Regulation of Soluble Guanylate Cyclase Activity by Porphyrins and Metalloporphyrins*

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Alterations of the chemical structure of protoporphyrin IX markedly altered the activation of soluble guanylate cyclase purified from bovine lung. Hydrophobic side chains at positions 2 and 4 and vicinal propionic acid residues at positions 6 and 7 of the porphyrin ring (protoporphyrin IX, mesoporphyrin IX) were essential for enzyme activation (K _1 = 7–8 nM; V _max = 6–8 μmol of cGMP/min/mg). Substitution of hydrophobic with polar groups (hematoporphyrin IX, coproporphyrin III), or with hydrogen atoms (deuteroporphyrin IX), and methylolation of propionate residues resulted in decreased enzyme stimulation. Stimulatory prophyrins increased the V _max and the apparent affinities of enzyme for MgGTP and uncomplexed Mg ^2+ . An open central core in the porphyrin ring was essential for enzyme activation. The pyrrolic nitrogen adect, N-phenylprotoporphyrin IX, was inhibitory and competitive with protoporphyrin IX (K _i = 73 nM). Similarly, metalloporphyrins inhibited enzymatic activity and ferro-protoporphyrin IX (K _i = 350 nM), zinc-protoporphyrin IX (K _i = 50 nM) and manganese-protoporphyrin IX (K _i = 9 nM) were competitive with protoporphyrin IX. Inhibitory prophyrins and metalloporphyrins also prevented enzyme activation by S-nitroso-N-acetylpenicillamine and NO. Guanylate cyclase reconstituted with such porphyrins required higher concentrations of protoporphyrin IX for further activation and were not activated by NO. Thus, porphyrins, metalloporphyrins, and NO appeared to interact at a common binding site on guanylate cyclase. This common site is likely that which normally binds heme and, therefore, NO-heme when the heme-containing enzyme is exposed to NO. Thus, NO and nitroso compounds may react with enzyme-bound heme to generate a modified porphyrin which structurally resembles protoporphyrin IX in its interaction with guanylate cyclase.

Activation of soluble guanylate cyclase by NO and related agents requires the presence of heme and has been attributed to the formation of NO-heme which directly activates the enzyme (1–5). Preparations of purified soluble guanylate cyclase known to be heme-deficient show little or no activation by nitroso compounds but full activation is restored after reconstitution of the enzyme with heme (4–6). Guanylate cyclase activation by phenylhydrazine is also heme-dependent but is independent of a mechanism involving NO (7). Instead, enzyme activation by phenylhydrazine is attributed to an iron-phenyl heme complex which forms as a result of an O2-dependent chemical reaction between phenylhydrazine and certain hemoproteins. Protoporphyrin IX, on the other hand, activates soluble guanylate cyclase by a heme-independent mechanism (8, 9). Despite the different requirements for enzyme activation, all of these substances activate guanylate cyclase in a kinetically similar manner (7, 9) and enzyme activation is inhibited by excess concentrations of the metalloporphyrin, heme (4, 5, 7–9). These observations led us to suggest that NO-heme and iron-phenyl heme, which structurally resemble protoporphyrin IX, activate guanylate cyclase through a protoporphyrin IX-like binding interaction (7, 9, 10).

The objective of the present study was to develop a better understanding of the mechanisms by which protoporphyrin IX activates and ferro-protoporphyrin IX (heme) inhibits soluble guanylate cyclase. To this end, the effects of alterations of the chemical structure of protoporphyrin IX on enzyme activation were examined. In addition, several metalloporphyrins of protoporphyrin IX were studied for their effects on guanylate cyclase activation by porphyrins, NO, and S-nitroso-N-acetylpencillamine. These studies help to explain how NO and other substances activate soluble guanylate cyclase and how guanylate cyclase activity could be regulated according to a unifying mechanism.

