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ACTIVATION OF HEPATIC GUANYLATE CYCLASE BY NITROSYL–HEME COMPLEXES

COMPARISON OF UNPURIFIED AND PARTIALLY PURIFIED ENZYME

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Abstract—The objective of this study was to evaluate the effects of several agents on activation of both unpurified and partially purified hepatic soluble guanylate cyclase by preformed NO (nitric oxide or nitrosyl)–heme complexes. Guanylate cyclase was activated by NO complexes of the heme compounds, hematin, hemoglobin, myoglobin, catalase and cytochrome *c*, and also by the reaction product of NO and ferredoxin, a non-heme, iron sulfur electron transfer protein. NO–lipoxygenase, which contains non-heme iron, did not activate guanylate cyclase. NO–heme complexes activated unpurified enzyme almost equally well in the presence of either Mg^{2+} or Mn^{2+}. However, activation of purified (350- to 750-fold) guanylate cyclase was markedly greater with Mg^{2+} than with Mn^{2+}. At concentrations that did not alter basal enzymatic activity, Ca^{2+} markedly inhibited guanylate cyclase activation in the presence of Mg^{2+} but not of Mn^{2+}. Hemoproteins inhibited activation of unpurified and purified enzyme by NO–heme complexes, and increasing the concentrations of the latter overcame the inhibition. Gel filtration studies indicated that uncomplexed and NO-complexed hematin bind to common or adjacent sites on guanylate cyclase. Whereas DL-dithiothreitol enhanced activation, ferricyanide, cystine, *o*-iodosobenzoic acid and ethacrynic acid inhibited activation of guanylate cyclase by NO–heme complexes. The data indicate that the effects of these diverse agents on guanylate cyclase activation by preformed NO–heme are similar to their effects on enzyme activation by NO and nitroso compounds, both of which readily form NO–heme complexes. Therefore, the effects of these diverse agents may be on guanylate cyclase rather than on NO–heme formation.

A variety of nitrogen-containing compounds, including azide, hydroxylamine, $NaNO_2$, organic nitrates, nitroso compounds and NO†, have been reported to activate guanylate cyclase [1–6]. Certain studies suggested that NO may be the common intermediate species responsible for enzyme activation [5, 6], Many of the nitrogen-containing activators of guanylate cyclase are capable of releasing NO under specified conditions [7] or of reacting with certain hemoproteins to form NO [8–11]. A recent report [11] indicated that, unlike unpurified preparations of hepatic guanylate cyclase, the partially purified enzyme was not activated by NO, nitroso compounds or azide unless hemoproteins were added back to the enzyme reaction mixtures. Heme reacted with these compounds to form NO–heme complexes, which were potent activators of guanylate cyclase [11]. Contrariwise, another group reported [12] that heme was not required for guanylate cyclase activation by NO and related agents. Observations from this laboratory [13] were essentially in agreement with the latter findings [12]; however, an absolute requirement for thiols was found for nitroprusside- and nitrosoguanidine-activation of guanylate cyclase. Additional studies revealed that nitroso compounds, $NaNO_2$ and organic nitrates activated guanylate cyclase by reacting with thiols to form *S*-nitrosothiols, which are potent enzyme activators [7, 13, 14]. Therefore, the data from various laboratories suggest that diverse substances may be either required for, or may influence, guanylate cyclase activation, depending upon the particular nitrogen-containing enzyme activator.

Based on the apparent requirement of heme for guanylate cyclase activation by NO, the formation of NO–heme complexes was suggested to be a requisite for enzyme activation [11]. Thiols, oxidants, reductants, hemoproteins and other substances are known to alter activation of guanylate cyclase by NO and related agents [2, 5, 6, 11, 15–17]. Conceivably, such alterations could have been due to effects on the enzyme and/or effects on NO–heme complex formation, especially in experiments with unpurified enzyme. The present study was performed, therefore, to evaluate the influence of several agents on activation of both unpurified and partially purified hepatic guanylate cyclase by preformed NO-heme complexes.

MATERIALS AND METHODS

Materials. [^{3}H]Cyclic GMP (2–3 Ci/mmole, ammonium salt) and α-[^{32}P]GTP (10–16 Ci/mmole, triethylammonium salt) were purchased from the

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† Abbreviations: NO, nitric oxide or nitrosyl; cyclic GMP, guanosine 3′,5′-monophosphate; MIX, 1-methyl-3-isobutylxanthine; and DTT, DL-dithiothreitol.

