Biochimica et Biophysica Acta, 673 (1981) 394—407

BBA 29558

SELECTIVE ALTERATIONS IN RESPONSIVENESS OF GUANYLATE CYCLASE TO ACTIVATION BY NITROSO COMPOUNDS DURING ENZYME PURIFICATION

LOUIS J. IGNARRO, BARBARA K. BARRY, DARLENE Y. GRUETTER, ELIOT H. OHLSTEIN, CARL A. GRUETTER, PHILIP J. KADOWITZ and WILLIAM H. BARICOS

Departments of Pharmacology and Biochemistry, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112 (U.S.A.)

(Received October 16th, 1980)

Key words: Guanylate cyclase; cyclic GMP; S-Nitrosocysteine; Nitric oxide; NO-heme complex

Summary

Partially purified, heme-free, hepatic soluble guanylate cyclase was activated by NO, *S*-nitrosocysteine and NO-heme complexes to the extent of 19—34-fold in the presence of 3 mM Mg^{2+}, but only up to 2-fold in the presence of 3 mM Mn^{2+}, when the GTP concentration was 1 mM. Even with Mg^{2+}, however, nitroprusside and nitrosoguanidine failed to activate guanylate cyclase. Dithiothreitol or cysteine and, to a much lesser extent, hematin or hemoglobin restored the capacity of nitroprusside and nitrosoguanidine to activate guanylate cyclase, and enhanced enzyme activation by NO. Restoration or enhancement of enzyme activation with thiols was unrelated to their reducing potential because nonthiol reductants such as dithionite and ascorbate failed to influence guanylate cyclase activation. Instead, thiols likely reacted with the nitroso compounds to form *S*-nitrosothiols, which were potent activators of guanylate cyclase. Similarly, heme-containing substances reacted with nitroso compounds to form NO-heme complexes, which are known to activate guanylate cyclase. Enzyme activation by nitroprusside and nitrosoguanidine was restored, and that by NO and NO-hemoglobin was enhanced, by addition of heated hepatic soluble fraction (devoid of guanylate cyclase activity). The heated soluble fraction appears to contain a partially heat-stable, thiol-containing component(s) of molecular weight greater than 5000, which may be in part responsible for the observed effects on enzyme activation. These data suggest that partial enzyme purification results in the removal of thiol components that are required for the full expression of guanylate cyclase activation.

Abbreviation: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Introduction

Recent experimental evidence suggests that activation of guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) results from complex oxidation-reduction reactions [1—8] which likely involve -SH groups [4,7—14]. NO, nitroso compounds and nitrogen-containing substances which form NO by reacting with certain hemoproteins all markedly activate unpurified guanylate cyclase [3,5,7,15—18]. Organic nitrates and nitrites, as well as $NaNO_2$, require the presence of cysteine or other thiols in order to activate guanylate cyclase [14]. Studies with partially purified enzyme have yielded conflicting views on the mechanism by which NO activates guanylate cyclase [19—21]. Craven and DeRubertis [19] reported that partially purified, heme-free, hepatic soluble guanylate cyclase resulted in the complete loss of responsiveness to NO and nitroso compounds, and that enzyme activation was restored upon addition of heme. In contrast, Braughler et al. [20,21] reported that activation of partially purified guanylate cyclase by NO and related agents displayed no apparent requirement for additional factors [20], but that enzyme activation by NO was enhanced by addition of dithiothreitol, methemoglobin and other agents [21]. However, neither determination of heme concentrations in enzyme preparations nor analysis of enzyme activation by preformed NO-heme complexes was performed by the latter investigators. In addition, no attempt was made to remove dithiothreitol from enzyme preparations prior to use and, therefore, dithiothreitol was present in enzyme reaction mixtures [20,21]. Moreover, the data reported by Craven and DeRubertis [19] and Braughler et al. [21] are subject to interpretive problems because in most of the experiments Mn^{2+} instead of Mg^{2+} was used to satisfy the divalent cation requirement of guanylate cyclase, a condition under which appreciable activation of partially purified enzyme by NO is more difficult to observe (Ref. 20; this report).

The objective of this investigation was to re-examine the conditions required for activation of guanylate cyclase by NO, nitroso compounds, NO-heme complexes and *S*-nitrosocysteine. The results of the present experiments with partially purified, heme-free, hepatic soluble guanylate cyclase indicate that meaningful interpretations are possible only when Mg^{2+}, and not Mn^{2+}, is employed in enzyme reaction mixtures. Moreover, generalizations among NO, nitroso compounds and NO-heme complexes with regard to requirements for enzyme activation are unwarranted because these requirements differ among the various compounds.

Materials and Methods

Materials

Methemoglobin (2× crystallized, bovine blood), catalase (purified, bovine liver), hematin, DL-dithiothreitol, L-cysteine, ascorbic acid, sodium dithionite, ethacrynic acid, *N*-ethylmaleimide, sodium nitroprusside and Sephadex G-25 (50—150 μm) were purchased from Sigma. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was obtained from Aldrich and nitric oxide (NO; 99.9%) and O_2-free N_2 (99.9%) were from Matheson Gas. DE52-cellulose (for column chromatography) was purchased from Whatman and Bio-Gel A-0.5m was obtained

