35.8 $\mu M^{-1}\ cm^{-1}$ respectively (Falk 1964).

Results and discussion

Membrane preparations of *A. brasilense* catalyzed an increase in protoheme production with an increase in incubation time (Table 1). This activity has been corrected for nonenzymatic activity by conducting parallel assays for corresponding periods of incubation using boiled membranes to substitute for the enzyme fraction. Although much has been written about the nonenzymatic formation of protoheme from protoporphyrin and Fe^{2+} (Falk 1964; Kassner and Walchak 1973), less than 0.005 μmol protoheme was produced in 1 h in the absence of active enzyme using the conditions described. In a related experiment, the quantity of membrane in the reaction was varied and the resulting level of activity was proportional to membrane protein added. A requirement for dithiothreitol was shown for this ferrochelatase reaction since reactions lacking it displayed only a tenth the reactivity; this was consistent with other reports (Labbe and Hubbard 1961; Porra et al. 1967).

Table 1. Ferrochelatase activity in reactions incubated for increasing periods of time

Incubation time (h)	Protoheme produced (μmol·h^{-1}·mg protein^{-1})
0	0
0.5	0.36±0.1
1.0	2.21±0.3
1.5	2.63±0.5
2.0	4.50±0.6

Corrections were made for nonenzymatic heme synthesis by subtracting the activity obtained from the reaction containing boiled membrane fraction from the test reaction containing native membranes. Conditions of the reaction were as presented in the Materials and methods

With respect to preference for protoporphyrin, the ferrochelatase from *A. brasilense* displayed greatest activity with deuteroporphyrin IX, lower levels of activity being observed with protoporphyrin IX and mesoporphyrin IX (Table 2). The preference for protoporphyrin IX over mesoporphyrin differs from other reports (Dailey 1982; Dailey and Lascelles 1974; Taketani and Tokunaga 1981); however, we found the observation to be reproducible in our system. Although the activ-

Table 2. Specificity of ferrochelatase from *A. brasilense* for porphyrins

Porphyrin added	Protoheme produced (μmol·h^{-1}·mg protein^{-1})
Mesoporphyrin IX	1.53±0.3
Protoporphyrin IX	2.42±0.2
Deuteroporphyrin IX	4.38±0.7

The 1.5-ml reaction contained 66.7 μM porphyrin and components as indicated in Materials and methods. Values given are an average of three experiments with the range of activities as indicated

ity with deuteroporphyrin IX was slightly greater than with protoporphyrin, we employed protoporphyrin IX in future experiments because it is the physiologically important porphyrin in protoheme synthesis. The optimal pH for the ferrochelatase activity in *A. brasilense* with 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol (Bistris) and 1,3-[tris(hydroxymethyl)methylamino]propane (Bistris-propane) buffers was 6.0 and 6.5, respectively (Fig. 1). Of significance was the low activity at pH 8 with the *A. brasilense* preparation because the pH optimum for nonenzymatic formation of protoheme was reported to be at pH 8 (Kassner and Walchak 1973).

In addition to Fe^{2+}, the ferrochelatase from *A. brasilense* was able to utilize various divalent cations as substrates. (This data is presented as absorbance change because the absorption coefficients of the metalloporphyrins are not available; Table 3.) Due to the close proximity of the spectral peaks of the various individual metalloporphyrin species (Fig. 2), the mixed activities of Fe^{2+} and another metal cation in the synthesis of metalloporphyrin requires measurements other than with visible spectra. A useful expression of activities catalyzed by ferrochelatase is presented in Fig. 3 where corrections have been made for nonenzymatic synthesis of metalloporphyrin. Ferrochelatase activity with Cu^{2+} was very high while activity with Co^{2+}, Ni^{2+}, Mg^{2+}, and Zn^{2+} was less than with Fe^{2+}.