New England Nuclear Corp., Boston, MA. Dowex-50(H^+) was purchased from the Sigma Chemical Co., St. Louis, MO (Dowex-50W, 50 × 4–400, hydrogen form) as was Sephadex G-25 (50–150 μm), and neutral alumina (Woelm) was obtained from ICN Nutritional Biochemicals, Cleveland, OH. DE52 cellulose for column chromatography and Bio-Gel A-0.5m were purchased respectively, from Whatman, Kent, U.K., and Bio-Rad Laboratories, Richmond, CA. NO gas (99.9%) was obtained from Matheson Gas, Rutherford, NJ. Heme and non-heme iron compounds were purchased from the Sigma Chemical Co., and their origins and purities were as follows: hemoglobin and methemoglobin, bovine blood, twice crystallized; myoglobin, equine skeletal muscle, crystalline; catalase, bovine liver, purified powder; hematin, bovine blood; cytochrome *c*, equine heart, 98 per cent purified; ferredoxin, *Clostridium pasteurianum*, Type V purity; lipoxygenase, soybean, Type I purified lipoxidase; ferritin, horse spleen. Bovine serum albumin, L-histidine, ferricyanide, ethacrynic acid, cystine, *o*-iodosobenzoic acid, and DTT were purchased from the Sigma Chemical Co. All H_2O used in these experiments was twice glass-distilled.

Preparation of unpurified and partially purified hepatic guanylate cyclase. Rats (mixed sex, 250–300 g, Sprague–Dawley) were fasted for 24 hr, decapitated, and exsanguinated; the livers were quickly perfused *in situ* (portal vein) with 10 ml of ice-cold 10 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose (sucrose-buffer). Livers were excised, minced finely with scissors, and pressed through a stainless steel grid containing holes (1.5 mm dia.) with the aid of a tissue press (Harvard Apparatus, Inc., Millis, MA). The pressed liver was filtered on Nitex No. 110 nylon filament bolting cloth (50 μm pore dia., Tobler, Ernst & Traber, Inc., Elmsford, NY) with 15 vol. of cold sucrose-buffer or until the filtrate was devoid of visible bloody color. Homogenates (20%, w/v) were prepared in cold sucrose-buffer with the aid of a Potter–Elvehjem tissue grinder (Teflon pestle; 0.008 in. clearance; 1,200 rpm). Homogenates were centrifuged at 1,000 *g* for 20 min at 4° (Sorvall) and the resulting supernatant fractions were further centrifuged at 105,000 *g* for 60 min at 4° (Beckman). High speed supernatant fractions (unpurified guanylate cyclase) were stored at −85°. Concentrations of protein ranged from 5 to 8 mg/ml. Guanylate cyclase was partially purified by a modification of procedures described previously [11]. High speed hepatic supernatant fractions were prepared as described above except that the pressed liver was homogenized in 10 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose, 2 mM DTT, and 0.5 mM EDTA. Guanylate cyclase was recovered from supernatant fractions by adding solid ammonium sulfate to 40% saturation (0–4°). The precipitate was collected by centrifugation, suspended in 20 ml of 10 mM Tris–HCl, pH 7.4, containing 2 mM DTT and 0.5 mM EDTA (DTT-buffer), and dialyzed against 2 liters of DTT-buffer for 16 hr at 0–4°. After centrifugation of the dialyzed mixture, the clear supernatant fraction was applied to a 1.5 × 25 cm column of DE52 cellulose equilibrated with DTT-buffer and the column was eluted with a linear NaCl gradient (0 to 0.3 M, 250 ml) in DTT-buffer (16 ml/hr; 4-ml fractions). Guanylate cyclase-rich fractions, eluting in 0.18 to 0.19 M NaCl, were pooled, concentrated by ultrafiltration, applied to a 1.5 × 25 cm column of Bio-Gel A-0.5 m equilibrated with DTT-buffer containing 0.1 M NaCl, and eluted with the same (16 ml/hr; 2-ml fractions). Fractions rich in guanylate cyclase activity were stored under O_2-free N_2 at −85°. Just prior to use, enzyme fractions were subjected to gel filtration (1.5 × 5 cm column of Sephadex G-25 equilibrated and eluted with 10 mM Tris–HCl, pH 7.4, containing 0.1 M NaCl) to remove DTT and EDTA. Specific basal activities (MgGTP substrate) of various unpurified and partially purified preparations of guanylate cyclase were 0.002 to 0.004 and 0.70 to 2.1 nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$, respectively, representing a 350- to 750-fold enzyme purification.

Heme determination. Fractions derived from each purification step were assayed for heme, after reduction with dithionite, by visible absorption spectroscopy [18]. Over 90 per cent of the heme was eliminated by liver perfusion and mince washing, and the remainder was removed during ammonium sulfate precipitation and chromatography on DE52 cellulose. No heme was detected in Bio-Gel fractions containing guanylate cyclase activity. Since the limit of sensitivity was 0.005 to 0.01 μM heme, and because enzyme fractions were diluted 100-fold in enzyme reaction mixtures, the concentration of heme in final enzyme incubates was less than 0.1 nM.