EXPERIMENTAL PROCEDURES

Materials—Reagents and other materials for the purification and assay of soluble guanylate cyclase from bovine lung have been described (8, 9). The synthesis and handling of S-nitroso-N-acetylpenicillamine have been described previously (11). N-Phenylprotoporphyrin IX was synthesized as described previously (7). N-Methylprotoporphyrin IX was kindly provided by Dr. Paul R. Ortiz de Montellano (University of California, San Francisco). The remaining porphyrins and metalloporphyrins were purchased from Porphyrin Products (Logan, UT) and were used as supplied, in the chromatographically pure form. Solutions of porphyrins were prepared fresh daily by dissolving them in dimethylsulfoxide followed by dilution in 40 mM TEA-HCl, pH 7.4. Solutions of metalloporphyrins were freshly prepared by dissolving them in either dimethylsulfoxide or 0.1 N NaOH followed by immediate dilution in 40 mM TEA-HCl, pH 7.4. All other chemicals were of the highest purity commercially available.

Enzyme Purification—Soluble guanylate cyclase was purified to apparent homogeneity from bovine lung exactly as described (7, 9). Enzyme purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7–9). Soluble guanylate cyclase, as purified for this study, contained stoichiometric amounts of bound heme (8, 9). Enzyme was stored in individual aliquots containing 1.8 μg of enzyme protein as previously described (9).

Guanylate Cyclase Assay—Guanylate cyclase activity was determined by measuring the formation of cyclic[32P]GMP from [α-32P]GTP as described previously (6, 12). Enzyme reactions were conducted at 37°C in a final volume of 0.2 ml and reaction mixtures

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1 The abbreviation used is: TEA-HCl, trimethanolamine-HCl.
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Generally contained 40 mM TEA-HCl, pH 7.4, 2 mM dithiothreitol, 1 mM GTP, 3 mM Mg2+, and 45 ng of enzyme protein. Exceptions are indicated in the appropriate table or figure legends. Samples in each enzymatic assay were run in duplicate or triplicate.

Special Preincubation-Incubation Conditions—Prior to conducting the experiments described in this study a large series of preliminary experiments were conducted to assess the linearity of product formation with enzyme in order to assess the linearity of product formation. This was necessary to ensure that binding interactions to reach equilibrium so that steady state velocities could be examined. Previous studies had revealed that whereas protoporphyrin IX converted these porphyrins from activators to inhibitors because of inherent limitations in experimental design (9). At lower concentrations, the dimethyl esters of protoporphyrin IX were weak competitive inhibitors of protoporphyrin IX (4, 5). Moreover, methylation of hematoporphyrin IX and deuteroporphyrin IX yielded porphyrin esters which contained guanylate cyclase by protoporphyrin IX. In the present study heme-containing, purified soluble guanylate cyclase was reconstituted with protoporphyrin IX. In the present study heme-containing enzyme was readily reconstituted with protoporphyrin IX (4, 5). Moreover, methylation of the propionic acid residues resulted in the formation of uncomplexed Mg2+ and MgGTP (or GTP) were calculated as described previously (9). The Km values for these porphyrins and metalloporphyrins which contain guanylate cyclase by protoporphyrin IX and deuteroporphyrin IX were calculated from slope replots of double reciprocal plots as shown in Figs. 3 and 4. All kinetic plots were fitted by least squares linear regression analysis.

Enzyme Reconstitution Experiments—Previous studies indicated that heme-deficient soluble guanylate cyclase could be readily reconstituted with either heme or protoporphyrin IX (4, 5). Moreover, heme-containing enzyme was readily reconstituted with protoporphyrin IX. In the present study heme-containing, purified soluble guanylate cyclase was reconstituted with protoporphyrin IX, deuteroporphyrin IX, N-phenylporphyrin IX, and metalloporphyrins according to the following procedure. An aliquot of guanylate cyclase (1.8 μg) in 120 μl of 25 mM TEA-HCl, pH 7.4, containing 5 mM dithiothreitol, 30% (v/v) glycerol, and approximately 1.3 mM NaCl was mixed with 380 μl of 50 mM TEA-HCl, pH 7.4, containing 2 mM dithiothreitol and 10 μM concentrations of one of the three porphyrins indicated above, 1 μM zinc protoporphyrin IX, or 0.1 mM manganese-protoporphyrin IX. The solution was kept at 0-4 °C for 20 min and then applied to a column (15 x 16 cm) of Sephadex G-25 that had been precariculated with blue dextran. The column was pre-equilibrated and eluted with 50 mM TEA-HCl, pH 7.4, containing 2 mM dithiothreitol and 0.1 mM NaCl. Guanylate cyclase eluted in the void volume, whereas the other porphyrins eluted at or near the last eluting peak as well as column washes, as reported previously (4). Addition of carrier protein to facilitate the elution of enzyme in order to prevent excessive dilution was avoided because the presence of such protein in assay mixtures could have confounded the interpretation of the data. For example, albumin is well known to bind porphyrins. Accordingly, the concentration of guanylate cyclase in the column eluates and reaction mixtures was lower than that routinely employed. Therefore, the data in Table III are expressed as picomole of cGMP/10 min/0.2 ml of assay.

