The Role of Arginyl Residues in Porphyrin Binding to Ferrochelatase*  

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The role of cationic amino acid residues in the binding of porphyrin substrates by purified bovine ferrochelatase (protopheme ferro-lyase, EC 4.99.1.1) have been examined via chemical modification with camphorquinone-10-sulfonic acid, phenylglyoxal, butanedione, and trinitrobenzene sulfonate. The data obtained show that modification of arginyl, but not lysyl, residues results in the rapid inactivation of ferrochelatase. The 2,4-disulfonate deuteroporphyrin, which is a competitive inhibitor of mammalian ferrochelatase, protects the enzyme against inactivation. Ferrous iron has no protective effect. Reaction with radiolabeled phenylglyoxal shows that modification of 1 arginyl residue causes maximum inhibition of enzyme activity. The inactivation does not follow simple pseudo-first order reaction kinetics, but is distinctly biphasic in nature. Comparison of the enzyme kinetics for modified versus unmodified enzyme show that modification with camphorquinone-10-sulfonic acid has no effect on the $K_m$ for iron but does alter the $K_m$ for porphyrin.

Ferrochelatase (protopheme ferro-lyase, EC 4.99.1.1) catalyzes the insertion of ferrous iron into protoporphyrin IX to form protoporphyrin. This mitochondrial, membrane-bound enzyme has now been purified and characterized from mammalian and avian sources (1-8). It has been shown that the bovine enzyme utilizes an ordered sequential reaction mechanism with iron binding prior to porphyrin binding (3) with the ferrous iron binding being mediated via two vicinal sulfhydryl groups (4).

Mammalian ferrochelatase is highly selective with respect to its porphyrin substrate. It utilizes only dicarboxylic porphyrins of the IX isomer and will not use the corresponding methyl esters (7, 9). Movement of even single protonate side chain results in substantial loss in acceptability as a substrate (9). There is also selectivity toward the substituents on the other two pyrrolic rings. The enzyme will use proto-, meso-, deuter-, and hematoporphyrin, but larger or charged substituents at the 2,4-position result in loss of reactivity (3, 7). Spectrofluorometric studies on the purified bovine enzyme suggest that the active site is a relatively protected, moderately hydrophobic region (8). All these data support a model for the active site that is a porphyrin-binding pocket similar in nature to those found in heme-binding proteins.

In an effort to understand the mechanism for the selectivity of ferrochelatase toward the number and position of porphyrin carboxyl groups, we have chosen to examine the role that cationic amino acid residues may play in porphyrin binding. In the present study we present evidence that mammalian ferrochelatase contains arginyl residues whose modification results in the loss of activity. The enzyme is protected from inactivation when porphyrin, but not iron, is present during reaction with arginyl reagents.

**MATERIALS AND METHODS**

Bovine liver ferrochelatase was purified and assayed as described previously (3, 10). Livers were obtained fresh from a local abattoir and were sliced and placed on ice immediately. Due to the size and nature of the abattoir, it was not possible to always obtain the same breed or sex of animal, and in some instances definitive identification was not possible. Mouse ferrochelatase was purified as described previously (11).

The reaction of ferrochelatase (1 µM) with camphorquinone-10-sulfonic acid (CQS) was carried out in 20 mM Tris borate, pH 9.0, 1% sodium cholate, 0.5 mM dithiothreitol, and 10 µg/ml phenylmethylysulfonyl fluoride. The stock solution of CQS (10 mg/ml) was made up in 0.1 M sodium borate immediately before use and the pH was adjusted to 9.0 with sodium hydroxide. The final concentration of CQS in the reaction mixture was 1.3 mM. All reactions were carried out at 25 °C, and the reaction of CQS was quenched by the rapid addition of arginine to yield a final concentration of 4 mM. This amount of arginine alone had no measurable effect on the enzymatic activity or kinetic properties of ferrochelatase. The enzyme was then assayed in the usual fashion. In reactions with cyclohexanediene and 2,3-butanedione, similar concentrations of reagents were used.