from Bio-Rad. Sources of other reagents employed have been described [14,22,23]. All H_2O used in these experiments was twice glass-distilled. Handling and preparation of dilutions of NO in O_2-free N_2, and the solutions of nitroprusside and MNNG have been described [22—24]. Solutions of dithiothreitol, ascorbate and sodium dithionite were prepared in ice-cold H_2O or ice-cold 50 mM Tris-HCl, pH 7, and were used within 1 h. Failure to keep dithionite solutions cold resulted in decomposition, with formation of elemental sulfur. Hemoglobin was prepared from methemoglobin by reduction with dithionite as follows. Methemoglobin (2 mM) in 50 mM Tris-HCl, pH 7, was evacuated and equilibrated with N_2 for 30 min at 4°C. Working at 4°C in a N_2 atmosphere (with the aid of a large inflatable plastic bag), dithionite (2 mg/ml) was added, and 5 min later the solution was passed through Sephadex G-25 (1.5 × 5 cm column), previously equilibrated with N_2-flushed buffer containing 0.1 M NaCl, in order to remove the dithionite. The collected eluate (void volume) was stored under N_2 until used (within 15 min). Complete reduction of methemoglobin to hemoglobin was verified by visible absorption spectroscopy [25]. The nitrosyl derivatives of hemoglobin (NO · hemoglobin) and catalase (NO-catalase) were synthesized, verified and their concentrations determined by a modification [26] of the procedure of Kon [27]. The *S*-nitroso derivative of L-cysteine (*S*-nitrosocysteine) was prepared and verified exactly as described previously [13,14,28].

Preparation and partial purification of hepatic soluble guanylate cyclase

Male, Sprague-Dawley rats (250—300 g) were fasted for 24 h, decapitated, exsanguinated, and livers rapidly perfused in situ (portal vein) with 20 ml ice-cold 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose (sucrose buffer). Livers were finely minced, pressed through a stainless steel grid (1.5 mm diam. holes) with the aid of a tissue press (Harvard Apparatus). The resulting mince was collected into cold sucrose buffer, pressed through a second grid (0.5 mm diam. holes) and the fine mince was filtered through Nitex No. 110 nylon filament bolting cloth (50 μm pore diam.; Tobler, Ernst & Traber, Inc.) with 10—15 vol. cold sucrose buffer, or until the filtrate was devoid of red color. The washed mince was homogenized (20% w/v) in sucrose-buffer containing 2 mM dithiothreitol and 0.5 mM EDTA (buffer A) with the aid of a Potter-Elvehjem glass tissue grinder equipped with a Teflon pestle (0.008 inch clearance; 1200 rev./min). Homogenates were centrifuged at 1000 × *g* for 20 min (4°C), and the resulting supernatant was centrifuged at 100 000 × *g* for 60 min (4°C). Guanylate cyclase was partially purified from the high-speed supernatant by a modification of previously reported procedures [19]. Briefly, to approximately 400 ml supernatant, solid ammonium sulfate was added (over 3 h at 0°C) to 40% saturation. The precipitate was collected (22 000 × *g* for 20 min at 4°C), suspended in 20 ml of buffer A and dialyzed against 2 l buffer A (16 h at 4°C with three changes of buffer). After centrifugation (22 000 × *g* for 20 min at 4°C) of the dialysate, the clear supernatant was applied to a DE52-cellulose column (1.5 × 25 cm) equilibrated with buffer A. The column was eluted with a linear NaCl gradient (0—0.3 M) in buffer A (flow rate: 16 ml/h; 4-ml fractions). Guanylate cyclase eluted as a sharp peak in 0.18—0.19 M NaCl. Fractions containing at least 15% of peak enzymatic activity were pooled, concen-

trated to 2.5—3 ml by ultrafiltration (Amicon; XM-50 membrane), and applied to a Bio-Gel A-0.5m column (1.5 × 25 cm) previously equilibrated with buffer A containing 0.1 M NaCl. The column was eluted with the same buffer (flow rate: 16 ml/h; 2 ml fractions). Fractions containing guanylate cyclase activity were stored at −85°C, and just prior to use aliquots were thawed and passed through Sephadex G-25 (1.5 × 5 cm column), equilibrated and eluted with 10 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl, in order to remove the dithiothreitol and EDTA.

Determination of heme content

Fractions eluted from the DE52-cellulose and Bio-Gel A-0.5m columns were assayed for heme, after reduction of samples with dithionite, by visible absorption spectroscopy [25]. Liver perfusion and filtration of liver mince were essential because these procedures removed over 90% of the hemoglobin. Most of the remainder was removed during ammonium sulfate precipitation. Remaining heme eluted from the DE52-cellulose column as two peaks (0.05—0.08 and 0.10—0.13 M NaCl) prior to guanylate cyclase (0.18—0.19 M NaCl). Heme was not detected in enzyme fractions eluting from Bio-Gel A-0.5m columns. Since the limit of sensitivity of the heme assay is 5—10 nM, and because enzyme fractions are diluted in enzyme reaction mixtures, less than 0.05—0.1 nM heme, if any at all, was present in guanylate cyclase reaction mixtures.

Guanylate cyclase assay

Guanylate cyclase activity was determined by a modification of methods described previously [14,22]. Reaction mixtures generally contained 40 mM Tris-HCl, pH 7.4, 1 mM GTP, 3 mM $Mg(C_2H_3O_2)_2$ and 7—30 μg protein (several exceptions are noted in Table I). Reaction mixtures for determination of guanylate cyclase activities in homogenate contained also 0.3 mM 1-methyl-3-isobutylxanthine, 10 mM creatine phosphate and 50 units of creatine phosphokinase whereas those for the soluble and ammonium sulfate fractions contained 0.3 mM 1-methyl-3-isobutylxanthine (Table II). Reaction mixtures containing enzyme purified through the agarose (Bio-Gel) and/or DE52-cellulose chromatography steps did not require addition of a phosphodiesterase inhibitor. Other assay conditions are indicated in figure and table legends; no preincubations involving enzyme were conducted. Verification of cyclic GMP as the only product formed from GTP in enzymatic assays was performed as previously described [22,28].