Formation of NO-heme and other NO complexes. NO-heme complexes were prepared by a modification of the procedure developed for hemoglobin by Kon [19]. Methemoglobin (0.05 to 0.2 mM), myoglobin (0.2 mM), catalase (0.05 mM), hematin (0.05 to 0.2 mM), and cytochrome *c* (0.05 mM) solutions in 50 mM Tris–HCl, pH 7.0, were equilibrated with O_2-free N_2 and reacted with NO [19] to form the corresponding nitrosyl (NO)–heme complexes [8, 10, 19–22]. Lipoxygenase (0.1 mM) was similarly reacted with NO to form NO–lipoxygenase [23]. Formation of NO complexes with the non-heme iron proteins, ferredoxin and ferritin, has not been described, although previous studies would suggest that such a reaction is likely [23–25]. Reactions between 0.05 mM ferredoxin or ferritin and NO were conducted as described above for heme-containing compounds. Amounts of NO–heme complexes formed in solution were determined by visible absorption spectroscopy [8, 10, 20, 26], employing the following extinction coefficients ($mM^{-1}\,cm^{-1}$): NO–hemoglobin $E^{545} = 12.6$, $E^{575} = 13$; methemoglobin $E^{545} = 5.8$, $E^{575} = 4.2$; NO–myoglobin $E^{422} = 125$; myoglobin $E^{581} = 14.6$; NO–catalase $E^{580} = 16.7$; and catalase $E^{505} = 11.4$. Amounts of NO complexes formed with hematin, cytochrome *c* or lipoxygenase were not estimated, and complete conversion was assumed [20–23]. No attempts were made to characterize the product of the reaction between NO and ferredoxin or ferritin.

Guanylate cyclase assay and other procedures. Guanylate cyclase activity and verification of cyclic GMP formation were performed exactly as described previously [13, 17]. α-[^{32}P]GTP (3–5 × 10^5 cpm) and [^{3}H]cyclic GMP (3–5 × 10^4 cpm) were included in all

Table 1. Effects of hematin and hemoglobin on basal and NO-stimulated activity of partially purified guanylate cyclase*

Additions (μM)	Guanylate cyclase activity [nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$]	
	Basal	1 μl NO
None	1.1 ± 0.08	19 ± 0.7
Hematin, 0.01	1.2 ± 0.09	18 ± 0.8
0.03	1.0 ± 0.10	25 ± 0.9
0.10	1.1 ± 0.05	38 ± 1.8
0.30	1.0 ± 0.05	27 ± 0.7
Hemoglobin, 0.01	1.2 ± 0.10	20 ± 0.5
0.03	1.2 ± 0.08	18 ± 0.8
0.10	1.0 ± 0.08	35 ± 1.9
0.30	1.1 ± 0.06	26 ± 0.7

* Reaction mixtures (1 ml) contained 1 mM GTP, 3 mM Mg^{2+}, 12–20 μg protein, and the additions indicated above, and were incubated for 10 min at 37°. Reactions were started, 1 min after warming mixtures to 37°, by the addition of enzyme fraction, followed within 2 sec with NO. Basal signifies omission of NO. Hemoglobin was prepared from methemoglobin by standard procedures involving reduction with dithionite at 4°, pH 7.0, under O_2-free N_2, and gel filtration (Sephadex G-25) to remove dithionite; complete conversion was verified spectrophotometrically. Data are means ± S.E.M. for six determinations from three separate experiments.

enzyme reaction mixtures. Protein concentrations of enzyme fractions were determined by the method of Lowry *et al.* [27], using bovine serum albumin as standard.

RESULTS

Effects of Mg^{2+}, Mn^{2+} and other metals on guanylate cyclase activity. Basal enzymatic activity and activity stimulated by NO, NO–catalase, NO–hemoglobin or NO–myoglobin (amount of cyclic GMP formed per mg protein) using either unpurified or partially purified hepatic guanylate cyclase were linear for at least 5 min at 37° under the experimental conditions employed (data not shown). Rates of cyclic GMP formation were proportional to protein concentrations when incubation times were limited to 5 min. Partially purified guanylate cyclase preparations were devoid of heme as described in Materials and Methods. The data in Table 1 illustrate that NO activated guanylate cyclase in the absence of added heme and that addition of either hematin or hemoglobin enhanced enzyme activation by NO without altering basal enzymatic activity. The amount (1 μl) of NO tested was predetermined to be that which caused maximal enzyme activation. At higher concentrations, both hematin and hemoglobin caused less enhancement (Table 1) or inhibited enzyme activation without altering basal enzymatic activity (data not shown). The observations that hematin and hemoglobin did not affect enzyme activation until their concentrations reached

Table 2. Effects of various metals on basal and NO–heme-stimulated activities of unpurified and partially purified guanylate cyclase*