For reaction with phenylglyoxal, enzyme concentration was 1 µM, phenylglyoxal was 1.5 mM, and the buffer was 0.1 M HEPES, pH 8.1, 1% sodium cholate, 0.5 mM dithiothreitol, and 10 µg/ml phenylmethylysulfonyl fluoride. The reaction was run at 25 °C and was quenched with arginine as described above. For experiments that used [14C]phenylglyoxal, the final specific activity in the reaction mixture was 25 mCi/mmol. To remove excess reagent before liquid scintillation counting, the samples were passed by centrifugal force through Sephadex G-25 gel filtration columns (12).

The reactions with trinitrobenzene sulfonate and methyl acetimidate (0.1 mM final concentration) were carried out at 25 °C in the HEPES buffer described above. The reaction was quenched by addition of 10 mM ammonium bicarbonate, and excess reagent was removed prior to the assay by centrifugal gel filtration (12) through a Sephadex G-25 column that had been equilibrated with 10 mM Tris acetate, pH 8.1, 0.1 M potassium chloride, 0.5 mM dithiothreitol, and 1.0% Brij 35 (w/v).

The amino acid composition of bovine ferrochelatase was determined after 6 N HCl hydrolysis. Amino acids were quantitated by high pressure liquid chromatography (13). Tryptophan content was determined by the fluorescence method of Pajot (14), and cysteine was quantitated with Ellmans reagent (15).

CQS was obtained from Pierce and trinitrobenzene sulfonate, phenylglyoxal, butanedione, and cyclohexanediene were from Sigma.

Porphyrins were purchased from Porphyrin Products, Logan, UT. [14C]Phenylglyoxal was from Amersham Corp. All other reagents were of the highest quality available.

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1 The abbreviations used are: CQS, camphorquinone-10-sulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
RESULTS

Rate of Inactivation of Ferrochelatase by Arginyl Reagents—
The arginyl-specific reagent CQS (16) was used to modify arginine residues. This reagent offers the advantage that it is stable at neutral pH in the absence of borate buffers, unlike other reagents which require the presence of borate to stabilize the complex. The kinetics of inactivation of ferrochelatase by CQS are shown in Fig. 1. Similar data were found for ferrochelatase from livers from a variety of cattle and mice. The data show a biphasic nature to the inactivation. The initial rapid phase is over within 2 min under the conditions employed. CQS is reported to be an arginyl-specific reagent that is removed by prolonged incubation with 0.2 M O-phenylenediamine at pH 8–9 (16). Unfortunately, incubation of other reagents which require the presence of borate to stabilize was stable at neutral pH in the absence of borate buffers, unlike phenylglyoxal were similar to that found with CQS, whereas butanedione gave about a 2-fold slower rate. All three reagents yielded a biphasic inactivation curve similar to that shown in Fig. 1 for CQS. Similar data were found for both bovine and mouse ferrochelatase.

The possibility that side reactions with amino groups of lysine were responsible for loss of activity is unlikely, since lysyl modification with trinitrobenzene sulfonate and methyl acetimidate had no effect on ferrochelatase activity (data not shown). Reaction of the enzyme as described under “Materials and Methods” for a period of 1 h resulted in no detectable loss of activity. Further reaction was not attempted, since denaturation due to chemical modification was not desirable.

Since the arginyl and sulfhydryl reactive reagents show their effect on enzyme activity within just a few minutes, it was believed that side reactions of arginyl-specific reagents with lysyl residues were unlikely to occur more rapidly than reaction with lysyl-specific reagents. Also, sulfhydryl group involvement is ruled out, since ferrous iron, which has previously been shown to protect reactive sulfhydryl residues of bovine ferrochelatase (4), has no protective effect.

In an effort to quantitate the numbers of essential arginyl residues, 14C-labeled phenylglyoxal was used and the number of residues modified versus the activity remaining was plotted (Fig. 2). Since two molecules of phenylglyoxal bind to one guanidinium group of arginine, a stoichiometry of 2 to 1 is expected. Because the inactivation reaction is biphasic, one might expect to see approximately 1.0 residue of arginine modified by the end of the first phase. What is found is that about 0.8 equivalent of arginine is reacted. Although this is a little lower than expected, we believe that the data do support the idea of a single arginine residue being involved. The reason for the apparently low stoichiometry may be attributable to the loss of radiolabel during the additional centrifugation step that is employed for the removal of unreacted labeled phenylglyoxal prior to liquid scintillation counting. Thus the data shown in Fig. 2 suggest that modification of 1 arginyl residue/enzyme molecule may be responsible for the rapid inactivation of ferrochelatase seen with phenylglyoxal.

