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

HARRY A. DAILEY,* JENNIE E. FLEMING, AND BERTILLE M. HARBIN

Department of Microbiology, University of Georgia, Athens, Georgia 30602

Received 17 June 1985/Accepted 27 September 1985

Purified ferrochelatase (protoheme ferrolyase; EC 4.99.1.1) from the bacterium *Rhodopseudomonas sphaeroides* was examined to determine the roles of cationic and sulphydryl residues in substrate binding. Reaction of the enzyme sulphydryl residues with N-ethylmaleimide or monobromobimane resulted in a rapid loss of enzyme activity. Ferrous, 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 sulphydryl 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 tetrapyrrrole 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 sulphydryl- 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

$^5$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 sulphydryl 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).
TABLE 1. Apparent $K_m$ values of $R$. sphaeroides ferrochelatase for 2,4-substituted porphyrins

<table>
<thead>
<tr>
<th>Porphyrin substrate</th>
<th>Apparent $K_m$ (µM)</th>
<th>$V_{max}$ (µmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoporphyrin</td>
<td>18</td>
<td>0.1</td>
</tr>
<tr>
<td>Mesoporphyrin</td>
<td>20</td>
<td>1.2</td>
</tr>
<tr>
<td>Deuteroporphyrin</td>
<td>95</td>
<td>3.2</td>
</tr>
<tr>
<td>2,4-Disulfonate deuteroporphyrin</td>
<td>52</td>
<td>2.9</td>
</tr>
<tr>
<td>2,4-bis-Glycol deuteroporphyrin</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>2,4-bis-Acetal deuteroporphyrin</td>
<td>56</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* In all assays ferrous iron was used as the substrate; the assays were carried out as described previously (2, 5).

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-m1 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 sulphydryl 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 sulphydryl 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...
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 acetylimidate 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 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-
ferrochelatase is activity documented for various residues in the arginyl press; in rochelatase, although the kinetics enzyme in binding between this in modified enzymes.otic remains binding, as position kinetics rate enzyme enzymes two porphyrin if with ferrous iron increases (2, examined a enzyme. Thus, suggest that the porphyrin binding site of this bacterial enzyme must differ significantly from the site found in the eucaryotic enzymes.

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 deuteroporphyrin 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. 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.](image)

![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.](image)
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 Mn²⁺ 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

This work was supported in part by Public Health Service grants AM31064 and AM33230 from the National Institutes of Health. H.A.D. was the recipient of Public Health Service Research Career Development Award AM01038 from the National Institutes of Health.

LITERATURE CITED