Cation additions (mM)	Guanylate cyclase activity [nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$]					
	Unpurified			Partially purified		
	Basal	NO–catalase	NO–hemoglobin	Basal	NO–catalase	NO–hemoglobin
Mn^{2+}, 3	0.032	0.46	0.44	4.4	9.8	9.2
Mn^{2+}, 1	0.008	0.59	0.57	2.6	13	12
+ Ca^{2+}, 0.1	0.009	0.60	0.56	2.5	14	13
+ La^{3+}, 0.01	0.010	0.055	0.048	2.4	5.7	4.9
Mg^{2+}, 1	0.001	0.19	0.17	0.54	11	5.3
Mg^{2+}, 3	0.003	1.2	1.2	0.98	34	12
+ Ca^{2+}, 0.1	0.003	0.35	0.31	0.94	9.7	4.8
+ La^{3+}, 0.01	0.004	0.030	0.026	0.99	2.2	2.0

* Reaction mixtures (1 ml) for unpurified enzyme contained 1 mM GTP, 0.3 mM MIX, 310–380 μg protein, and the cations indicated above. Those for partially purified enzyme contained 1 mM GTP, 18–32 μg protein, and the cations indicated. Mixtures were incubated for 5 min at 37°. Reactions were started, 1 min after warming mixtures to 37°, by addition of enzyme fraction, followed within 2 sec with either 1 μM NO–catalase or 1 μM NO–hemoglobin. Basal signifies omission of NO–heme complex. Data are means of duplicate determinations from three separate experiments. S.E.M. varied by no more than 8 per cent of the corresponding mean (not shown). Omission of Mn^{2+} or Mg^{2+} from any of the above reaction mixtures yielded undetectable product formation.

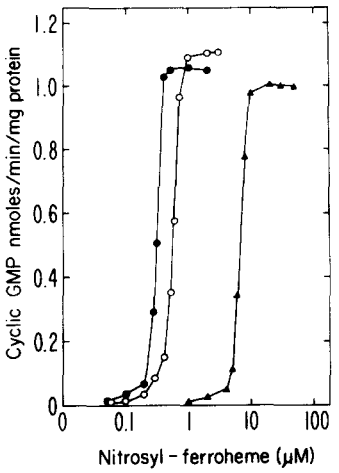

Fig. 1. Relationship between concentrations of nitrosyl (NO)–ferroheme complexes and activation of unpurified guanylate cyclase. Reaction mixtures (1 ml) contained 0.3 mM GTP, 3 mM Mg^{2+}, 0.3 mM MIX, 380 μg protein and the NO–ferroheme complexes indicated, and were incubated for 5 min at 37°. Reactions were started, 1 min after warming mixtures to 37°, by addition of enzyme fraction, followed within 2 sec with NO–ferroheme complex as indicated. Key: (●) NO–catalase; (○) NO–hemoglobin; and (▲) NO–myoglobin. Concentrations indicated on the abscissa are on log scale. Basal guanylate cyclase activity was 0.004 ± 0.001 nmole cyclic GMP·min^{-1}·(mg protein)$^{-1}$]. Data represent the averages of duplicate determinations from a single experiment. Two to three additional experiments, each using a different batch of enzyme fraction (280–355 μg protein), yielded qualitatively similar data.

approximately 0.03 and 0.10 μM, respectively, support the argument that, if any heme was present in enzyme preparations, it did not influence enzyme activation by NO.

Unpurified guanylate cyclase was activated by NO–catalase or NO–hemoglobin in the presence of either Mg^{2+} or Mn^{2+} (Table 2), but not in the presence of Ca^{2+} or La^{3+} (not shown). Mg^{2+} was more effective than Mn^{2+} in supporting stimulated enzymatic activity, whereas the reverse was true for basal enzymatic activity. In the presence of Mg^{2+}, both Ca^{2+} and La^{3+} inhibited activation of guanylate cyclase without affecting basal enzymatic activity (Table 2). In the presence of Mn^{2+}, however, guanylate cyclase activity was unaffected by Ca^{2+}, whereas La^{3+} inhibited enzyme activation. Activation of partially purified guanylate cyclase by NO–catalase and NO–hemoglobin was much more prominent in the presence of Mg^{2+} and of Mn^{2+} (Table 2). Whereas increasing the concentration ratio of Mg^{2+} to GTP 3-fold also increased enzyme activation, increasing that of Mn^{2+} to GTP actually decreased enzyme activation. Neither basal nor stimulated (NO–heme complexes) activity of partially purified enzyme was detectable when either Ca^{2+} or La^{3+} replaced Mg^{2+} or Mn^{2+} (data not shown). The effects of Ca^{2+} and La^{3+} on activity of partially purified guanylate cyclase were qualitatively similar to those observed with unpurified enzyme (Table 2).