Substrate Protection against Inactivation—The ability of both substrates to protect ferrochelatase from inactivation by CQS and cyclohexanedione was examined. The data for both were similar, so only the CQS inactivation data are shown graphically. Ferrous iron at concentrations that protect the enzyme from inactivation by sulfhydryl-specific reagents (4) had no protective effect (data not shown). The effect of the porphyrin on inactivation was studied using the competitive inhibitor 2,4-disulfonic acid deuteroporphyrin (3). This, rather than a substrate porphyrin, such as deuteroporphyrin, was employed to prevent catalysis. Because low levels of contaminating iron are always present in the buffers and assay reagents, it was believed that some catalysis would occur that might affect the data. The competitive inhibitor has a Ki similar to the Ke of deuteroporphyrin. The chemical modification reaction is diluted 10-fold into the assay reaction, and control reactions are run to correct for any effect of the inhibitor on unmodified enzyme. The data shown in Fig. 1 clearly demonstrate that porphyrin provides protection.

![Figure 1](image1.png)

**Fig. 1. Inactivation of ferrochelatase by camphorquinone-10-sulfonic acid.** Experimental details are outlined under “Materials and Methods.” The solid circles were data obtained from two different ferrochelatase preparations each done in duplicate. The open circles are the same two enzyme preparations, but the reaction with CQS was carried out in the presence of 150 μM 2,4-disulfonic acid deuteroporphyrin. Incubation in the presence of 100 μM ferrous citrate yielded curves to be shown by the solid circles. Results are expressed as the enzyme activity remaining at time t (E0) divided by the activity at time 0 (E0).

![Figure 2](image2.png)

**Fig. 2. Inactivation of ferrochelatase by phenylglyoxal.** A, inactivation of ferrochelatase by phenylglyoxal as a function of time. Reaction conditions are given in the text. B, inactivation as a function of the number of arginyl residues modified with phenylglyoxal. Details are given in the text.
against inactivation by CQS. Assuming that the reacting arginyl residue is involved in substrate binding, then the concentration of 2,4-disulfonic acid deuteroporphyrin that yields approximately 50% protection against inactivation should be similar to the $K_i$. Since 30% activity remains in this experiment in the absence of added porphyrin, then by definition 50% protection occurs when approximately 65% of the control activity remains. The data shown in Fig. 3 agree with this assumption. A concentration of 50 μM disulfonic acid deuteroporphyrin provided 50% protection against inactivation, and the $K_i$ for this compound is reported to be 70 μM for bovine ferrochelatase (3).

Kinetics of Modified Ferrochelatase—Ferrochelatase was modified with CQS or cyclohexanedione for 5 min. The $K_m$ and $V_{max}$ of these modified enzyme preparations were determined for both iron and porphyrin substrates. The data are shown in Fig. 4, A and B. The data obtained for both CQS and cyclohexanedione were very similar, so only the data for the CQS-modified enzyme preparation is shown in the figures. The data clearly show that the modification decreases the $V_{max}$, but has no effect on the $K_m$ with respect to iron. With deuteroporphyrin as substrate both $V_{max}$ and $K_m$ are altered.

Amino Acid Composition of Bovine Ferrochelatase—The amino acid composition of bovine ferrochelatase is shown in Table I. These data are the average of three determinations.

**DISCUSSION**

Arginyl residues have been implicated in the binding of a variety of anionically charged substrates by enzymes (18–20) and are frequently found in heme-binding proteins as one of the residues involved in binding protoheme (21). Ferrochelatase utilizes only dicarboxylate porphyrins of the IX isomer type as substrate. The high degree of specificity exhibited by the enzyme for the placement and number of anionic moieties suggests that ferrochelatase may utilize complementary charge pair interactions in binding or aligning its porphyrin substrate.

