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Ferrochelatase from *Rhodopseudomonas sphaeroides*: Substrate Specificity and Role of Sulfhydryl and Arginyl Residues

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Purified ferrochelatase (protoheme ferrolyase; EC 4.99.1.1) from the bacterium *Rhodopseudomonas sphaeroides* was examined to determine the roles of cationic and sulfhydryl residues in substrate binding. Reaction of the enzyme sulfhydryl residues with *N*-ethylmaleimide or monobromobimane resulted in a rapid loss of enzyme activity. Ferrous iron, but not porphyrin substrate, had a protective effect against inactivation by these two reagents. Quantitation with ^{3}H-labeled *N*-ethylmaleimide revealed that inactivation required one to two sulfhydryl groups to be modified. Modification of arginyl residues with either 2,3-butanedione or camphorquinone 10-sulfonate resulted in a loss of ferrochelatase activity. A kinetic analysis of the modified enzyme showed that the K_m for ferrous iron was not altered but that the K_m for the porphyrin substrate was increased. These data suggested that arginyl residues may be involved in porphyrin binding, possibly via charge pair interactions between the arginyl residue and the anionic porphyrin propionate side chain. Modification of lysyl residues had no effect on enzyme activity. We also examined the ability of bacterial ferrochelatase to use various 2,4-disubstituted porphyrins as substrates. We found that 2,4-bis-acetal- and 2,4-disulfonate deuteroporphyrins were effective substrates for the purified bacterial enzyme and that *N*-methylprotoporphyrin was an effective inhibitor of the enzyme. Our data for the ferrochelatase of *R. sphaeroides* are compared with previously published data for the eucaryotic enzyme.

The tetrapyrrole biosynthetic pathway in the facultative photosynthetic bacterium *Rhodopseudomonas sphaeroides* has been extensively studied (9). Part of the interest in this organism is due to its ability to dramatically induce porphyrin production for bacteriochlorophyll without a parallel increase in the production of heme (15). To understand how regulation of heme and chlorophyll production may occur, workers in our laboratory have focused on the terminal step in the heme biosynthetic pathway, the insertion of ferrous iron into protoporphyrin IX to form protoheme. This terminal step is catalyzed by the membrane-bound enzyme ferrochelatase. We previously purified and partially characterized ferrochelatase from *R. sphaeroides* (2) and examined aspects of iron metabolism as they relate to heme biosynthesis in this organism (5, 16, 17).

In the present study, additional characteristics of the purified enzyme were examined in an effort to obtain a better understanding of how ferrochelatase functions in this facultative photosynthetic organism. Previous data have shown that ferrochelatase from *R. sphaeroides* differs dramatically in size and some properties from other bacterial (7; Dailey, Methods Enzymol., in press) and eucaryotic ferrochelatases (3, 4, 6, 10; Dailey, Fleming, and Harbin, Methods Enzymol., in press). Here we present data that demonstrate the sensitivity of the enzyme to sulfhydryl- and arginyl-specific reagents and show that this bacterial ferrochelatase can utilize as substrates 2,4-disubstituted porphyrins that are competitive inhibitors of the mammalian enzyme (6).

MATERIALS AND METHODS

Ferrochelatase from *R. sphaeroides* was purified and assayed as previously described (2; Dailey, in press). For some experiments ferrochelatase was assayed by using the ^{59}Fe procedure (5). In all assays except the radioactive assay deuteroporphyrin was the porphyrin substrate used; in the radioactive assay protoporphyrin was used. Experiments with *N*-methylprotoporphyrin and 2,4-disubstituted porphyrins were performed as described previously for bovine ferrochelatase (4, 6).