Effects of NO complexes of various iron-containing agents on guanylate acyclase activity. NO–catalase, NO–hemoglobin and NO–myoglobin activated unpurified hepatic guanylate cyclase in a concentration-dependent manner (Fig. 1). NO–catalase was slightly more potent than NO–hemoglobin, which was about 10-fold more potent than NO–myoglobin. The nitrosyl (NO) complexes of hematin and cytochrome *c* (two heme iron-containing agents) and of ferredoxin (which contains non-heme iron) activated unpurified guanylate cyclase in a concentration-dependent manner (Table 3). However, the NO complex of the non-heme iron protein (lipoxygenase) failed to activate the enzyme. Qualitatively similar observations were made with partially purified guanylate cyclase, except that the minimally effective concentrations of NO complexes were 5- to 10-fold lower than those required for activation of unpurified enzyme. In addition, reactions of NO with ferritin, $FeCl_2$, and complexes of Fe^{2+}–histidine or Fe^{2+}–albumin, which are well known to form paramagnetic nitrosyl-complexed species [24, 25], failed to activate unpurified or partially purified guanylate cyclase (data not shown).

Effects of hemoproteins on activation of guanylate cyclase by NO–heme complexes. Due to the very

Table 3. Effects of NO complexes of various iron-containing agents on activity of unpurified guanylate cyclase*

	Guanylate cyclase activity [nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$]		
	Concentration (μM) of NO complex		
NO complex	0.2	1.0	5.0
Hematin	0.12 ± 0.008	0.79 ± 0.04	1.1 ± 0.04
Cytochrome *c*	0.19 ± 0.01	1.3 ± 0.03	1.5 ± 0.03
Ferredoxin	0.060 ± 0.007	0.56 ± 0.02	0.58 ± 0.04
Lipoxygenase	0.003 ± 0.001	0.004 ± 0.001	0.002 ± 0.001

* Reaction mixtures (1 ml) contained 0.3 mM GTP, 3 mM Mg^{2+}, 0.3 mM MIX, 280–350 μg protein, and the NO complexes indicated above and were incubated for 5 min at 37°. Reactions were started, 1 min after warming mixtures to 37°, by addition of enzyme fraction, followed within 2 sec with NO complex. NO complexes were prepared as described in Materials and Methods. Basal enzymatic activity (omission of NO complexes) was 0.003 + 0.001 nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$]. Data are means ± S.E.M. for six determinations from three separate experiments.

Table 4. Effects of hemoproteins on activation of unpurified guanylase cyclase by NO–catalase, NO–hemoglobin and NO–myoglobin*

Additions (μM)	Guanylate cyclase activity [nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$]					
	NO–catalase		NO–hemoglobin		NO–myoglobin	
	0.5 μM	1 μM	0.4 μM	1 μM	4 μM	10 μM
None	1.8 ± 0.09	1.8 ± 0.05	1.6 ± 0.08	1.8 ± 0.05	1.4 ± 0.07	1.5 ± 0.04
Methemoglobin, 2	1.1 ± 0.06	1.7 ± 0.04	1.7 ± 0.06	1.8 ± 0.05	1.4 ± 0.06	1.4 ± 0.05
25	0.41 ± 0.02	1.1 ± 0.03	0.59 ± 0.04	1.0 ± 0.04	0.04 ± 0.004	0.82 ± 0.03
50	0.02 ± 0.002	0.85 ± 0.04	0.05 ± 0.003	0.71 ± 0.03		0.34 ± 0.01
Myoglobin, 2	1.9 ± 0.10				1.5 ± 0.05	
25	0.14 ± 0.01	1.7 ± 0.05	1.5 ± 0.05	1.7 ± 0.06	0.07 ± 0.004	1.5 ± 0.05
50		1.7 ± 0.04	0.12 ± 0.005	1.7 ± 0.04		1.4 ± 0.04
Catalase, 2	1.9 ± 0.08	1.8 ± 0.05	1.7 ± 0.07	1.7 ± 0.06	1.3 ± 0.04	1.5 ± 0.05
25	0.71 ± 0.03	0.90 ± 0.04	0.97 ± 0.04	1.7 ± 0.05	0.08 ± 0.006	0.99 ± 0.03

* Reaction mixtures (1 ml) contained 0.3 mM GTP, 3 mM Mg^{2+}, 0.3 mM MIX, 280–380 μg protein, and the additions indicated above, and were incubated for 5 min at 37°. Reactions were started, 1 min after warming mixtures to 37°, by addition of enzyme fraction, followed within 2 sec with NO–heme complex as indicated. Basal guanylate cyclase activity was 0.004 ± 0.001 [nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$] and was not altered by the hemoproteins indicated. Data are means ± S.E.M. for eight determinations from four separate experiments.