Preparation and reactions of heated hepatic soluble fraction

Heated hepatic soluble fraction (Tables V and VI) was prepared by heating (55°C, 15 min) the 100 000 × *g* (60 min) supernatant from homogenates prepared as described above, with the exception that dithiothreitol and EDTA were omitted. Gel filtration was performed at 4°C with 1.5 × 5 cm columns of Sephadex G-25, equilibrated and eluted with 50 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl. 200-μl aliquots of 400 μl collected eluate (void volume) were added to enzyme reaction mixtures. In certain cases (Table V) 1 ml of heated soluble fraction was mixed with 0.1 ml of either 50 mM dithiothreitol or 50 mM ethacrynic acid, incubated at 25°C for 5 min, and 50 μl aliquots were

subjected to gel filtration as described above. In other cases (Table VI) 1 ml aliquots of heated soluble fraction were mixed with 10 μl NO, 1 mM MNNG or 1 mM nitroprusside, each with or without 0.1 ml of 50 mM ethacrynic acid or 10 mM *N*-ethylmaleimide, incubated at 25°C for 5 min in an O_2-free N_2 atmosphere, and subjected to gel filtration as described above.

Results

Differences between Mg^{2+} and Mn^{2+} in guanylate cyclase activation by NO and nitroso compounds

Partial purification of hepatic guanylate cyclase resulted in a 265—438-fold increase, or a 100—164-fold increase, in basal specific activity (1 mM GTP, 3 mM Mg^{2+} or Mn^{2+}) over homogenate or soluble fraction, respectively. Partially purified enzyme was devoid of heme, as indicated in Materials and Methods. Basal enzymatic activity using 1 mM GTP plus either 3 mM Mg^{2+} (1.1 ± 0.2 nmol/min per mg protein) or 3 mM Mn^{2+} (3.6 ± 0.5 nmol/min per mg protein) was linear for 10 min (3—24 μg protein). Double reciprocal plots of velocity versus GTP concentration (0.01—1 mM) gave K_m values of 25 μM (with excess Mg^{2+}) and 11 μM (with excess Mn^{2+}). Although optimal divalent cation concentrations in the presence of 1 mM GTP were 3 mM Mg^{2+} or 3 mM Mn^{2+} for basal activity, those for NO-activated guanylate cyclase were 3 mM Mg^{2+} or 0.3—1 mM Mn^{2+}. NO-stimulated guanylate cyclase activity, using 1 mM GTP plus either 3 mM Mg^{2+} or 1 mM Mn^{2+}, was linear for 10 min. Neither basal nor NO-stimulated enzymatic activity was detected in the absence of GTP and/or metal. Unlike unpurified enzyme [16,29], partially purified hepatic guanylate cyclase clearly preferred Mg^{2+} to Mn^{2+} in supporting enzyme activation by NO or by nitroso compounds plus dithiothreitol (Table I). Enzyme activation

TABLE I

EFFECTS OF Mg^{2+} AND Mn^{2+} CONCENTRATIONS AND DITHIOTHREITOL ON BASAL AND STIMULATED HEPATIC GUANYLATE CYCLASE ACTIVITY

Reaction mixtures, containing various concentrations of GTP and Mg^{2+} or Mn^{2+}, and 1 μl NO or 0.1 mM MNNG in the absence or presence of 2 mM dithiothreitol, were incubated in 1-ml volumes containing 9 μg protein for 10 min at 37°C. Reactions were initiated 1 min after warming reaction mixtures at 37°C by addition of enzyme followed immediately (within 2 s) with either NO or MNNG. Basal signifies addition of neither NO nor MNNG. Data represent the means of duplicate determinations from three separate experiments conducted with one batch of enzyme. S.E. varied by no more than 5% of the corresponding mean (not shown). Two additional experiments, each with a different batch of enzyme, yielded similar results. Values given are guanylate cyclase activity expressed as nmol cyclic GMP/min per mg protein.

GTP (mM)	Mg^{2+} or Mn^{2+} (mM)	Basal	NO	MNNG	+ Dithiothreitol		
					Basal	NO	MNNG
0.3	0.3 Mg^{2+}	0.19	1.8	0.19	0.18	3.4	1.0
0.3	1 Mg^{2+}	0.44	7.1	0.49	0.41	13	3.3
0.3	3 Mg^{2+}	0.65	16	0.71	0.66	24	6.3
1	1 Mg^{2+}	0.54	7.7	0.63	0.49	12	9.2
1	3 Mg^{2+}	0.98	17	1.1	0.93	42	24
0.3	0.3 Mn^{2+}	0.68	2.8	0.69	0.73	6.8	4.9
0.3	1 Mn^{2+}	2.0	2.0	2.1	1.9	4.1	4.3
0.3	3 Mn^{2+}	3.1	3.0	3.0	3.0	3.8	4.6
1	1 Mn^{2+}	2.1	6.9	2.7	2.2	11	10
1	3 Mn^{2+}	3.5	5.7	3.7	3.8	9.1	7.0

increased as the Mg^{2+}/GTP concentration ratio was raised but decreased as the Mn^{2+}/GTP concentration ratio was raised. Even at optimal concentration ratios of 1 mM Mn^{2+}/1 mM GTP or 0.3 mM Mn^{2+}/0.3 mM GTP (not all data are illustrated), Mn^{2+} was markedly less effective than Mg^{2+} in supporting enzyme activation by NO (Table I) or by *S*-nitrosocysteine or NO · hemoglobin (data not shown). In contrast, Mn^{2+} was still more effective than Mg^{2+} in supporting basal enzymatic activity (Table I). In the remainder of these experiments Mg^{2+} was employed instead of Mn^{2+}.