RESULTS

Effect of Structural Modifications of Porphyrins on Guanylate Cyclase Activation—Table I lists the porphyrins and metalloporphyrins studied, the major differences in their chemical structures, and descriptive effects on guanylate cyclase activity. The structural modifications examined involve the vinyl side chains at positions 2 and 4, the vicinal propionic acid residues at positions 6 and 7, pyrrolic nitrogen substitutions, and the divalent metal in metalloporphyrins. Fig. 1 illustrates two examples of double reciprocal plots of velocity against porphyrin concentration. Calculated values for apparent Ks for several porphyrins are listed in Table II. All enzymatic reactions were conducted with a constant enzyme concentration since a previous study showed that the apparent Ks for protoporphyrin IX varied directly with enzyme concentration (9). Substitution of ethyl for vinyl groups, forming mesoporphyrin IX, had no appreciable influence on Ks, but replacement of these hydrophobic groups with more polar hydroxyethyl groups, as in hematoporphyrin IX, resulted in a 10-fold increase in the Ks (Table II). Replacement with hydrogens (deuteroporphyrin IX) resulted in a marked decrease in activity. A further marked increase in Ks (4 μM) and decrease in Vmax (0.72 μmol/min/mg) were obtained when propionic acid residues were present at positions 2 and 4 (coproporphyrin III). Substitution of highly polar groups (disulfonate or bisglycol deuteroporphyrin IX) resulted in inactive porphyrins.

Methylation of the propionic acid residues resulted in the weakly active protoporphyrin IX dimethyl ester (Table II). Moreover, methylation of hematoporphyrin IX and deuteroporphyrin IX converted these porphyrins from activators to inhibitors. The precise location of the propionate groups at positions 6 and 7 may be important for enzyme activation because coproporphyrin I, with propionate groups at positions 6 and 8, was completely inactive. Substitutions on the pyrrolic nitrogens had a profound effect on enzyme activation. N-Methylhematoporphyrin IX was less active than protoporphyrin IX and N-phenylproporphyrin IX inhibited enzymatic activity.

The activation of guanylate cyclase by protoporphyrin IX is characterized by a marked increase in the Vmax as well as a decrease in the apparent Ks and Km for both MgGTP and uncomplexed Mg2+ (9). Fig. 2 illustrates double reciprocal plots of velocity against GTP concentration at a fixed excess concentration of Mg2+ for three porphyrins. The calculated values for Km, Ks, and Vmax for several porphyrins are listed in Table II. Deuteroporphyrin IX and the dimethyl ester of protoporphyrin IX were the least active in altering these kinetic parameters. Similar effects were observed on the apparent Ks and Km for uncomplexed Mg2+ (Table II). Values for the latter kinetic parameters were more variable than those for MgGTP because of inherent limitations in experimental design (9). Therefore, the kinetic parameters for uncomplexed Mg2+ should be regarded as approximations.

Inhibition of Guanylate Cyclase Activity by Structurally Modified Porphyrins and Metalloporphyrins—Inhibition of the propionic acid residues on the less active hematoporphyrin IX and deuteroporphyrin IX yielded porphyrin esters which directly inhibited guanylate cyclase activity at concentrations of 10-100 μM. At lower concentrations, the dimethyl esters of hematoporphyrin IX (Ks = 0.7 μM) and deuteroporphyrin IX (Ks = 1.1 μM) were weak competitive inhibitors of protoporphyrin IX. N-Phenylporphyrin IX was also inhibitory.
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### TABLE I

