Regulation of activity of purified guanylate cyclase from liver that is unresponsive to nitric oxide

Su-Chen TSAI, Ronald ADAMIK, Vincent C. MANGANIELLO and Martha VAUGHAN

Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20205, U.S.A.

(Received 7 April 1983/Accepted 21 July 1983)

Guanylate cyclase was purified from rat liver supernatant. Electrophoresis under denaturing conditions revealed one major peptide of M_r approx. 69 000. On the basis of the Stokes radius (4.7 nm) and $s_{20,w}$ (6.4S), the calculated M_r value of the native enzyme was 133 000, i.e. it is apparently a homodimer. Kinetics of inactivation by diamide (which was reversible with dithiothreitol) suggested that oxidation of a single class of thiol sites was involved. In the absence of other additions, cyclase activity assayed with Mn^{2+} was over 7 times that assayed with Mg^{2+} ; maximal effects were observed with approx. 5 mM of each (with 1 mM-GTP). The purified enzyme was markedly activated by nitrosylhaemoglobin. Relative activation was much greater in assays with Mg^{2+} than with Mn^{2+} , although maximal activities were similar. When assayed with Mg^{2+} , the enzyme exhibited a single K_m (0.35 mM) for GTP; with Mn^{2+} , plots of $1/v$ versus $1/[S]$ were non-linear. Activator or nitrosylhaemoglobin increased V_{max} , but did not alter K_m in the presence of either Mg^{2+} or Mn^{2+} . The enzyme was inhibited by Na_3VO_4 , Na_2WO_4 and $Na_2B_4O_7$. Reduction from V^V to V^{IV} abolished the inhibitory effect of vanadate. $Na_2B_4O_7$ (2 mM) inhibited activity with Mn^{2+} , but not with Mg^{2+} . In assays with Mg^{2+} , but not with Mn^{2+} , FMN, NAD^+ and NADH (each 0.5 mM) inhibited activation by protoporphyrin IX and nitrosylhaemoglobin. Rotenone (0.6 mM) inhibited activity with protoporphyrin IX to a greater extent than with nitrosylhaemoglobin. Methylene Blue (1 mM) inhibited activation by nitrosylhaemoglobin, protoporphyrin IX and activator. It appears that this enzyme purified from rat liver lacks haem (and perhaps other components) required for activation by NO, and it should be particularly useful for elucidating the mechanism of action of NO, protoporphyrin IX and other activators.

NO and compounds from which it can arise (e.g. nitroprusside, azide) activate soluble guanylate cyclase from virtually all mammalian tissues (for review see Mittal & Murad, 1977). Craven & DeRubertis (1978) first demonstrated the importance of haem and haemoproteins in NO activation. Although it has been suggested that the purified enzyme can be directly activated by NO (Braugher *et al.*, 1979a, b), more recent reports indicate the presence of haem in purified preparations of guanylate cyclase that respond to NO, nitroprusside or protoporphyrin IX (Gerzer *et al.*, 1981a,b; Ignarro *et al.*, 1982a,b).

As a step towards elucidating the mechanisms of action of NO and other activators, we have purified soluble guanylate cyclase from liver in a form that is

activated little, if at all, by NO and apparently lacks haem. We report here some physical and kinetic characteristics of, and effects of nitrosylhaemoglobin and a heat-stable activator on, the purified enzyme. We have also compared effects of Hb and activator on NO activation as well as effects of a number of inhibitors on enzyme activity.

Materials and methods

Materials

Nitroprusside, haematin, haemoglobin, protoporphyrin IX, phenylmethanesulphonyl fluoride, GTP-agarose, GTP, cyclic GMP, cytochrome *c*, ferredoxin, FMN, NAD^+ , NADH, Methylene Blue and reduced glutathione were from Sigma Chemical Co.; rotenone was from Aldrich Chemical Co.; diamide was from Calbiochem; DEAE-cellulose was from Whatman; phenyl-Sepharose and Affi Blue

* Abbreviations used: Hb, haemoglobin; SDS, sodium dodecyl sulphate.

Sephacrose were from Pharmacia; [α - 32 P]GTP and cyclic [3 H]GMP (purified on t.l.c.) were from New England Nuclear; Ultrogel AcA-34 was from LKB; DEAE-Bio-Gel was from BioRad; NO (99%) was from Matheson Gas; Na_2WO_4 , $\text{Na}_2\text{B}_4\text{O}_7$ and Na_3VO_4 (ortho) were from Fisher Scientific; Na_3VO_4 (V^{V}) was reduced to the V^{IV} level by addition of 10-fold concentrated ascorbate for 30 min (Cantley *et al.*, 1977).