When these cations were evaluated as potential inhibitors to Fe^{2+} in the ferrochelatase assay, inhibition was displayed by Cu^{2+}, Co^{2+} and Zn^{2+} (Table 4). Cations which had no effect on the catalytic incorporation of $^{59}Fe^{2+}$ into protoheme included Ni^{2+} and Mg^{2+}. This role of Cu^{2+} as inhibitor is consistent with the observations by Jones and Jones (1970), Dailey and Lascelles (1974), Taketani and Tokunaga (1981), Dailey (1982), but is in contrast to the stimulation re-

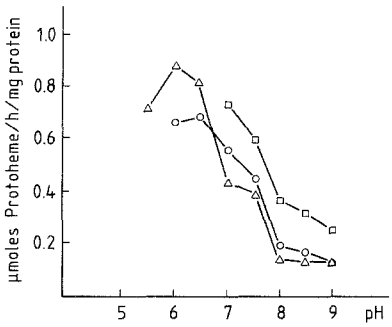

Fig. 1. Effect of pH on ferrochelatase activity. Reaction mixtures contained 66.7 µM protoporphyrin, 1 µM dithiothreitol, 0.1 µM $FeSO_4$, 6 mg membrane protein and Tris/HCl (□), Bistris/HCl (△) or Bistris-propane/HCl (○) at 66.7 µM

Table 3. Enzymatic incorporation of metal cations into protoporphyrin

Metal salts added	Wavelength at which absorbance was recorded (nm)	Metal cation incorporation ($\Delta A \cdot h^{-1} \cdot$ mg protein^{-1})
$CuCl_2$	570	0.021
$FeSO_4$	557	0.014
$NiSO_4$	557	0.008
$ZnSO_4$	557	0.005
$CoCl_2$	557	0.003
$MgCl_2$	557	0

Additions of metal salts were made to give a final concentration of 12 µM for each cation. The activity for Fe^{2+} was 0.7 µmol protoheme produced $\cdot h^{-1} \cdot$ mg protein^{-1}. Values of metal cations incorporated were obtained by subtraction of controls containing boiled membranes from the test reaction

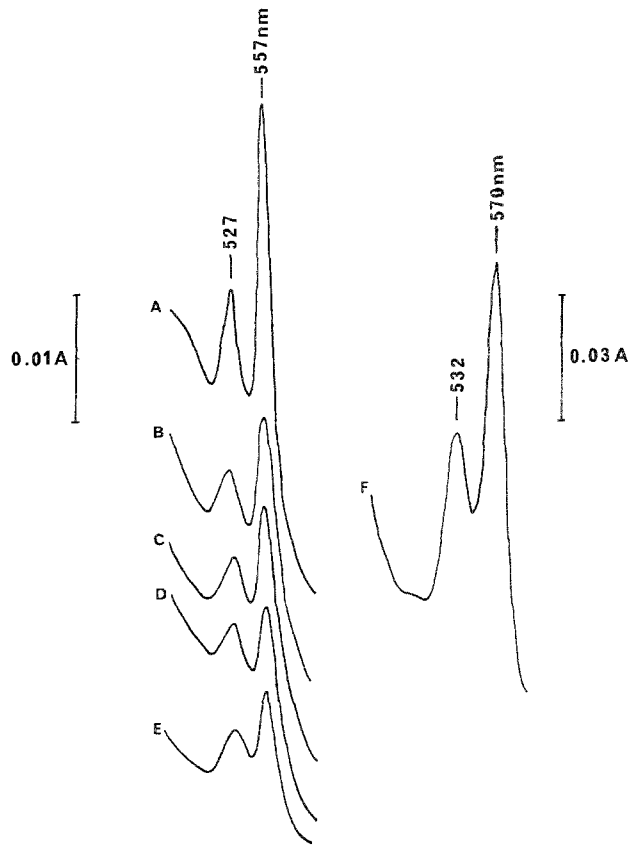

Fig. 2A–F. Characteristic spectrophotometric scans of metalloporphyrins. Reactions contained 66.7 µM Tris/HCl, pH 7.5, 66.7 µM protoporphyrin, 6 mg membrane protein, 1 µM dithiothreitol, and 12 µM of (A) $FeSO_4$, (B) $NiSO_4$, (C) $CoCl_2$, (D) $ZnSO_4$, (E) $MgCl_2$, and (F) $CuCl_2$. Alkaline pyridine hemochromagens were reduced with sodium hydrosulfite

Table 4. Inhibition of ferrochelatase by metal cations

Metal salts added with $FeSO_4$	Ferrochelatase activity (%)
None	100
$MgCl_2$	151
$NiSO_4$	114
$CoCl_2$	8
$CuCl_2$	7
$ZnSO_4$	1

Fe^{2+} added was 0.6 μM and other cations were added to a final concentration of 120 μM. Ferrochelatase 100% activity was the production of 0.18 μmol protoheme·h^{-1}·mg $protein^{-1}$

ported by Dailey (1977) and Wagner and Tephy (1975).