Dissimilar changes in responsiveness of guanylate cyclase to activators during enzyme purification

NO, nitroprusside, MNNG, *S*-nitrosocysteine and NO-catalase activate unpurified preparations of guanylate cyclase [3,5,7,13,14,16,17,26,28]. Although NO retained its capacity to activate partially purified enzyme, MNNG and nitroprusside required addition of dithiothreitol in order to cause activation (Tables I and II). Cysteine (2 mM) elicited effects that were virtually identical to those of dithiothreitol (data not shown). In order to better understand these changes in enzyme responsiveness, activation of guanylate cyclase at various stages of enzyme purification was monitored (Table II). Whereas NO, *S*-nitrosocysteine and NO-catalase activated guanylate cyclase at each stage of purification, MNNG and nitroprusside completely lost their capacities to cause activation by the agarose step. Enzyme activation (agarose step) by MNNG and nitroprusside was restored by addition of dithiothreitol, which also enhanced enzyme activation by NO and NO-catalase (Table II). Neither dithiothreitol (Table II) nor cysteine (not shown) altered basal guanylate cyclase activity at any step. Associated with these purification steps was a steady decrease in percent activation over corresponding basal activity by each of the activators, even in the presence of dithiothreitol. These data suggest that endogenous substances required for the full expression of guanylate cyclase activation were removed during enzyme purification.

Independent effects of heme and thiols on restoration of guanylate cyclase activation by nitroso compounds

In view of conflicting reports [19—21] on the requirement of heme for guanylate cyclase activation by NO, the effects of hematin and hemoglobin on activation of heme-free, partially purified enzyme were evaluated. NO and *S*-nitrosocysteine activated guanylate cyclase without addition of heme, although hematin or hemoglobin enhanced enzyme activation (Fig. 1). Minimally effective concentrations were 10 nM hematin and 20 nM hemoglobin, with maximal enhancement occurring with 50—100 nM. Higher concentrations inhibited enzyme activation. The observation that neither hematin nor hemoglobin enhanced enzyme activation until concentrations of 10—20 nM were reached (Fig. 1) supports our finding, based on direct heme determinations, that partially purified guanylate cyclase preparations were devoid of heme (see Materials and Methods). Neither hematin nor hemoglobin (1 nM—10 μM) altered basal guanylate cyclase activity (1 mM GTP, 3 mM Mg^{2+}), which remained at 0.9—1.0 nmol cyclic GMP/min per mg protein. Dithiothreitol had been claimed to enhance the effect of heme on NO activation of guan-

TABLE II

CHANGES IN BASAL AND STIMULATED HEPATIC GUANYLATE CYCLASE ACTIVITY DURING PARTIAL PURIFICATION

Reaction mixtures, containing 1 mM GTP and 3 mM Mg^{2+}, with or without 2 mM dithiothreitol (DTT), were incubated in 1-ml volumes for 10 min at 37°C as described in Materials and Methods. Amounts of protein added to reaction mixtures were 1.1 mg (homogenate), 404 μg (soluble), 269 μg (ammonium sulfate), 35 μg (DE52-cellulose) and 24 μg (agarose). Reactions were initiated 1 min after warming reaction mixtures at 37°C by addition of enzyme fraction followed immediately (within 2 s) with one of the activators. Basal signifies omission of activator. Data represent the means of duplicate determinatons from two to three separate experiments conducted with a single batch of each enzyme fraction. S.E. varied by no more than 10% of the corresponding mean (not shown). One additional experiment, with a different batch of enzyme fractions, yielded similar results. Values given are guanylate cyclase activity expressed as nmol cyclic GMP/min per mg protein.

Enzyme fraction	Basal		1 μl NO		0.1 mM MNNG		0.1 mM nitroprusside		10 μM *S*-Nitrosocysteine		1 μM NO-catalase	
	−DTT	+DTT	−DTT	+DTT	−DTT	+DTT	−DTT	+DTT	−DTT	+DTT	−DTT	+DTT
Homogenate	0.003	0.003	0.62	0.61	0.52	0.58	0.012	0.40	0.57	0.58	0.60	0.59
Soluble	0.009	0.010	2.4	2.4	1.1	2.5	0.79	1.6	2.0	2.0	2.4	2.3
Ammonium sulfate	0.088	0.085	3.3	3.9	1.0	1.9	0.96	1.2	3.6	3.7	4.3	4.1
DE52-cellulose	0.34	0.32	11	14	0.55	5.8	0.43	3.1	12	14	17	15
Agarose	0.91	0.87	17	32	0.93	14	0.90	6.5	25	29	31	51

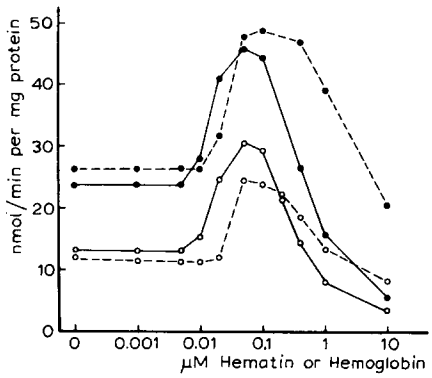

Fig. 1. Effects of hematin and hemoglobin on activation of hepatic guanylate cyclase by NO and *S*-nitrosocysteine. Reaction mixtures, containing 1 mM GTP and 3 mM Mg^{2+}, without or with hematin (——) or hemoglobin (- - - -), were incubated in 1 ml volumes containing 10—14 μg protein for 10 min at 37°C. Reactions were initiated 1 min after warming reaction mixtures at 37°C by addition of enzyme followed immediately (within 2 s) with 1 μl NO (○) or 10 μM *S*-nitrosocysteine (●). Hemoglobin was prepared from methemoglobin by reduction with dithionite. Data represent the means of duplicate determinations from three separate experiments, each using a different batch of enzyme. S.E. varied by no more than 5% of the corresponding mean (not shown).