**Structural modifications of protoporphyrin IX and their effects on guanylate cyclase**

<table>
<thead>
<tr>
<th>Porphyrin or metalloporphyrin</th>
<th>R</th>
<th>R'</th>
<th>Effect on guanylate cyclase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoporphyrin IX</td>
<td>-CH=CH₂</td>
<td>-CH₂CH₃COOH</td>
<td>Activation</td>
</tr>
<tr>
<td>Mesoporphyrin IX</td>
<td>-CH₂CH₃</td>
<td>-CH₂CH₃COOH</td>
<td>Activation</td>
</tr>
<tr>
<td>Hematoporphyrin IX</td>
<td>-CHOHCH₃</td>
<td>-CH₂CH₃COOH</td>
<td>Activation</td>
</tr>
<tr>
<td>Deuteroporphyrin IX</td>
<td>-H</td>
<td>-CH₂CH₃COOH</td>
<td>Activation</td>
</tr>
<tr>
<td>Deuteroporphyrin IX disulfonate</td>
<td>-SO₃</td>
<td>-CH₂CH₃COOH</td>
<td>Inactive</td>
</tr>
<tr>
<td>Deuteroporphyrin IX bisglycol</td>
<td>-CHOCH₂OH</td>
<td>-CH₂CH₃COOH</td>
<td>Inactive</td>
</tr>
<tr>
<td>Coproporphyrin III</td>
<td>-CH₂CH₃COOH</td>
<td>-CH₂CH₃COOH</td>
<td>Activation</td>
</tr>
<tr>
<td>Protoporphyrin IX dimethyl ester</td>
<td>-CH₂CH₃</td>
<td>-CH₂CH₃COOHCH₃</td>
<td>Activation</td>
</tr>
<tr>
<td>Hematoporphyrin IX dimethyl ester</td>
<td>-CHOHCH₃</td>
<td>-CH₂CH₃COOHCH₃</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Deuteroporphyrin IX dimethyl ester</td>
<td>-H</td>
<td>-CH₂CH₃COOHCH₃</td>
<td>Inhibition</td>
</tr>
<tr>
<td>N-Methylprotoporphyrin IX</td>
<td>-CH=CH₂</td>
<td>-CH₂CH₃COOH</td>
<td>Activation</td>
</tr>
<tr>
<td>Ferro-protoporphyrin IX</td>
<td>-CH₂CH₃</td>
<td>-CH₂CH₃COOH</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Zinc-protoporphyrin IX</td>
<td>-CH=CH₂</td>
<td>-CH₂CH₃COOH</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Manganese-protoporphyrin IX</td>
<td>-CH=CH₂</td>
<td>-CH₂CH₃COOH</td>
<td>Inhibition</td>
</tr>
</tbody>
</table>

*Positions 6 and 8.*

and lower concentrations were competitive with protoporphyrin IX (Fig. 3) and had an apparent $K_I$ of 73 nM. N-Phenylprotoporphyrin IX also inhibited enzyme activation by S-nitroso-N-acetylpenicillamine and NO (not shown).

Metallation drastically altered the effects of protoporphyrin IX on guanylate cyclase. Ferro-protoporphyrin IX inhibits guanylate cyclase activity (8) and is a competitive inhibitor ($K_I = 350$ nM) of protoporphyrin IX (9). Zinc-protoporphyrin IX ($K_I = 50$ nM) and manganese-protoporphyrin IX ($K_I = 9$ nM) were much more potent than heme as competitive inhibitors of protoporphyrin IX. The effects of manganese-protoporphyrin IX are illustrated in Fig. 4. These metalloporphyrins also inhibited enzyme activation by S-nitroso-N-acetylpenicillamine and NO. Guanylate cyclase was reconstituted with zinc- or manganese-protoporphyrin IX (see "Experimental Procedures"). Both reconstituted forms of the enzyme retained 70–80% of the basal activity of unreconstituted enzyme but were completely unresponsive to NO or $S$-nitroso-$N$-acetylpenicillamine whereas high concentrations of protoporphyrin IX (10 μM) caused significant activation (not shown).

**Activator-Activator Interactions with Guanylate Cyclase—**

In order to assess whether two stimulatory porphyrins could interact at a common binding site on guanylate cyclase, the effect of a weakly active porphyrin on enzyme activation by protoporphyrin IX was examined. Deuteroporphyrin IX inhibited enzyme activation by protoporphyrin IX in what appeared to be a competitive manner (Fig. 5). Similar inhibitory effects were observed with the dimethyl ester of protoporphyrin IX. A similar examination was made on the interactions between deuteroporphyrin IX and S-nitroso-N-acetylpenicillamine, which activates guanylate cyclase by a heme-dependent mechanism involving the formation of NO-heme (4, 5). Fig. 5 illustrates that deuteroporphyrin IX inhibited enzyme activation by S-nitroso-$N$-acetylpenicillamine.