Examination of affinities of ferrochelatase from *A. brasilense* was conducted through kinetic evaluations. The Michaelis-Menton expression of Fe^{2+} (Fig. 3) and Cu^{2+} (Fig. 4) were used to calculate the apparent K_m of 20.1 μM for Fe^{2+} and 21.9 μM for Cu^{2+}. This affinity for Fe^{2+} is in excellent agreement with the apparent K_m of 20 μM for *Spirillum itersonii* (Dailey 1977; Dailey and

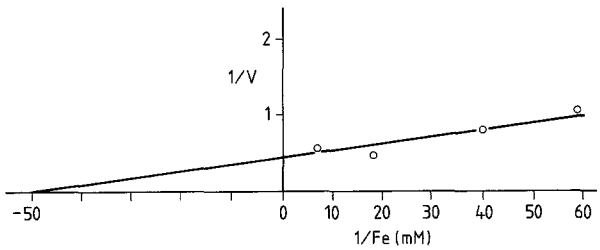

Fig 3. Double-reciprocal plot of ferrochelatase activity at different levels of Fe^{2+}. Reactions contained 66.7 μM Tris/HCl, pH 7.5, 66.7 μM protoporphyrin, 5 mg membrane protein, 1 μM dithiothreitol, and $FeSO_4$ at the concentrations indicated

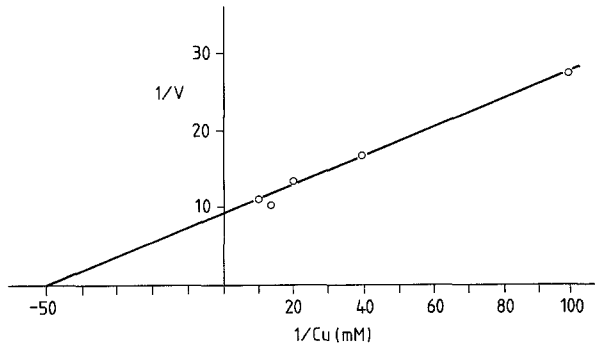

Fig. 4. Double-reciprocal plot of ferrochelatase activity at different levels of Fe^{2+}. Reactions were as described for Fig. 4 except that $CuCl_2$ was substituted for iron

Table 5. Stimulation of ferrochelatase activity by fatty acids

Addition to the reaction Acid	amount (mg)	Protoheme produced (%)
None	-	100
Oleic		
	0.5	226
	1.0	218
	1.5	144
Stearic		
	0.5	175
	1.0	175
	1.5	192
Pimelic		
	0.5	118
	1.0	87
	1.5	85

Oleic and stearic acids were dissolved in ethanol and a volume of 10 μl was added to respective assay mixtures. Pimelic acid was dissolved in water. The activity of the control (100%) was 2.12 μmol protoheme·h^{-1}·mg $protein^{-1}$

Lascelles 1974) and 22 μM for *Rhodospirillum spheroides* (Dailey 1982).

Another characteristic of ferrochelatase examined was the stimulation by fatty acids. With the addition of 0.5 mg oleic or stearic acid, ferrochelatase activity was stimulated; however, with the addition of pimelic acid, no increase in enzyme activity was seen (Table 5). The stimulation of ferrochelatase in the membranes from *A. brasilense* by lipids is consistent with reports on other biological systems (Taketani and Tokunaga 1981; Wagner and Tephly 1975; Sawada et al. 1969). It should be noted that fatty acids did not stimulate ferrochelatase purified from *S. itersonii* (Dailey 1977) or from *R. spheroides* (Dailey 1982).