The enzyme was modified with *N*-ethylmaleimide, monobromobimane, and iodoacetamide by using the procedures described previously for bovine ferrochelatase (3). All reactions were done at 24°C. *N*-ethylmaleimide was freshly prepared as a 100 mM stock solution in 95% ethanol; iodoacetamide and monobromotrimethylammoniobimane were prepared as 50 mM stock solutions in deionized, glass-distilled water; and monobromobimane was prepared as a 50 mM stock solution in high-pressure liquid chromatography grade acetonitrile. All reagents were kept in foil-wrapped tubes at 0 to 5°C. The actual reaction mixtures contained purified ferrochelatase (concentration, about 1 μM) in a solution containing 10 mM Tris acetate (pH 8.1), 20% glycerol, and 1.0% (wt/vol) sodium cholate, and the modification reaction was started by adding reagent (usually about 5 to 10 μl/ml). Addition of an equivalent amount of water, ethanol, or acetonitrile had no effect on the activity of the enzyme. Samples (100 μl) were removed at various times, and the reaction was quenched by rapid addition of and mixing with 20 μl of 50 mM dithiothreitol. Ferrochelatase activity was determined directly with these samples. In the experiments in which excess ^{3}H-labeled *N*-ethylmaleimide had to be removed, the quenched reaction mixture was passed, by using centrifugation, through a Sephadex G-25 column (19) that had been equilibrated with the buffer described above. For quantitation of reacted enzyme sulfhydryl groups, ^{3}H-labeled *N*-ethylmaleimide was used, and the concentration of enzyme was determined by using the following relationship: millimolar extinction coefficient (E_{mM}) = 76 at 278 nm (Dailey, in press).

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TABLE 1. Apparent K_m values of *R. sphaeroides* ferrochelatase for 2,4-substituted porphyrins[a]

Porphyrin substrate	Apparent K_m (μM)	V_{max} (μmol/min per mg)
Protoporphyrin	18	0.1
Mesoporphyrin	20	1.2
Deuteroporphyrin	95	3.2
2,4-Disulfonate deuteroporphyrin	52	2.9
2,4-bis-Glycol deuteroporphyrin	ND[b]	ND
2,4-bis-Acetal deuteroporphyrin	56	2.8

[a] In all assays ferrous iron was used as the substrate; the assays were carried out as described previously (2, 5).
[b] ND, Not determined (Activity was below the limits of detection).

For modification of arginyl residues, 2,3-butanedione was used (21). The reaction mixture contained approximately 1 μM ferrochelatase in 1.0% sodium cholate–50 mM sodium borate (pH 7.5)–0.1 mM 2,3-butanedione. After incubation for a predetermined time, the reaction was stopped by adding 4 mM arginine. Samples (100 μl) were separated from excess reagent by centrifugation through a 1-ml Sephadex G-25 column (19) equilibrated with a solution containing 20 mM Tris acetate (pH 8.1), 0.5 mM dithiothreitol, and 10 μg of phenylmethylsulfonyl fluoride per ml. Control assays without butanedione were run in the same fashion. Modification with camphorquinone 10-sulfonate was carried out in the same way.

All porphyrins except *N*-methylprotoporphyrin were purchased from Porphyrin Products, Logan, Utah; *N*-methylprotoporphyrin was produced as described by Kunze et al. (13). Monobromobimane was obtained from Calbiochem-Behring, La Jolla, Calif., and ^{3}H-labeled *N*-ethylmaleimide was obtained from New England Nuclear Corp., Boston, Mass. All other reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Substrate specificity. Since ferrochelatase from mammalian sources has been shown to exhibit a high degree of specificity toward the nature of the substituents at the 2 and 4 positions on the porphyrin ring, 2,4-disubstituted porphyrins were examined for their ability to serve as substrates for *R. sphaeroides* ferrochelatase (Table 1). Both 2,4-disulfonic and 2,4-bis-acetal deuteroporphyrins were substrates for the bacterial ferrochelatase, while 2,4-bis-glycol deuteroporphyrin was not used as a substrate when it was examined at concentrations as high as 100 μM.

N-Methylprotoporphyrin, which has been shown to be a tightly binding competitive inhibitor of bovine ferrochelatase (4), was a strong inhibitor of *R. sphaeroides* ferrochelatase and exhibited noncompetitive inhibition with respect to iron (Fig. 1). It was not possible to determine a K_i for *N*-methylprotoporphyrin with respect to porphyrin. This was because the high porphyrin concentrations required to determine a K_i for a tightly binding competitive inhibitor (4) were found to be inhibitory to the bacterial ferrochelatase. Even deuteroporphyrin, which is not inhibitory to the mammalian ferrochelatase at concentrations as high as 250 μM, was inhibitory to the bacterial enzyme at concentrations greater than 100 μM. The data obtained at lower porphyrin concentrations (Fig. 1) yielded an apparently noncompetitive Lineweaver-Burk plot, which is characteristic of a tightly binding competitive inhibitor at low substrate concentrations (23).