steep slope of the concentration–effect curves of NO–heme complexes (Fig. 1), it was difficult to construct reproducible curves from data obtained in the presence of various concentrations of hemoprotein. Therefore, it was difficult to assess the nature of the inhibition. The following experimental design, however, yielded data (Table 4) which were helpful in understanding the inhibitory effects of the hemoproteins. Two concentrations of each NO–heme complex were tested. The smaller of the two represents that which barely elicited maximal enzyme activation (predetermined experimentally; data not shown). Slightly smaller amounts of NO–heme complex elicited highly variable data because of the extremely narrow concentration–effect relationship (Fig. 1). Hemoprotein concentrations tested were 2–50 μM, except for those of catalase which were limited to 2 and 25 μM because of solubility/denaturation problems. At the concentrations tested, hemoproteins failed to alter basal guanylate cyclase activity. The data in Table 4 indicate that inhibition by hemoproteins of enzyme activation was concentration dependent, being partially overcome by increasing concentrations of NO–heme. The simplest interpretation of these observations is that hemoproteins and NO–heme complexes interacted with the same or closely adjacent binding sites on guanylate cyclase. In effect, the inhibition of enzyme activation may have been competitive with NO–heme in the sense that higher concentrations of NO-heme complex overcame the inhibition (Table 4).

Table 5. Effect of hematin on the isolation of activated partially purified guanylate cyclase from reaction mixtures by gel filtration*

Preincubation reaction mixture for gel filtration	Guanylate cyclase activity of incubates [nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$]	
	− NO	+ NO
Enzyme alone	2.1 ± 0.1	36 ± 3
Enzyme + 1 μl NO	2.0 ± 0.2	38 ± 4
Enzyme + 0.5 μM hematin	2.1 ± 0.1	49 ± 6
Enzyme + 0.5 μM NO–hematin	48 ± 5	49 ± 3
Hematin-treated enzyme + 0.5 μM NO–hematin	14 ± 2	47 ± 5
NO–hematin-treated enzyme + 0.5 μM hematin	25 ± 3	46 ± 4

* Partially purified guanylate cyclase (10–20 μg protein) was preincubated (0.5 ml) for 5 min at 0° in 40 mM Tris–HCl, pH 7.4, containing NO, hematin or NO–hematin, in an atmosphere of O_2-free N_2. Preincubations involving hematin- or NO-hematin-treated enzyme signify initial 5-min preincubations of enzyme plus either 0.5 μM hematin or 0.5 μM NO–hematin followed by an additional 5 min of preincubation after addition of either NO–hematin or hematin respectively. Preincubates were applied to 1.5 × 5 cm columns of Sephadex G-25 previously equilibrated with O_2-free 40 mM Tris–HCl, pH 7.4, containing 0.1 M NaCl, and columns were eluted with the same buffer at 4° under N_2. Aliquots of 0.2 ml of 1-ml eluates (in the void volume) were tested in enzyme incubations. Reaction mixtures (1 ml) contained 0.1 mM GTP, 3 mM Mg^{2+}, and appropriate column eluates in the absence and presence of 1 μl NO, and were incubated for 10 min at 37°. Reactions were started 1 min after warming mixtures at 37° by addition of column eluates, followed within 2 sec with NO (where indicated). Data are means ± S.E.M. for six determinations from three separate experiments.

To understand better the nature of the interactions of guanylate cyclase, heme and NO–heme complex, an experiment was designed to determine the reversibility of activation of guanylate cyclase by NO and NO–hematin. Partially purified (650- to 750-fold purification) hepatic guanylate cyclase was preincubated alone or with NO, hematin, NO–hematin or combinations of the latter two, and was subjected to gel filtration to remove unreacted or unbound NO, hematin and/or NO–hematin. Eluted enzyme was then incubated in reaction mixtures with or without NO (Table 5). Enzyme preincubated with NO could not be recovered in the activated state by gel filtration, although the recovered enzyme remained sensitive to re-activation by NO. Enzyme preincubated with hematin was slightly more sensitive to activation by NO (Table 5), thus suggesting that hematin was bound to guanylate cyclase and eluted with enzyme from gel filtration columns, as was suggested previously [11]. Guanylate cyclase preincubated with NO–hematin was recovered in the activated state, and no further activation was achieved with NO. Hematin-treated enzyme was less responsive than untreated enzyme to activation by NO-hematin, and enzyme treated with NO-hematin was inhibited upon addition of hematin (Table 5). Analysis of elutes by visible absorption spectroscopy [18] revealed that heme was present in the void volume after gel filtration of hematin and/or NO–hematin only when guanylate cyclase preparations were included (not shown). The data suggest that hematin and NO-hematin were bound to guanylate cyclase, perhaps at the same or closely adjacent sites, and may have competed with each other for such sites. The duration of the activated state of guanylate cyclase preincubated with NO–hematin was not determined.