The data presented above demonstrate that at least 1 arginyl residue is involved in porphyrin binding to ferrochelatase, this then appears to be analogous to protoheme binding in some globin molecules (21). The stoichiometry of inactivation suggests that 1 rapidly reacting arginyl residue is involved, but the biphasic inactivation curve suggests that a second, more slowly reacting species may also be involved. Although the data presented here do not identify this other species, it is doubtful that it is a lysyl residue, since modification of lysyl residues with trinitrobenzene sulfonate had no effect on enzyme activity. Interestingly, the rate of the second slower phase appears similar to the slow rate of inactivation seen in the presence of porphyrin. This may suggest that the slower rate of inactivation is nonspecific in nature and not involved directly in porphyrin binding.

The previous observations that ferrochelatase utilizes only dicarboxylate porphyrins of the IX isomer as substrates (7, 9) strongly suggest that 2 amino acid residues are involved in porphyrin binding and alignment. Interestingly, the kinetic data suggest that the modified arginyl residue is not the only factor involved in porphyrin binding, since the arginyl modification actually decreases the apparent $K_m$.

If arginyl groups were involved in porphyrin, but not iron, binding, then modification of these residues would be expected to alter porphyrin specificity, and consequently the $K_m$. The previous observations that ferrochelatase utilizes only dicarboxylate porphyrins of the IX isomer as substrates (7, 9) strongly suggest that 2 amino acid residues are involved in porphyrin binding and alignment. Interestingly, the kinetic data suggest that the modified arginyl residue is not the only factor involved in porphyrin binding, since the arginyl modification actually decreases the apparent $K_m$.

![Fig. 3. Protective effect of porphyrin against inactivation by CQS. Ferrochelatase was inactivated by CQS as described under "Materials and Methods" in the presence of 2,4-disulfonic acid deuteroporphyrin at concentrations ranging from 0 to 300 μM. The incubation with CQS was for 5 min before the addition of excess arginine. Activity is expressed as the per cent of activity of an unreacted control.](image)

![Fig. 4. Effect of arginyl modification on ferrochelatase kinetics. Bovine ferrochelatase was reacted with CQS for 5 min as described in the text before addition of excess arginine. Data are shown as Lineweaver-Burk plot of kinetics of unmodified enzyme (●) and modified enzyme (○). The units of $u$ are nmol of deuteroporphyrin formed min⁻¹ nmol of ferrochelatase⁻¹. In panel A the variable substrate is ferrous iron, and in panel B the variable substrate is deuteroporphyrin.](image)

**TABLE I**

Amino acid composition of bovine ferrochelatase. Data from 24-h hydrolysis of ferrochelatase. Each number is the average of three determinations.

| Residue        | Number |
|----------------|--------|
| Aspartic acid  | 34.0   |
| Glutamic acid  | 14.0   |
| Serine         | 32.5   |
| Threonine      | 42.8   |
| Arginine       | 51.6   |
| Lysine         | 55.3   |
| Alanine        | 39.6   |
| Glycine        | 96.0   |
| Histidine      | ND*    |
| Cysteine       | 7.2    |
| Methionine     | 10.5   |
| Tyrosine       | 4.0    |
| Tryptophan     | 7.6†   |
| Phenylalanine  | 21.9   |
| Proline        | 20.5   |
| Leucine        | 16.5   |
| Isoleucine     | 17.5   |
| Valine         | 25.4   |

* ND, not determined.
† Quantitated with dithionitrobenzoic acid on enzyme denatured in 1% sodium dodecyl sulfate.
‡ Quantitated by fluorescence.
to cause a decreased $V_{\text{max}}$ for iron and an unaltered $K_m$ for iron. The data obtained experimentally support this hypothesis. Arginy1 group modification could have several effects on the enzyme kinetics with respect to the porphyrin substrate. If the major factor in porphyrin binding is the complementary charge pairing between substrate and enzyme, then modification of arginyl residues would cause an increase in $K_m$ without an alteration in $V_{\text{max}}$. However, due to the hydrophobic, planar nature of the porphyrin macrocycle, it might be anticipated that the arginyl group is only partially responsible for substrate binding.