Effect of -SH modification on *R. sphaeroides* ferrochelatase. The ability of the sulfhydryl reagents *N*-ethylmaleimide, iodoacetamide, and monobromobimane to inhibit ferrochelatase activity was examined (Fig. 2). Iodoacetamide at a concentration of 1 mM had almost no effect on enzyme activity, while monobromobimane and *N*-ethylmaleimide both caused rapid inactivation of ferrochelatase. Unlike the bovine ferrochelatase reaction (3), the inactivation reaction was not a pseudo-first-order reaction. By using ^{3}H-labeled *N*-ethylmaleimide, it was possible to determine the number of residues reacted. Figure 3 shows that complete inactivation of *R. sphaeroides* ferrochelatase occurred when more than one residue (possibly two) was modified.

The ability of the two substrates to protect ferrochelatase against inactivation by *N*-ethylmaleimide was examined (Fig. 4). Ferrous iron at a concentration of 100 μM had a protective effect, as might be expected if sulfhydryl residues are involved in iron binding, as they have been suggested to be for bovine ferrochelatase (3). However, the presence of 100 μM deuteroporphyrin caused an increased rate of inactivation by *N*-ethylmaleimide. These data are unlike those reported for either bovine or chicken ferrochelatase (4, 10). Mg^{2+} at a concentration of 100 μM and 1 mM EDTA had no effect on the inactivation of the enzyme by *N*-ethylmaleimide (data not shown).

Effect of modification with 2,3-butanedione. Since cationic residues may be involved in binding the porphyrin substrate

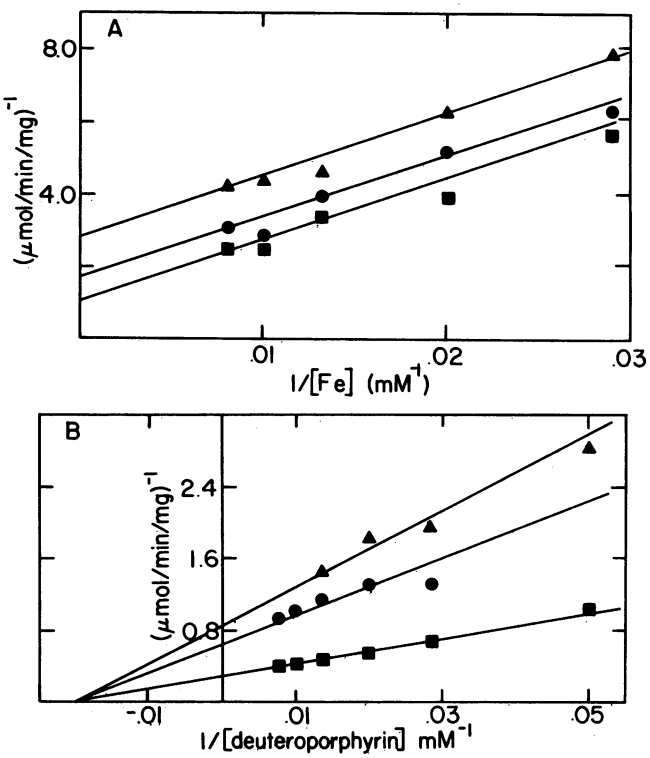

FIG. 1. Kinetics of inhibition of *R. sphaeroides* ferrochelatase by *N*-methylprotoporphyrin: Lineweaver-Burk plots for ferrochelatase with increasing concentrations of *N*-methylprotoporphyrin. The units of activity and the concentration of *N*-methylprotoporphyrin used were the same for both experiments. Symbols: ▲, 20 nM *N*-methylprotoporphyrin; ●, 10 nM *N*-methylprotoporphyrin; ■, no *N*-methylprotoporphyrin added. (A) Inhibition with respect to ferrous iron. (B) Inhibition with respect to deuteroporphyrin.