Effects of ferricyanide and thiol reactive agents on guanylate cyclase activity. Activation of partially purified guanylate cyclase by NO–heme complexes was enhanced by DTT, and inhibited by ferricyanide, cystine, *o*-iodosobenzoic acid, ethacrynic acid and methemoglobin (Table 6). The concentrations of NO–heme complexes tested were predetermined to be approximately 5-fold larger than those required to elicit maximum enzyme activation under the incubation conditions employed (data not shown). Although each of the NO–heme complexes exhibited widely different activation maxima, inclusion of DTT in reaction mixtures increased activation maxima to a common ceiling (Table 6). In this regard it is of interest that each of the NO–heme complexes activated unpurified guanylate cyclase, in the absence of added thiols, to a common ceiling (Fig. 1). Whereas methemoglobin, ferricyanide and DTT did not alter basal guanylate cyclase activity, cystine, *o*-iodosobenzoic acid and ethacrynic acid inhibited it. Finally, partially purified guanylate cyclase [basal activity: 0.95 ± 0.02 nmole cyclic GMP·min^{-1}·(mg protein)$^{-1}$] was activated by 1 μM NO–cytochrome *c* [52 ± 4 nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$], 1 mM NO–hematin [25 ± 3 nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$], and 1 μM NO–ferredoxin [18 ± 2 nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$].

DISCUSSION

Partially purified (350- to 750-fold) hepatic soluble guanylate cyclase was activated by NO, and this activation was enhanced up to 2-fold by hematin or hemoglobin. Therefore, contrary to the suggestion offered previously [11], heme was apparently not required to support activation of guanylate cyclase by NO, although heme enhanced enzyme activation by NO [28]. NO–heme complexes were suggested to be the active intermediate species of NO and nitroso compounds responsible for guanylate cyclase activation [11, 29]. This view was based on the apparent requirement of heme for enzyme activation by NO but not by NO–heme, a requirement which could not be confirmed by another group [12]. The latter group, however, did not determine the heme content of their guanylate cyclase preparations. Experiments in the present report with heme-free guanylate cyclase support the suggestion [12] that heme is not absolutely required for enzyme activation by NO. The objective of this study was to

Table 6. Effects of methemoglobin, ferricyanide and thiol reactive agents on basal and NO–heme-stimulated activity of partially purified guanylate cyclase*

Additions	Guanylate cyclase activity [nmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$]			
	Basal	NO–catalase (1 μM)	NO–hemoglobin (1 μM)	NO–myoglobin (10 μM)
None	0.77 ± 0.1	44 ± 2	16 ± 0.7	7.1 ± 0.3
Methemoglobin (25 μM)	0.81 ± 0.1	4.8 ± 0.3	0.93 ± 0.1	1.4 ± 0.1
Ferricyanide (50 μM)	0.74 ± 0.2	15 ± 0.7	6.2 ± 0.4	3.7 ± 0.1
DTT (2 mM)	0.80 ± 0.1	68 ± 3	64 ± 5	60 ± 4
Cystine (2 mM)	0.18 ± 0.03	12 ± 1	3.6 ± 0.5	1.4 ± 0.1
o-Iodosobenzoic acid (10 μM)	0.17 ± 0.02	10 ± 2	4.0 ± 0.3	1.6 ± 0.2
Ethacrynic acid (10 μM)	0.35 ± 0.06	19 ± 2	7.1 ± 0.8	3.3 ± 0.5

* Reaction mixtures (1 ml) contained 1 mM GTP, 3 mM Mg^{2+}, 25–78 μg protein, and the additions indicated above, and were incubated for 5 min at 37°. Reactions were started, 1 min after warming mixtures to 37°, by addition of enzyme fraction, followed within 2 sec with NO–heme complexes as indicated. Basal signifies omission of NO–heme complex. Data are means ± S.E.M. for four to six determinations from two to three experiments.

evaluate the effects of several agents on guanylate cyclase activation by preformed NO–heme complexes, rather than by NO itself, to avoid possible interpretive problems resulting from interactions between NO and heme to form NO-heme complexes during enzyme assay, especially when testing unpurified enzyme fractions.

Although basal guanylate cyclase activity is generally much greater in the presence of Mn^{2+} than of Mg^{2+}, enzymatic activity stimulated by NO, nitroso compounds and related agents is almost equally supported by Mn^{2+} and Mg^{2+} [30–32]. This was particularly true for unpurified enzyme fractions but not for partially purified enzyme preparations when the enzyme activators were preformed NO–heme complexes (Table 2). For example, NO–catalase activated partially purified guanylate cyclase 20- to 35-fold and 2- to 5-fold in the presence of Mg^{2+} and Mn^{2+} respectively. We were unable to confirm a previous report [11] that NO–hemoglobin activated partially purified hepatic guanylate cyclase 16- to 60-fold in the presence of Mn^{2+}. This remarkable difference between unpurified and partially purified guanylate cyclase will require further clarification. In agreement with many previous reports, neither Ca^{2+} nor La^{3+} substituted for Mg^{2+} or Mn^{2+} in supporting guanylate cyclase activity. At concentrations which did not modify basal enzymatic activity, Ca^{2+} markedly inhibited activation of guanylate cyclase by NO–heme complexes in the presence of Mg^{2+} but not Mn^{2+}. La^{3+} inhibited enzyme activation in the presence of either cation although inhibition was much greater in the presence of Mg^{2+}. Recent studies in this laboratory on activation of vascular smooth muscle guanylate cyclase by NO and other vasodilators suggest that Ca^{2+} inhibition of Mg^{2+}-dependent enzyme activation is attributable to competition between Ca^{2+} and Mg^{2+} for common binding sites on GTP, perhaps rendering GTP less suitable as substrate for activated guanylate cyclase [32].