What is found for ferrochelatase is a decreased $K_m$ and a decreased $V_{\text{max}}$ for the porphyrin substrate (Fig. 4B). A possible explanation for these data is that the actual initial association of porphyrin with the enzyme does not depend upon charge pairing, but the charge pairing is critical for proper orientation of the porphyrin for efficient catalysis. The arginyl modification with CQS alters the enzyme in some fashion that allows for efficient porphyrin binding, but the bound porphyrin is improperly aligned for iron insertion. Such a situation would result in a decrease in the $V_{\text{max}}$ since, even at infinite porphyrin concentrations, the bound porphyrin macrocycle would be aligned in a catalytically inefficient orientation and it may allow for a decreased observed $K_m$. This argument and the data presented above would further suggest that the observed $K_m$ for the porphyrin does not simply reflect a dissociation constant for the porphyrin, but also contains contributions from the rate of enzyme substrate breakdown to products. To insure that the experimental data were not due to an aberration caused by CQS alone, arginyl residues were also modified with cyclohexanedione, butanedione, and phenylglyoxal. The kinetics of the modified enzyme preparations were essentially identical to those found with CQS-modified ferrochelatase.

The data gathered to date show that the porphyrin-binding site on ferrochelatase is highly selective with regard to the size, position, and charge of substituents on all four of the pyrrole rings (3, 7, 9) as well as the size of any N-alkyl substituent (22). A model compatible with these data would be one where the porphyrin-binding site on ferrochelatase resembled the heme-binding pocket of globins and cytochromes (23). Such a model would place the porphyrin in a relatively nonpolar environment that is poorly accessible to outside hydrophilic molecules, which is what has been reported for the bovine enzyme (8). Future study of ferrochelatase, including determination of its primary structure, may reveal further similarities or differences between this enzyme and other porphyrin-binding proteins.

REFERENCES
1. Taketani, S., and Tokunaga, R. (1981) J. Biol. Chem. 256, 12748-12753
2. Taketani, S., and Tokunaga, R. (1982) Eur. J. Biochem. 127, 443-447
3. Dailey, H. A., and Fleming, J. E. (1983) J. Biol. Chem. 258, 11453-11459
4. Dailey, H. A. (1984) J. Biol. Chem. 259, 2711-2715
5. Hanson, J. W., and Dailey, H. A. (1984) Biochem. J. 222, 695-700
6. Harbin, B. M., and Dailey, H. A. (1985) Biochemistry 24, 366-370
7. Dailey, H. A., and Smith, A. (1984) Biochem. J. 223, 441-445
8. Dailey, H. A. (1985) Biochemistry 24, 1287-1291
9. Honeybourne, C. L., Jackson, J. T., and Jones, O. T. G. (1979) FEBS Lett. 96, 207-210
10. Dailey, H. A. (1982) J. Biol. Chem. 257, 14714-14718
11. Dailey, H. A., Fleming, J. E., and Harbin, B. M. (1986) Methods Enzymol. 123, 401-408
12. Penefsky, H. S. (1977) J. Biol. Chem. 252, 2891-2899
13. Wiedmeier, V. T., Porterfield, S. F., and Hendrich, C. E. (1982) J. Chromatogr. 231, 410-417
14. Pajot, P. (1976) Eur. J. Biochem. 63, 263-269
15. Anderson, W. L., and Wetlauer, D. B. (1975) Anal. Biochem. 67, 493-502
16. Pand, C. S., Peliz, M., and Glass, J. D. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 885-889
17. Riordan, J. F. (1973) Biochemistry 12, 3915-3923
18. Zakim, D., Hochman, Y., and Kenny, W. C. (1983) J. Biol. Chem. 258, 6430-6434
19. Enoch, H. G., and Strittmatter, P. (1978) Biochemistry 17, 4927-4932
20. Takata, Y., and Fujiioka, M. (1983) J. Biol. Chem. 258, 7374-7378
21. Perutz, M. F. (1979) Annu. Rev. Biochem. 48, 327-386
22. DeMatteis, P., Gibbs, A. H., and Smith, A. G. (1980) Biochem. J. 189, 645-648
23. Argos, P., and Rossmann, M. G. (1979) Biochemistry 18, 4951-4960