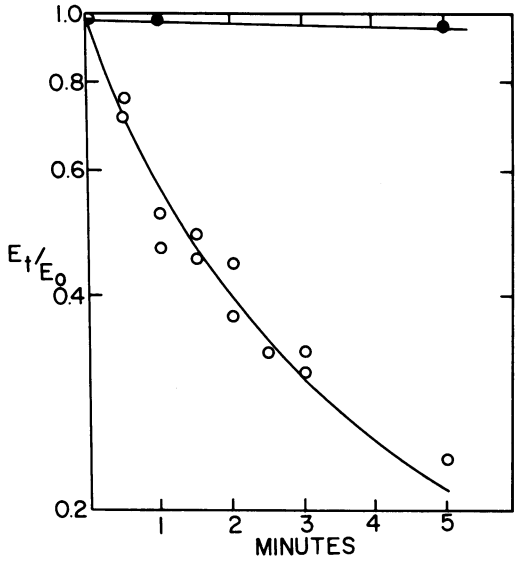

FIG. 2. Inactivation of ferrochelatase by sulfhydryl reagents. Symbols: ●, inactivation by iodoacetamide; ○, inactivation by *N*-ethylmaleimide. Monobromobimane gave inactivation kinetics identical to those shown for *N*-ethylmaleimide. E_t, Enzyme activity at time t; E_0, enzyme activity at zero time. The final concentration of each sulfhydryl reagent was 1 mM. Details are given in the text.

in bovine ferrochelatase (Dailey et al., in press; Dailey and Fleming, manuscript in preparation) the possible role of these residues in the bacterial enzyme was examined. Modification of lysyl groups with trinitrobenzene sulfonate or methyl acetimidate had no visible effect on enzyme activity (data not shown). Reaction of the enzyme with the arginyl reagents 2,3-butanedione and camphorquinone 10-sulfonate

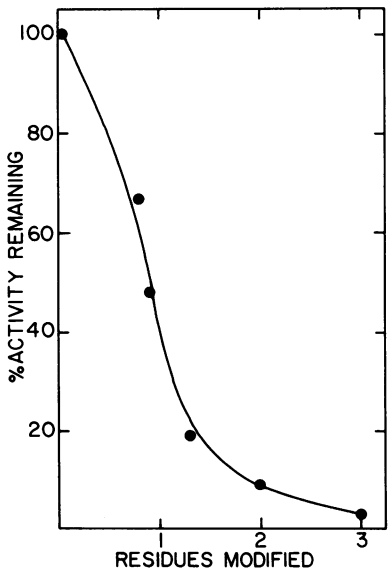

FIG. 3. Stoichiometry of inactivation of *R. sphaeroides* ferrochelatase by *N*-ethylmaleimide. Purified ferrochelatase (1 μM) was reacted with 1 mM ^{3}H-labeled *N*-ethylmaleimide as described in the text. The graph shows the percentage of activity remaining versus the number of sulfhydryl residues modified.

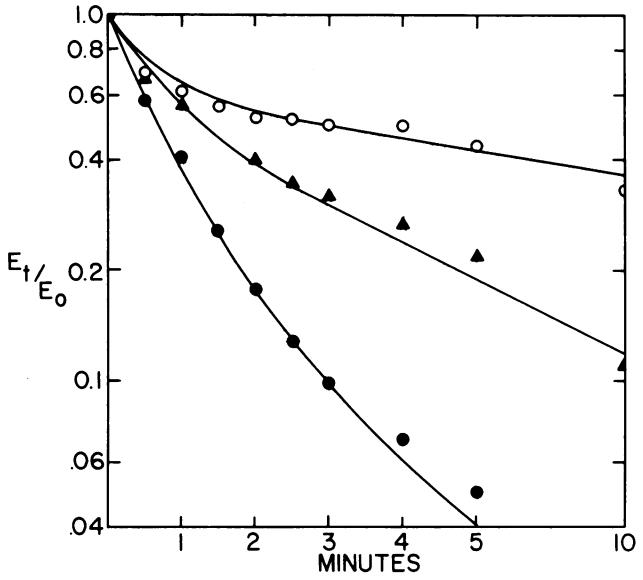

FIG. 4. Protection by substrates against inactivation of ferrochelatase by *N*-ethylmaleimide. The concentration of *N*-ethylmaleimide was 1 mM, and the enzyme concentration was approximately 1 μM. The experimental details are described in Materials and Methods. Symbols: ○, data obtained for inactivation in the presence of 100 μM ferrous iron; ▲, data obtained without any added substrate; ●, data obtained in the presence of 100 μM deuteroporphyrin. E_t, Enzyme activity at time t; E_0, enzyme activity at zero time.

inhibited activity to similar extents. Because of the similarities in the activities of these compounds, only the data obtained with 2,3-butanedione are shown.

Figure 5 shows the rapid rate of inactivation of ferrochelatase with 2,3-butanedione. Neither ferrous iron nor porphyrin substrates had a protective effect against this inactivation. With the bovine enzyme only the competitive porphyrin inhibitors, such as 2,4-disulfonic acid deuteroporphyrin, gave protection against inactivation by butanedione, but for the enzyme from *R. sphaeroides* these porphyrins were substrates and did not give significant protection against inactivation by arginyl reagents.