Previous studies showed that guanylate cyclase is readily reconstituted with either heme or protoporphyrin IX and that the latter form of the enzyme is recovered in the maximally activated state after gel filtration chromatography (9). Table III illustrates the effects of two porphyrins and S-nitroso-$N$-acetylpenicillamine on guanylate cyclase that had been reconstituted with one of three different porphyrins. Protoporphyrin IX-reconstituted enzyme was recovered in the maximally activated form, with respect to activation by protoporphyrin IX. Deuteroporphyrin IX-reconstituted enzyme was recovered in a lower activated state, which was maximal for
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FIG. 1. Relationship between enzyme reaction velocity and porphyrin concentration. Enzymatic reactions (0.2 ml) were conducted for 10 min at 37 °C in 40 mM TEA-HCl, pH 7.4, containing 2 mM dithiothreitol, 1 mM GTP, 3 mM Mg²⁺, 45 ng of guanylate cyclase, and various concentrations of protoporphyrin IX (●) or mesoporphyrin IX (○) as indicated. Porphyrins were added to reaction mixtures immediately after enzyme. Velocities are expressed as micromole of cGMP/min/mg.

TABLE II
Effects of structural modifications of protoporphyrin IX on kinetic parameters for guanylate cyclase

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Kinetic parameters</th>
<th>Kinetic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K⁺⁺ GTP</td>
<td>Uncomplexed Mg²⁺</td>
</tr>
<tr>
<td></td>
<td>K⁺⁺ K⁺ V₉₉₉₉⁺ K⁺⁺ K⁺ V₉₉₉₉⁺</td>
<td></td>
</tr>
<tr>
<td>None (basal activity)</td>
<td>122 194 0.13 91 190 0.14</td>
<td></td>
</tr>
<tr>
<td>Protoporphyrin IX (1 mM)</td>
<td>7.7 50 47 7.7 40 52 8.0</td>
<td></td>
</tr>
<tr>
<td>Mesoporphyrin IX (1 mM)</td>
<td>7.7 45 41 6.2 47 58 6.2</td>
<td></td>
</tr>
<tr>
<td>Hematoporphyrin IX (10 μM)</td>
<td>76 50 46 7.1 43 53 7.5</td>
<td></td>
</tr>
<tr>
<td>Deuteroporphyrin IX (100 μM)</td>
<td>2940 77 96 2.5 61 88 2.7</td>
<td></td>
</tr>
<tr>
<td>Protoporphyrin IX di-CH₃ ester (100 μM)</td>
<td>1818 91 125 1.8 70 116 1.9</td>
<td></td>
</tr>
<tr>
<td>N-Methylprotoporphyrin IX (10 μM)</td>
<td>115 48 52 3.0</td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as nanomolar.
* Obtained from intercept replots of primary plots; expressed as micromolar.
* Obtained from slope replots of primary plots; expressed as micromolar.
* Preincubated with enzyme at 37 °C for 10 min prior to initiation of reaction with MgGTP.

deuteroporphyrin IX. Neither reconstituted enzyme was further activated by the respective porphyrin (Table III). High concentrations of deuteroporphyrin IX slightly inhibited protoporphyrin IX-reconstituted guanylate cyclase whereas high concentrations of protoporphyrin IX stimulated deuteroporphyrin IX-reconstituted enzyme. High concentrations of S-nitroso-N-acetylpenicillamine or NO, however, did not further activate either form of reconstituted enzyme. N-Phenylprotoporphyrin IX-reconstituted enzyme was recovered in an uninhibited form but was refractory to further activation by S-nitroso-N-acetylpenicillamine or low concentrations of porphyrins. Attempts were made to reconstitute guanylate cyclase with NO-heme by reacting the enzyme with S-nitroso-N-acetylpenicillamine, NO or NO-heme itself. However, the activated form of the enzyme after gel filtration was unstable, showing a gradual loss of activity at 37 °C and could not be further studied with confidence.