Hemoproteins were reported to inhibit activation of guanylate cyclase by NO, nitroso compounds and related agents [11, 16, 17, 29, 33]. At first, these observations were seemingly at odds with the findings that hemoproteins reacted with NO and related agents to form NO–heme complexes, which were potent activators of guanylate cyclase [11, 29]. However, the present data, as well as those of a recent report [29], illustrate clearly that hemoproteins did in fact inhibit enzyme activation by preformed NO–heme complexes. Thus, the inhibitory effect of hemoproteins may be related to an interaction with binding sites on guanylate cyclase that interact also with NO–heme complexes. This interpretation is supported by the observations that increasing the concentration of NO–heme complexes overcame the inhibition by hemoproteins (Table 4). Unfortunately, a more direct analysis of the kinetics of inhibition by hemoproteins was not possible because of the extremely narrow concentration–effect (activation) relationship characteristic of NO–heme complexes. Similar problems were encountered by another group who recently reported [29] that various hemoproteins inhibited guanylate cyclase activation by NO–catalase. Gel filtration experiments with preincubates of partially purified (650- to 750-fold) guanylate cyclase containing NO or NO–hematin indicated that the activated form of the enzyme was recovered by gel filtration only when NO–hematin, but not NO, was used as enzyme activator. In agreement with a previous report [11], hematin was found to bind to guanylate cyclase. Moreover, NO–hematin was found in the present study to bind to the enzyme, and this most likely accounted for the recovery of NO–hematin-activated guanylate cyclase by gel filtration. Perhaps more interesting, however, is the observation that hematin, at concentrations equal to those of NO–hematin, partially inhibited the recovery of NO–hematin-activated guanylate cyclase by gel filtration. These data support our interpretation that heme is capable of competing with NO-heme for common or adjacent binding sites on guanylate cyclase.

Oxidants such as ferricyanide and methylene blue, as well as thiol alkylating agents such as ethacrynic acid, were reported to inhibit, whereas thiols enhanced, activation [2, 5, 6, 11, 14, 15, 17, 28, 29] of guanylate cyclase by NO and/or related agents. Similarly, these agents altered activation of partially purified guanylate cyclase by preformed NO–heme complexes (Table 6). Therefore, it is unlikely that the effects of oxidants and thiol reactive agents on enzyme activation by NO resulted from changes in reactivity between NO and heme to form NO–heme complexes. For example, thiols were claimed to enhance heme-supported NO activation of guanylate cyclase by facilitating the reaction between NO and heme to form NO–heme [11]. However, the present findings that DTT markedly enhanced enzyme activation by preformed NO–heme complexes suggest that alternative mechanisms are possible. One possible mechanism is a reaction between NO, nitroso compounds or NO–heme and thiols to form *S*-nitrosothiols, which are very potent activators of guanylate cyclase [7, 13, 14]. Similarly, inhibition of NO activation of guanylate cyclase by ferricyanide may not necessarily be attributed solely to inhibition of NO–heme formation as was suggested earlier [33]. Instead, as an electron acceptor, ferricyanide may directly interfere with enzyme activation, but not with basal enzymatic activity, assuming that the activation process involves electron transfer between activator (which is paramagnetic) and enzyme [3, 11]. Cystine, *o*-iodosobenzoic acid (—SH oxidant) and ethacrynic acid inhibited both basal and NO–heme-stimulated guanylate cyclase activity. These observations support the view that catalytic site —SH groups are involved in the expression of both basal and stimulated guanylate cyclase activity [34, 35].

In addition to NO complexes of heme-containing compounds, including cytochrome *c*, NO reacted with the non-heme iron sulfur protein, ferredoxin, to form a potent activator of guanylate cyclase. The NO complexes of lipoxygenase or of Fe^{2+} in solution with histidine, on the other hand, failed to activate guanylate cyclase. One property common to both cytochrome *c* and ferredoxin is the capacity to function as electron transfer molecules. Why the NO complexes of these agents, but not the native molecules themselves, activate guanylate cyclase is not understood. Presumably, the paramagnetic

nature of the NO complex together with the electron transfer property of these proteins enable them to activate the enzyme. The observations from this and another laboratory [12] that NO activates guanylate cyclase in the absence of heme suggest that guanylate cyclase itself may possess properties of an electron transfer protein.

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