Ferrochelatase which had been modified with 2,3-butanedione for 5 min was assayed to determine its K_m and V_{max} values for both iron and deuteroporphyrin. Figure 6 shows that modification did not affect the apparent K_m for iron, but resulted in a decreased V_{max}, as would be expected if modification did not affect the avidity of the enzyme for iron. The apparent K_m for deuteroporphyrin was increased by butanedione modification, while the V_{max} was not altered. These data support a model in which porphyrin binding, but not iron binding, involves arginyl residues and modification of these residues results in decreased affinity for the porphyrin substrate.

DISCUSSION

To date, ferrochelatase has been purified to homogeneity from a variety of mammalian and avian sources (4, 10, 22; Dailey et al., in press), but has been purified to homogeneity from only a single bacterial species, *R. sphaeroides* (2). Previously reported data for this enzyme clearly demonstrated that it differs significantly in size and in its reaction to various divalent cations, especially Mn^{2+}, from the eucary-

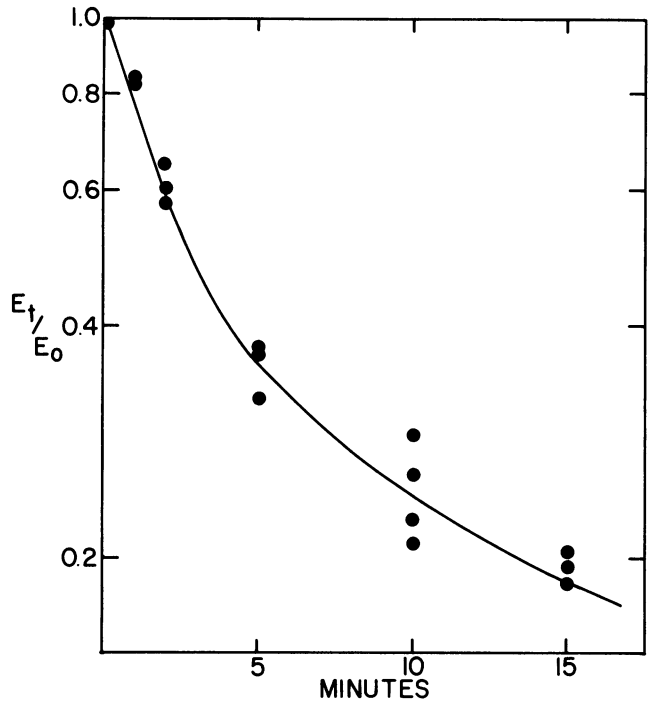

FIG. 5. Inactivation of ferrochelatase by 2,3-butanedione. The experimental details are described in Materials and Methods. The data shown were obtained from two separate preparations of purified enzyme. E_t, Enzyme activity at time t; E_0, enzyme activity at zero time.

otic enzymes (2; Dailey, in press). Because of its important position in the tetrapyrrole biosynthetic pathway in a facultative photosynthetic organism such as *R. sphaeroides*, we examined a variety of other properties of the enzyme.

Our data support a role for sulfhydryl groups in iron binding, as has been proposed previously for bovine ferrochelatase (3). However, a major difference between these two enzymes is the effect of the porphyrin substrate on the rate of inactivation by *N*-ethylmaleimide. For the *R. sphaeroides* enzyme deuteroporphyrin causes a substantial increase in the rate of inactivation. This might be explained if porphyrin binding occurs prior to iron binding, but the enzyme kinetics suggest an ordered sequential mechanism, with ferrous iron binding occurring prior to porphyrin binding (2, 12). Thus, the mechanism by which deuteroporphyrin increases the reactivity of catalytically important sulfhydryl residues remains unknown. However, the fact that it does react in this fachion points out a significant difference between this enzyme and the previously examined eucaryotic enzymes.

The effects of butanedione and other arginyl reagents on ferrochelatase activity and the kinetics of the butanedione-modified enzyme suggest that arginyl residues are involved in binding of the porphyrin substrate, but not the iron substrate. The inactivation observed for the *R. sphaeroides* enzyme is similar to that reported for the bovine ferrochelatase, although the kinetics vary slightly (Dailey et al., in press; Dailey and Fleming, in preparation). The role of arginyl residues in the binding of tetrapyrroles has been well documented for various globins and related heme-binding proteins (20), so their importance for porphyrin binding in ferrochelatase is not unexpected.