Interaction between MgGTP or MnGTP and Protopor-
MnProt. IX, manganese-protoporphyrin IX.

The hydrophobic vinyl side chains at positions 2 and 4 and the negatively charged carboxyl groups of the vicinal propionic acid residues at positions 6 and 7 in heme as well as the coordination of heme iron to the apoprotein, contribute to the formation of stable hemoprotein complexes (15). Protoporphyrin IX, which contains no metal, however, binds tightly to guanylate cyclase with an apparent Kd of 1.4 nM (9). Substitution of the two vinyl groups with less hydrophobic or more polar groups resulted in only weakly active or inactive porphyrins. Deuteroporphyrin IX, which lacks substituents on positions 2 and 4, caused only partial enzyme activation. Therefore, hydrophobic interactions between porphyrins and guanylate cyclase are essential for maximal enzyme activation. These observations are strikingly similar to those made with analogous structural modifications of ferro-protoporphyrin IX with respect to binding native human globin (16). As has been suggested for hemoglobin (15, 17), the hydrophobic binding sites in guanylate cyclase may lie buried in the interior of the molecule. Hydrophilic substitutions on porphyrins are not likely to interact with interiorized hydrophobic sites in hemoproteins (18).

Propionic acid residues at positions 6 and 7 of heme form electrostatic bonds with basic groups such as arginine in the apoprotein to form stable hemoprotein complexes (19). A similar interaction between protoporphyrin IX and guanylate cyclase is possible in view of the observations that the dimethyl ester of protoporphyrin IX was only a weak enzyme activator and the dimethyl esters of hematoporphyrin IX and deuteroporphyrin IX were enzyme inhibitors. Moreover, the location of vicinal propionate groups at positions 6 and 7 appears to be critical for enzyme activation. These propionic acid groups, which are ionized at pH 7.4, may form ionic interaction with positively charged groups in guanylate cyclase and thereby contribute to the binding of porphyrins. This view is consistent with previous findings that isoelectric precipitation (5, 20) or isoelectric focusing (6) of guanylate cyclase results in the detachment of heme and renders the enzyme unresponsive to NO in the absence of added heme.

Structural alterations (methylation) at positions 6 and 7 of porphyrins produced more marked changes in guanylate cyclase activity, as illustrated in Fig. 4. The hydrophobic vinyl side chains at positions 2 and 4 and the negatively charged carboxyl groups of the vicinal propionate groups at positions 6 and 7 contribute to the binding of porphyrins. Substitution of the two vinyl groups with less hydrophobic or more polar groups resulted in only weakly active or inactive porphyrins. Deuteroporphyrin IX, which lacks substituents on positions 2 and 4, caused only partial enzyme activation.
cyclase as well as x-ray crystallographic studies should enable would be unlikely for N-phenylprotoporphyrin the observations that protoporphyrin uents, which likely exhibit considerably less conformational contrast to the enzyme binding sites for the 6- and 7-substit-

Phosphorylase as well as x-ray crystallographic studies should enable would be unlikely for N-phenylprotoporphyrin

-phenylprotoporphyrin IX, suggesting that all of these porphyrins interact or bind at a common site on guanylate cyclase. The coordinately bound metal influences the binding of metalloporphyrins to the enzyme as indicated by the wide range of $K_i$ values. Metal binding likely involves coordination to a nearby amino acid residue. An important question that remains to be answered is why metallation of protoporphyrin IX converts the latter from a potent activator to an inhibitor of guanylate cyclase. One possibility is that metal coordination to guanylate cyclase may inhibit enzymatic activity. Disruption of metal coordination with continued porphyrin binding to the enzyme could expose the active site, perhaps through a localized conformational change, and result in enzyme activation. This view is consistent with the observations that guanylate cyclase activation by NO has an absolute requirement for heme and is attributed to the formation of NO-heme (4–6, 10). That is, unlike the iron in heme which is positioned within the plane of the planar porphyrin ring, the iron in NO-heme protrudes out of plane (21–24), and this could result in the loss of coordination of heme iron to guanylate cyclase, as appears to occur in other nitrosyl-hemoproteins (22). It is perhaps for this reason that NO-heme closely resembles protoporphyrin IX with respect to the activation of guanylate cyclase (9).