ylate cyclase by reducing heme iron to the ferro state, thereby facilitating the formation of NO · heme, which activates guanylate cyclase [19]. However, dithiothreitol enhanced guanylate cyclase activation by NO and MNNG in the absence of heme (Table I). Furthermore, dithiothreitol enhanced enzyme activation by already preformed NO-heme complexes (Tables II and III). Moreover, dithiothreitol and cysteine react with NO to form the corresponding *S*-nitrosothiols [13,14,28], which are potent activators of heme-free, partially purified hepatic guanylate cyclase [28]. Finally, whereas dithiothreitol enhanced enzyme activation by NO, MNNG and NO · hemoglobin, the more effective heme iron reductant, dithionite, failed to enhance enzyme activation (Table III). The combination of dithiothreitol and hematin elicited effects that were only additive at best, and dithionite failed to alter the effects of hematin (Table III).

TABLE III

EFFECTS OF DITHIOTHREITOL AND DITHIONITE, IN THE ABSENCE OR PRESENCE OF HEMATIN, ON ACTIVATION OF HEPATIC GUANYLATE CYCLASE BY NO AND NITROSO COMPOUNDS

Reaction mixtures, containing 1 mM GTP and 3 mM Mg^{2+}, with or without agent(s) listed under Additions, were incubated in 1-ml volumes, containing 7—11 μg of protein, for 10 min at 37°C. Reactions were initiated 1 min after warming reaction mixtures at 37°C by addition of enzyme followed immediately (within 2 s) with one of the activators. Data represent the means ± S.E. of duplicate determinations from two to three separate experiments, each using a different batch of enzyme. Values given are guanylate cyclase activity expressed as nmol cyclic GMP/min per mg protein.

Additions	NO (1 μl)	MNNG (0.1 mM)	*S*-Nitrosocysteine (10 μM)	NO · hemoglobin (1 μM)
None	12 ± 1	0.91 ± 0.1	22 ± 2	10 ± 0.9
0.05 μM hematin	26 ± 3	2.8 ± 0.4	42 ± 4	11 ± 1
2 mM dithiothreitol	30 ± 2	16 ± 1	25 ± 3	43 ± 5
Hematin + 2 mM dithiothreitol	39 ± 4	18 ± 2	41 ± 3	41 ± 3
10 μM dithionite	13 ± 2	0.85 ± 0.1	21 ± 2	11 ± 1
Hematin + 10 μM dithionite	27 ± 3	1.8 ± 0.2	36 ± 4	—

Basal enzymatic activity was not altered by 1 nM—10 μM hematin, 2 mM dithiothreitol, 10 μM dithionite, or combinations of these agents (data not shown). 10 μM dithionite was employed because larger concentrations were inhibitory, and 1 μM was no different from 10 μM dithionite. Ascorbate (0.5—5 mM), another reductant, failed to alter basal or stimulated (NO, NO · hemoglobin) guanylate cyclase activity in the absence or presence of hematin (data not shown). These data indicate that the enhancing effect of heme on guanylate cyclase activation is independent of thiols.

The lack of requirement of thiols for the formation of NO · hemoglobin is exemplified by the reaction between methemoglobin and nitroso compounds at neutral pH (Table IV). Reaction mixtures were applied to Sephadex G-25 columns and eluates constituting the void volume activated guanylate cyclase in the absence of added thiols (Table IV). Active eluates contained NO · hemoglobin and methemoglobin as determined by visible absorption spectroscopy (data not shown; see Materials and Methods for procedure). Nitroprusside, MNNG or *S*-nitrosocysteine alone failed to elute in the void volume, as determined by failure of eluates to activate guanylate cyclase in the presence of 2 mM dithiothreitol (activity did not differ from basal activity; 0.61—0.72 nmol cyclic GMP/min per mg protein).

Effects of heated hepatic soluble fraction on activation of hepatic guanylate cyclase

Addition of heated hepatic soluble fraction (devoid of guanylate cyclase activity) to reaction mixtures restored the capacity of nitroprusside and MNNG to activate guanylate cyclase, and enhanced enzyme activation by NO and NO · hemoglobin without affecting activation by *S*-nitrosocysteine (Table V). Gel filtration (Sephadex G-25) of heated hepatic soluble fraction failed to alter its effects on enzyme activation. Reaction of the heated fraction with dithiothreitol or ethacrynic acid, followed by gel filtration to remove the latter agents, enhanced or inhibited, respectively, the effects of the heated fraction

TABLE IV

EFFECTS OF REACTION MIXTURES CONTAINING NITROSO COMPOUNDS AND METHEMOGLOBIN, AFTER GEL FILTRATION, ON HEPATIC GUANYLATE CYCLASE ACTIVITY

Combinations of agents, or NO · hemoglobin alone, were incubated in 1 ml 50 mM Tris-HCl, pH 7.4 (after flushing buffer with N_2 for 30 min), for 30 s at 25°C, and 200-μl aliquots were passed through 1.5 × 5 cm columns of Sephadex G-25 at 4°C, previously equilibrated with N_2-flushed buffer containing 0.1 M NaCl. 50 μl eluate (0.4 ml collected in void volume) were added to guanylate cyclase reaction mixtures containing 1 mM GTP, 3 mM Mg^{2+} and 10 μg of protein in a final volume of 1 ml, and incubated for 10 min at 37°C. Initial concentrations of NO · hemoglobin and methemoglobin were adjusted so that final concentrations (after gel filtration) in guanylate cyclase reaction mixtures were 1 μM. Initial concentrations of nitroprusside, MNNG and *S*-nitrosocysteine were 10-fold greater than that of methemoglobin. Data represent the means ± S.E. of duplicate determinations from two separate experiments. Values given are guanylate cyclase activity expressed as nmol cyclic GMP/min per mg protein.