One characteristic of the enzyme which we studied that sets it apart from the eucaryotic enzymes examined is its ability to use as substrates porphyrins that are competitive inhibitors of the bovine enzyme. Both 2,4-bis-acetal deuterporphyrin and 2,4-disulfonate deuteroporphyrin are competitive inhibitors of the bovine enzyme, with K_m values that are approximately equal to or less than the K_m for deuteroporphyrin. For *R. sphaeroides* ferrochelatase both of these porphyrins are good substrates and have apparent K_m values less than the K_m of deuteroporphyrin. This suggests that the porphyrin binding site of this bacterial enzyme must differ significantly from the site found in the eucaryotic enzymes.

A feature common to all ferrochelatases examined to date, including the *R. sphaeroides* enzyme, is their extreme sensitivity to *N*-methylprotoporphyrin (1, 4, 8, 11, 18). Unfortunately, it was not possible in this study to determine the K_i with respect to the porphyrin substrate since the assay porphyrin concentrations that would be required are inhibitory to the enzyme. However, the available data are com-

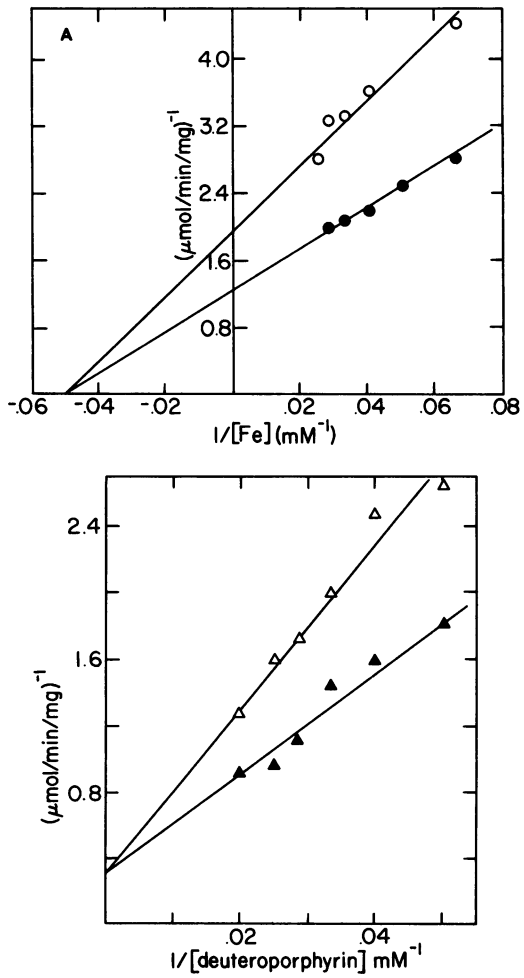

FIG. 6. Kinetics of 2,3-butanedione-modified *R. sphaeroides* ferrochelatase activity. The units were the same for both experiments. Symbols: ○ and △, data obtained with modified ferrochelatase; ● and ▲, data obtained with unmodified ferrochelatase. (A) Kinetics of modified enzyme with respect to ferrous iron. (B) Kinetics of modified ferrochelatase with respect to deuteroporphyrin.

patible with the hypothesis that *N*-methylprotoporphyrin is a tightly binding competitive inhibitor of the bacterial ferrochelatase (23).

Overall it is clear that ferrochelatases from both bacterial and eucaryotic sources catalyze the insertion of ferrous iron into a IX isomer porphyrin. All of the enzymes examined to date appear to contain reactive sulfhydryl residues that are necessary for iron binding and arginyl residues that are involved in prophyrin binding. Additionally, *N*-methylprotoporphyrin is an effective inhibitor of all ferrochelatases. Differences occur in the sensitivity to inhibition by Mn^{2+} and in the porphyrin specificity at the 2 and 4 positions. The reason why ferrochelatase from *R. sphaeroides* is three times larger than the eucaryotic enzymes is currently unknown. To date we have been unable to attribute any regulatory properties to *R. sphaeroides* ferrochelatase that might account for its increased size. While regulatory mechanisms involving ferrochelatase cannot be ruled out, it is possible that ferrochelatase itself is not subject to regulation at this branch point. Analogous situations in other systems in which branch point enzymes are not regulated have been described by La Porte et al. (14) for control of branch points. Final elucidation of the question of control may require both genetic and biochemical approaches.

ACKNOWLEDGMENTS

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