Like NO, S-nitroso-N-acetylpenicillamine activates soluble guanylate cyclase in a heme-dependent manner, involving the formation of the nitrosyl adduct of enzyme-bound heme (4, 5, 10). This NO-dependent heme binding site appears to be the same site which binds protoporphyrin IX because metalloporphyrins, N-phenylprotoporphyrin IX, and weakly active porphyrins markedly inhibited enzyme activation by NO or S-nitroso-N-acetylpenicillamine. In addition, guanylate cyclase reconstituted with one porphyrin was refractory to activation by a second porphyrin or NO or S-nitroso-N-acetylpenicillamine. Enzyme reconstitution with zinc- or manganese-protoporphyrin IX rendered the enzyme completely unresponsive to NO. The latter observations suggest that heme, which is required for enzyme activation by NO, was displaced from its binding site by metalloporphyrins with a higher affinity for the enzyme. Collectively, these studies suggest that nitroso and porphyrin activators interact at a common site on guanylate cyclase to cause activation.

These conclusions are consistent with previous observations that protoporphyrin IX, NO, nitroso compounds, and NO-heme activate guanylate cyclase by kinetically identical mechanisms (9). Moreover, iron-phenyl heme complexes, the active intermediates of phenylhydrazine, activate guanylate cyclase in a kinetically similar manner (7). NO, nitroso compounds, phenylhydrazine, and related activators appear to react with enzyme-bound heme to generate a modified porphyrin which structurally resembles protoporphyrin IX in its interaction with guanylate cyclase. Therefore, the direct modification of heme-bound guanylate cyclase may be one unifying mechanism involved in the regulation of guanylate cyclase activity and tissue cyclic GMP formation by NO, nitroso compounds, phenylhydrazine, and other agents which generate free radicals.

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**REFERENCES**


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### Table III

<table>
<thead>
<tr>
<th>Test agents*</th>
<th>Unreconstituted</th>
<th>Protoporphyrin IX</th>
<th>Deuteroporphyrin IX</th>
<th>N-Phenylprotoporphyrin IX</th>
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<tbody>
<tr>
<td>None</td>
<td>12</td>
<td>504</td>
<td>282</td>
<td>14</td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td>0.1 µM</td>
<td>538</td>
<td>493</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>550</td>
<td>496</td>
<td>388</td>
</tr>
<tr>
<td>Deuteroporphyrin IX</td>
<td>1 µM</td>
<td>84</td>
<td>487</td>
<td>261</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>212</td>
<td>369</td>
<td>243</td>
</tr>
<tr>
<td>S-Nitroso-N-acetylpenicillamine, 100</td>
<td>700</td>
<td>510</td>
<td>254</td>
<td>42</td>
</tr>
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</table>

*Unreconstituted and reconstituted forms of guanylate cyclase were prepared as described under “Experimental Procedures.” Test agents were added to enzyme reaction mixtures at 37 °C immediately after enzyme. Reaction mixtures (0.2 ml) also contained 40 mM TEA-HCl, pH 7.4, 2 mM dithiothreitol, 1 mM GTP, and 3 mM Mg$^2+$ and were incubated at 37 °C for 10 min.

Each value is the mean of triplicate determinations from one of two separate experiments.

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clase activation than did larger alterations at positions 2 and 4. Since several functional groups of various sizes are apparently accommodated by enzyme binding sites for the 2- and 4-porphyrin substituents, it would appear that these sites exhibit a high degree of conformational flexibility. This is in contrast to the enzyme binding sites for the 6- and 7-substituents, which likely exhibit considerably less conformational flexibility. Knowledge of the amino acid sequence of guanylate cyclase as well as X-ray crystallographic studies should enable a more precise interpretation of the present observations.