Reaction mixtures applied to Sephadex G-25	Guanylate cyclase activity
None	0.66 ± 0.04
NO · hemoglobin	11 ± 2
Nitroprusside + methemoglobin	5.4 ± 0.6
MNNG + methemoglobin	3.3 ± 0.5
S-Nitrosocysteine + methemoglobin	14 ± 2

TABLE V

EFFECT OF HEATED HEPATIC SOLUBLE FRACTION ON ACTIVATION OF HEPATIC GUANYLATE CYCLASE

Reaction mixtures, containing 1 mM GTP and 3 mM Mg^{2+}, were incubated in 1-ml volumes containing 8—24 μg protein for 10 min at 37°C. Reactions were initiated 1 min after warming reaction mixtures of 37°C by addition of enzyme followed immediately (within 2 s) with one of the activators. Heated soluble fraction (HSF) was prepared, reacted with dithiothreitol or ethacrynic acid, and chromatographed on Sephadex G-25, as described in Materials and Methods. Aliquots of 50 μl of the heated soluble fraction (203—240 μg protein) were added to enzyme reaction mixtures or applied to Sephadex G-25. Basal signifies omission of activator. Data represent the mean ± S.E. of duplicate determinations from three to four separate experiments. Values given are guanylate cyclase activity expressed as nmol cyclic GMP/min per mg protein.

Additions	Basal	NO (1 μl)	Nitroprusside (0.1 mM)	MNNG (0.1 mM)	*S*-Nitrosocysteine (10 μM)	NO · hemoglobin (1 μM)
None	0.91 ± 0.1	13 ± 2	0.93 ± 0.1	0.97 ± 0.1	20 ± 2	10 ± 1
Heated soluble fraction (HSF)	0.95 ± 0.2	40 ± 3	12 ± 1	19 ± 2	24 ± 2	33 ± 2
HSF (Sephadex G-25)	0.90 ± 0.2	49 ± 5	11 ± 1	15 ± 2	—	30 ± 3
HSF + 2 mM dithiothreitol (Sephadex G-25)	0.88 ± 0.1	52 ± 4	18 ± 2	42 ± 4	24 ± 3	58 ± 4
HSF heated at 90°C, 10 min	0.88 ± 0.2	28 ± 3	5.1 ± 0.7	8.2 ± 0.9	—	22 ± 2
HSF + 50 mM ethacrynic acid (Sephadex G-25)	0.93 ± 0.2	15 ± 2	0.85 ± 0.1	0.90 ± 0.1	—	11 ± 1

on guanylate cyclase activation by NO, nitroprusside, MNNG and NO · hemoglobin (Table V). Cysteine gave effects that were similar to those of dithiothreitol (data not shown). Further heating (90°C, 10 min) of the heated hepatic soluble fraction reduced but did not abolish its enhancing effects on enzyme activation. Heating (55°C, 15 min) or reacting hemoglobin solutions (10 μM) with either dithiothreitol or ethacrynic acid followed by gel filtration failed to alter the capacity of hemoglobin (100 nM final concentration) to enhance guanylate cyclase activation by 1 μl NO or 10 μM *S*-nitrosocysteine (data not shown).

Thus, the heated hepatic soluble fraction possesses properties that are distinct from those of hemoglobin and are similar to those of thiols with regard to effects on guanylate cyclase activation. Since the heated soluble fraction likely contains thiol substances, and because thiols react with NO to form *S*-nitrosothiols [13,14,28], the heated fraction was reacted with NO, MNNG or nitroprusside in order to determine whether a substance could be formed which would activate guanylate cyclase. Such a finding could explain the enhancing effect of the heated soluble fraction on guanylate cyclase activation by NO and certain nitroso compounds. The data in Table VI indicate that the above reaction mixtures, after gel filtration to remove the low molecular weight reactants (NO, MNNG, nitroprusside), contained substances of molecular weight in excess of 5000 which activated guanylate cyclase. When the heated soluble fraction was reacted with ethacrynic acid (Table VI) or *N*-ethylmaleimide (data not shown) prior to reaction with NO, MNNG or nitroprusside, the formation of a substance(s) capable of activating guanylate cyclase was markedly diminished. These observations suggest that thiol-containing substances in the heated soluble fraction reacted with NO, MNNG or nitroprusside to form compounds that activate guanylate cyclase.

TABLE VI

ACTIVATION OF HEPATIC GUANYLATE CYCLASE BY HEATED HEPATIC SOLUBLE FRACTION AFTER REACTION OF THE LATTER WITH NO OR NITROSO COMPOUNDS

Reaction mixtures containing 1 mM GTP and 3 mM Mg^{2+} were incubated in 1-ml volumes containing 14—30 μg protein for 10 min at 37°C. Reactions were initiated 1 min after warming reaction mixtures at 37°C by addition of enzyme followed immediately (within 2 s) with one of the substances listed under Additions. Heated soluble fraction (HSF) was prepared, reacted with NO, MNNG or nitroprusside in the absence or presence of ethacrynic acid, and chromatographed on Sephadex G-25 as described in Materials and Methods. 200-μl aliquots of 0.4 ml of collected eluates (void volume), containing 190—220 μg protein, were added to reaction mixtures as indicated above. Data represent the means ± S.E. of duplicate determinations from three separate experiments, each using a different batch of enzyme. Values given are guanylate cyclase activity expressed as nmol cyclic GMP/min per mg protein.