The markedly high activity of ferrochelatase in membranes from *A. brasilense* is significant because it represents the most active system reported. The amount of protoheme synthesis reported here with *A. brasilense* was 2.17 μmol protoheme·h^{-1}·mg $protein^{-1}$ while *R. sphaeroides* the activity was 0.29 μmol protoheme·h^{-1}·mg $protein^{-1}$ (Jones and Jones 1970) and for *S. itersonii* it was 0.006 μmol protoheme·h^{-1}·mg $protein^{-1}$ (Dailey and Lascelles 1974). The rate of protoheme syntheses with 0.5 mg oleic acid added to membranes from *A. brasilense* was 4.8 μmol·h^{-1}·mg $protein^{-1}$ which is greater than the 3.5 μmol·h^{-1}·mg $protein^{-1}$ reported with the purified ferrochelatase from rat liver mitochondria (Taketani and Tokunaga 1981). While the reason for the elevated level of ferrochelatase in *A. brasilense* is unclear at this time, the organism may

prove to be extremely useful for studying heme biosynthesis.

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References

Bugany H, Flohe L, Weser U (1971) Kinetics of metal chelatase from rat liver mitochondria. FEBS Lett 13:92–94

Dailey HA (1977) Purification and characterization of membrane bound ferrochelatase from *Spirillum itersonii*. J Bacteriol 132:302–307

Dailey HA (1982) Purification and characterization of membrane bound ferrochelatase from *Rhodopseudomonas spheroides*. J Biol Chem 257:14714–14718

Dailey HA, Lascelles J (1974) Ferrochelatase activity in wild type and mutant strains of *Spirillum itersonii*. Arch Biochem Biophys 160:523–529

Falk JE (1964) Porphyrins and metalloporphyrins. Elsevier Publishing Co., Amsterdam

Goldin BR, Little HN (1969) Metalloporphyrin chelatase from barley. Biochim Biophys Acta 171:321–332

Gornall GG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751–766

Jacobs NJ, Jacobs JM (1976) Nitrate, fumarate, and oxygen as electron acceptors for a late step in microbial heme synthesis. Biochim Biophys Acta 449:1–9

Jones MS, Jones OTG (1970) Ferrochelatase of *Rhodopseudomonas spheroides*. Biochem J 119:453–462

Jones MS, Jones OTG (1969) The structural organization of haem synthesis in rat liver mitochondria. Biochem J 113:507–514

Kassner RJ, Walchak DH (1973) Heme formation from Fe(II) and porphyrin in the absence of ferrochelatase activity. Biochim Biophys Acta 304:294–303

Labbe RF, Hubbard N (1961) Metal specificity of the iron-protoporphyrin chelating enzyme from rat liver. Biochim Biophys Acta 52:130–135

Porra RJ, Jones OTG (1963) Studies on ferrochelatase 1. Assay and properties of ferrochelatase from a pig liver mitochondrial extract. Biochem J 87:181–185

Porra RJ, Ross BD (1965) Heme synthase and cobalt porphyrin synthase in various micro-organisms. Biochem J 94:557–562

Porra RT, Vitolos K, Labbe RF, Newton NA (1967) Studies on ferrochelatase. The effects of thiols and other factors on the determination of activity. Biochem J 104:321–327

Riethmueller G, Tuppy H (1964) Hämsynthetase (Ferrochelatase) in *Saccharomyces cerevisiae* nach aerobem und anaerobem Wachstum. Biochem Z 340:413–420

Sawada H, Takeshita M, Sugita Y, Yoneyama Y (1969) Effect of lipid on protoheme ferro-lyase. Biochim Biophys Acta 178:145–155

Shagam JY, Barton LL, Reed WP, Chiovetti R (1988) Fluorescein isothiocyanate-labeled lectin analysis of the surface of the nitrogen-fixing bacterium *Azospirillum brasilense* by flow cytometry. Appl Environ Microbiol 54:1831–1837

Taketani S, Tokunaga R (1981) Rat liver ferrochelatase. Purification, properties and stimulation by fatty acids. J Biol Chem 256:12748–12753

Wagner GS, Tephly TR (1975) A possible role of copper in the regulation of heme biosynthesis through ferrochelatase. Adv Exp Med Biol 58:343–354

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