An open central core in the porphyrin ring is essential for maximal activation of guanylate cyclase. The addition of a methyl group to the pyrrolic nitrogen resulted in decreased enzyme activation and a more bulky phenyl group abolished enzyme activation. N-Phenylproporphyrin IX was a competitive inhibitor of protoporphyrin IX. Thus, although both porphyrins appear to bind at a common site on guanylate cyclase, they elicit opposing effects on enzymatic activity. The reason for these extraordinary differences in properties between protoporphyrin IX and the two pyrrole adducts is presently unknown. Resolution of this important question could provide some insight into the mechanism by which porphyrin IX activates guanylate cyclase. One plausible mechanism is that enzyme-bound protoporphyrin IX may bind MgGTP and/or Mg$^2+$ and, thereby, facilitate their binding to guanylate cyclase. Preliminary experiments indicated that albumin-bound protoporphyrin IX binds MgGTP and MnGTP but not free GTP. This possibility is consistent with the observations that protoporphyrin IX and structurally related porphyrins cause a 2-3-fold increase in apparent affinities of guanylate cyclase for both MgGTP and uncomplexed Mg$^2+$. Such an interaction with substrate or metal would be unlikely for N-phenylprotoporphyrin IX because of the projection of the phenyl moiety into the open central core of the porphyrin ring. Thus, N-phenylprotoporphyrin IX is a competitive inhibitor of protoporphyrin IX rather than an activator of guanylate cyclase.

Metalloporphyrins also displayed inhibitory rather than stimulatory effects on guanylate cyclase. The metalloporphyrins were competitive inhibitors of protoporphyrin IX, suggesting that all of these porphyrins interact or bind at a common site on guanylate cyclase. The coordinately bound metal influences the binding of metalloporphyrins to the enzyme as indicated by the wide range of $K_i$ values. Metal binding likely involves coordination to a nearby amino acid residue. An important question that remains to be answered is why metallation of protoporphyrin IX converts the latter from a potent activator to an inhibitor of guanylate cyclase. One possibility is that metal coordination to guanylate cyclase may inhibit enzymatic activity. Disruption of metal coordination with continued porphyrin binding to the enzyme could expose the active site, perhaps through a localized conformational change, and result in enzyme activation. This view is consistent with the observations that guanylate cyclase activation by NO has an absolute requirement for heme and is attributed to the formation of NO-heme (4–6, 10). That is, unlike the iron in heme which is positioned within the plane of the planar porphyrin ring, the iron in NO-heme protrudes out of plane (21–24), and this could result in the loss of coordination of heme iron to guanylate cyclase, as appears to occur in other nitrosyl-hemoproteins (22). It is perhaps for this reason that NO-heme closely resembles protoporphyrin IX with respect to the activation of guanylate cyclase (9).

Like NO, S-nitroso-N-acetylpenicillamine activates soluble guanylate cyclase in a heme-dependent manner, involving the formation of the nitrosyl adduct of enzyme-bound heme (4, 5, 10). This NO-dependent heme binding site appears to be the same site which binds protoporphyrin IX because metalloporphyrins, N-phenylprotoporphyrin IX, and weakly active porphyrins markedly inhibited enzyme activation by NO or S-nitroso-N-acetylpenicillamine. In addition, guanylate cyclase reconstituted with one porphyrin was refractory to activation by a second porphyrin or NO or S-nitroso-N-acetylpenicillamine. Enzyme reconstitution with zinc- or manganese-protoporphyrin IX rendered the enzyme completely unresponsive to NO. The latter observations suggest that heme, which is required for enzyme activation by NO, was displaced from its binding site by metalloporphyrins with a higher affinity for the enzyme. Collectively, these studies suggest that nitroso and porphyrin activators interact at a common site on guanylate cyclase to cause activation.

These conclusions are consistent with previous observations that protoporphyrin IX, NO, nitroso compounds, and NO-heme activate guanylate cyclase by kinetically identical mechanisms (9). Moreover, iron-phenyl heme complexes, the active intermediates of phenylhydrazine, activate guanylate cyclase in a kinetically similar manner (7). NO, nitroso compounds, phenylhydrazine, and related activators appear to react with enzyme-bound heme to generate a modified porphyrin which structurally resembles protoporphyrin IX in its interaction with guanylate cyclase. Therefore, the direct modification of heme-bound guanylate cyclase may be one unifying mechanism involved in the regulation of guanylate cyclase activity and tissue cyclic GMP formation by NO, nitroso compounds, phenylhydrazine, and other agents which generate free radicals.
Modulation of Guanylate Cyclase by Porphyrins

Regulation of soluble guanylate cyclase activity by porphyrins and metalloporphyrins.
L J Ignarro, B Ballot and K S Wood


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