Additions	Guanylate cyclase activity
None (basal activity)	0.81 ± 0.06
Heated soluble fraction (HSF)	0.83 ± 0.09
HSF reacted with NO	18 ± 1.7
HSF reacted with ethacrynic acid + NO	1.6 ± 0.2
HSF reacted with MNNG	32 ± 4.1
HSF reacted with ethacrynic acid + MNNG	1.9 ± 0.2
HSF reacted with nitroprusside	15 ± 1.5
HSF reacted with ethacrynic acid + nitroprusside	1.6 ± 0.1

Discussion

The results of this study indicate that partially purified, heme-free, hepatic soluble guanylate cyclase can be activated by NO, *S*-nitrosocysteine and NO-heme complexes, but not by nitroprusside or MNNG, in the absence of added heme or thiols. Nitroprusside and MNNG require addition of thiols, but not heme, in order to activate guanylate cyclase. Enzyme activation by NO is enhanced by addition of thiols or heme, whereas activation by NO · hemoglobin is enhanced only by thiols. Enzyme activation by *S*-nitrosocysteine, on the other hand, is enhanced by heme, but not by thiols. Therefore, it appears that thiols and heme can independently enhance guanylate cyclase activation.

Although either MnGTP or MgGTP can serve as substrate for guanylate cyclase, MnGTP is preferred for the full expression of basal activity whereas MgGTP is clearly preferred for enzyme activated by NO or MNNG plus dithiothreitol. Using optimal concentrations of GTP and Mn^{2+} or Mg^{2+}, enzyme activation by NO is over 4-fold greater with MgGTP than with MnGTP. Moreover, optimal concentration ratios of Mg^{2+} and GTP differ markedly from those of Mn^{2+} and GTP. Whereas raising the concentration ratio of Mg^{2+} to GTP increases, raising that of Mn^{2+} to GTP decreases or abolishes the capacity of NO (or MNNG plus dithiothreitol) to activate guanylate cyclase. Therefore, the choice between Mg^{2+} and Mn^{2+}, when the objective is to study activation of guanylate cyclase, must be carefully considered. For example, these observations may account, at least in part, for the findings that NO only slightly activated partially purified guanylate cyclase [21], because 4 mM Mn^{2+} plus 1 mM GTP were used in enzyme reaction mixtures. We were unable to confirm the findings in a previous report [19] that MNNG activated guanylate cyclase 16—60-fold in the presence of Mn^{2+}, hematin and dithiothreitol.

Conflicting reports on the requirement of heme for activation of guanylate cyclase by NO [19—21], and the requirement of thiols for enzyme activation by nitroso compounds [7,23,30], prompted an analysis of guanylate cyclase activation by NO and nitroso compounds at various stages of enzyme purification. Partial purification results in a marked decrease (from 267-fold to 20-fold) in enzyme activation by NO and an abolition of activation by nitroprusside and MNNG. The decrease in responsiveness of guanylate cyclase to activation is not likely due to the removal of heme because enzyme activation by NO-heme complexes is also markedly decreased, and addition of heme to reaction mixtures only slightly enhances enzyme activation by NO and nitroso compounds. On the other hand, removal of thiols may account, at least in part, for the decreased activation of guanylate cyclase during enzyme purification because addition of dithiothreitol or cysteine to reaction mixtures restores enzyme activation by nitroprusside and MNNG, and enhances enzyme activation by NO and preformed NO · hemoglobin. These observations, along with those that glyceryl trinitrate specifically requires cysteine and $NaNO_2$ requires one of many thiols in order to activate guanylate cyclase [14], suggests that a thiol component(s) present in the crude hepatic soluble fraction is removed by the enzyme purification procedures. Craven and DeRubertis [19] reported previously that very large amounts (10 mg/ml enzyme reaction mixture) of heated (95°C for 15 min) hepatic soluble fraction enhanced activation of guanylate cyclase by NO and

related compounds and, without performing any characterization studies, concluded that heme was responsible for the effect of the heated soluble fraction. However, our interpretation that thiols are involved is supported by the observations that, like dithiothreitol, the heated hepatic soluble fraction (devoid of guanylate cyclase activity) not only restores the capacity of nitroprusside and MNNG, but enhances that of preformed NO · hemoglobin, to activate partially purified hepatic guanylate cyclase. Indeed, preincubation of heated hepatic soluble fraction with ethacrynic acid or *N*-ethylmaleimide, both of which react covalently with -SH groups, abolishes the property of the former to enhance or support guanylate cyclase activation by NO and nitroso compounds. In addition, preincubation of heated hepatic soluble fraction with dithiothreitol enhances the proficiency of the former to increase enzyme activation by nitroprusside, MNNG and NO · hemoglobin. Moreover, NO, MNNG and nitroprusside react with a component(s) of the heated hepatic soluble fraction to form a product(s) which markedly activates guanylate cyclase, and this reaction is practically abolished by ethacrynic acid. Finally, treatment of hemoglobin solutions with either dithiothreitol or ethacrynic acid failed to alter its effects on guanylate cyclase activation. On the basis of experiments reported here, it appears that the hepatic soluble fraction contains a partially heat-stable, thiol-containing component(s) (molecular weight(s) exceeding 5000) which can enhance activation of guanylate cyclase by NO and nitroso compounds. Studies are in progress to isolate and characterize the responsible component(s).

Thiols and heme appear to affect guanylate cyclase activation independently of one another. Although thiols and heme enhance enzyme activation by NO, MNNG and nitroprusside, enzyme activation by NO-heme complexes is enhanced only by thiols whereas enzyme activation by *S*-nitrosocysteine is enhanced only by heme. The observations that dithiothreitol and cysteine enhance guanylate cyclase activation by preformed NO-heme complexes, and that the reductants dithionite and ascorbate fail to substitute for dithiothreitol in enhancing enzyme activation by NO and nitroso compounds, make it unlikely that thiols elicit their effects by facilitating the formation of NO-heme complexes as suggested previously [19]. Indeed, experiments reported here illustrate that MNNG, nitroprusside and *S*-nitrosocysteine react with methemoglobin in the absence of thiols to form NO-hemoglobin, which is known to activate guanylate cyclase [19]. A plausible alternative explanation for these observations is that thiols and heme react independently with NO to form *S*-nitrosothiols and NO-heme complexes, respectively, each of which markedly activates guanylate cyclase [13,14,19,28].

The results of this study indicate that partially purified, heme-free, hepatic guanylate cyclase has no absolute requirement of heme for enzyme activation by NO and nitroso compounds. Although NO, *S*-nitrosocysteine and NO-heme complexes require no additional factors, nitroprusside and MNNG require addition of thiols to activate guanylate cyclase. Heme, although not required, can enhance enzyme activation by NO and nitroso compounds. Thus, until the precise mechanisms of guanylate cyclase activation by NO and each of the nitroso compounds are elucidated, generalizations on the absolute requirements for enzyme activation cannot be made.

Acknowledgments

This work was supported in part by Research Grant AM 17692 and HL 18070 from the USPHS. L.J.I. is the recipient of Research Career Development Award No. 1-K04 AM 00076 from the USPHS. C.A.G. is supported by Individual N.I.H. Postdoctoral Fellowship HL 05597.

References

1 Haddox, M.K., Furcht, L.T., Gentry, S.R., Moser, M.E., Stephenson, J.H. and Goldberg, N.D. (1976) Nature 262, 146—148
2 White, A.A., Crawford, K.M., Patt, C.S. and Lad, P.J. (1976) J. Biol. Chem. 251, 7304—7312
3 DeRubertis, F.R. and Craven, P.A. (1976) Science 193, 897—899
4 Goldberg, N.D. and Haddox, M.K. (1977) Annu. Rev. Biochem. 46, 823—896
5 Mittal, C.K., Kimura, H. and Murad, F. (1977) J. Biol. Chem. 252, 4384—4390
6 Goldberg, N.D., Graff, G., Haddox, M.K., Stephenson, J.H., Glass, D.B. and Moser, M.E. (1978) Adv. Cyclic Nucl. Res. 9, 101—130
7 Bohme, E., Graf, H. and Schultz, G. (1978) Adv. Cyclic Nucl. Res. 9. 131—143
8 Craven, P.A. and DeRubertis, F.R. (1978) Biochim. Biophys. Acta 524, 231—244
9 Hardman, J.G. and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6363—6370
10 Thompson, W.J., Williams, R.H. and Little, S.A. (1973) Biochim. Biophys. Acta 302, 329—337
11 Garbers, D.L. and Hardman, J.G. (1975) J. Biol. Chem. 250, 2482—2486
12 Durham, J.P. (1976) Eur. J. Biochem. 61, 535—544
13 Ignarro, L.J., Edwards, J.C., Gruetter, D.Y., Barry, B.K. and Gruetter, C.A. (1980) FEBS Lett. 110, 275—278
14 Ignarro, L.J. and Gruetter, C.A. (1980) Biochim. Biophys. Acta 631, 221—231
15 Kimura, H., Mittal, C.K. and Murad, F. (1975) Nature 257, 700—702
16 Katsuki, S., Arnold, W., Mittal, C. and Murad, F. (1977) J. Cyclic Nucl. Res. 3, 23—35
17 Arnold, W.P., Mittal, C.K., Katsuki, S. and Murad, F. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3203—3207
18 Deguchi, T. (1977) J. Biol. Chem. 252, 596—601
19 Craven, P.A. and DeRubertis, F.R. (1978) J. Biol. Chem. 253, 8433—8443
20 Braughler, J.M., Mittal, C.K. and Murad, F. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 219—222
21 Braughler, J.M., Mittal, C.K. and Murad, F. (1979) J. Biol. Chem. 254, 12 450—12 454
22 Gruetter, C.A., Barry, B.K., McNamara, D.B., Gruetter, D.Y., Kadowitz, P.J. and Ignarro, L.J. (1979) J. Cyclic Nucl. Res. 55, 211—224
23 Ohlstein, E.H., Barry, B.K., Gruetter, D.Y. and Ignarro, L.J. (1979) FEBS Lett. 102, 316—320
24 Gruetter, C.A., Barry, B.K., McNamara, D.B., Kadowitz, P.J. and Ignarro, L.J. (1980) J. Pharmacol. Exp. Ther. 214, 9—15
25 Rossi-Fanelli, A., Antonini, E. and Caputo, A. (1958) Biochim. Biophys. Acta 30, 608—615
26 Edwards, J.C., Barry, B.K., Gruetter, D.Y., Ohlstein, E.H. and Ignarro, L.J. (1981) Biochem. Pharmacol., in the press
27 Kon, H. (1968) J. Biol. Chem. 243, 4350—4357
28 Ignarro, L.J., Barry, B.K., Gruetter, D.Y., Edwards, J.C., Ohlstein, E.H., Gruetter, C.A. and Baricos, W.H. (1980) Biochem. Biophys. Res. Commun. 94, 93—100
29 Kimura, H., Mittal, C.K. and Murad, F. (1976) J. Biol. Chem. 24, 7769—7773
30 DeRubertis, F.R. and Craven, P.A. (1977) J. Biol. Chem. 252, 5804—5814