Purification of guanylate cyclase

Guanylate cyclase was purified from rat liver by a modification of our published procedure (Tsai *et al.*, 1978, 1980). Results for a representative preparation are shown in Table 1. Liver (880 g) was homogenized in 1800 ml of 0.25 M-sucrose in 10 mM-Tris/HCl buffer, pH 7.4, containing 2 mM-dithiothreitol and 1 mM-EDTA (buffer A), with 0.5 mM-phenylmethanesulphonyl fluoride. The homogenate was centrifuged (7000 g, 60 min), and the supernatant was applied to DE-52 DEAE-cellulose (1.5 litres of gel), which was washed successively with 2 litres of buffer A, 2 litres of buffer A containing 75 mM-NaCl, and 2 litres of buffer A containing 175 mM-NaCl. Guanylate cyclase was eluted with 2 litres of buffer A containing 200 mM-NaCl and directly applied to phenyl-Sepharose (700 ml). The phenyl-Sepharose was washed with 2 litres of buffer A, then with 1.5 litres of 300 mM-Tris/HCl buffer, pH 8.8, containing 0.25 M-sucrose, 2 mM-dithiothreitol, 1 mM-EDTA and 7.5% (v/v) propylene glycol. Guanylate cyclase was eluted with buffer A containing 20% (v/v) dimethylformamide and immediately transferred to a Sephadex G-25 column (4 cm $\times$ 38 cm) to remove dimethylformamide. The guanylate cyclase was then applied to a column (4 cm $\times$ 40 cm) of DEAE-Bio-Gel and eluted with a linear gradient of NaCl (50–350 mM). The enzyme fractions were pooled, concentrated with Aquacide (Calbiochem) to 30 ml, dialysed against buffer A, and applied to a column (2.5 cm $\times$ 80 cm) of Ultrogel AcA-34 equilibrated and eluted with the same buffer. Active fractions were pooled and dialysed against a solution containing 10% (v/v) glycerol,

0.5 M-sucrose, 2 mM-dithiothreitol in 10 mM-Tris/HCl buffer, pH 7.4 (buffer B), and then stored at -65°C . At this stage (step 5, Table 1), activity was stable for months at -65°C .

The enzyme from step 5 was applied to 45 ml of GTP-agarose in a fritted-glass filter, which was equilibrated with 50 mM-sucrose in 20 mM-Tris/HCl buffer, pH 7.6, containing 2 mM-dithiothreitol and 1 mM- NaN_3 (buffer C). The pass-through fraction, containing most of the guanylate cyclase, was collected and the gel was washed with 68 ml of buffer C. The pooled fraction plus wash (70% of applied activity) was made 3 mM in MgCl_2 and transferred to 54 ml of GTP-agarose. The gel was washed with 5 vol. of buffer C containing 3 mM- MgCl_2 and 4 vol. of buffer C containing 25 mM-NaCl. Guanylate cyclase was then eluted with 3 vol. of buffer C containing 100 mM-NaCl, immediately dialysed against buffer B (45 min at 2°C) and stored at -65°C . (Recovery of this step was unusually low for the preparation indicated in Table 1.)

The enzyme was further purified by using preparative polyacrylamide-gel electrophoresis (Tsai *et al.*, 1978). Specific activities of peak fractions from electrophoresis (corresponding to 106–110 in Fig. 1a) were 1.5–2.0 $\mu\text{mol}/\text{min}$ per mg of protein. Polyacrylamide-slab-gel electrophoresis in the presence of SDS of samples from fractions indicated the presence of a major protein band of M_r approx. 69000 (Fig. 1b) that correlated with enzyme activity. The purified guanylate cyclase preparations contained small amounts of one or two other peptides that migrated more slowly than the enzyme on electrophoresis in the presence of SDS and have proved to be very difficult to remove from rat liver (Fig. 1b) or calf liver (results not shown) preparations. The purified enzyme exhibited a Stokes radius of 4.7 nm and an $s_{20,w}$ of 6.4 S, from which we have calculated an M_r value of 133000 for the native enzyme. When stored in buffer B at -65°C , the purified enzyme from preparative electrophoresis (10–30 μg of protein/ml) was stable for several months. It was however, extremely unstable at 0°C . Therefore, for most of the experiments reported in

Table 1. *Purification of guanylate cyclase from rat liver*

Preparation from 880 g of rat liver was as described in the Materials and methods section. Guanylate cyclase activity was assayed with 5 mM- MnCl_2 and activator.

Step	Fraction	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Recovery (%)
1	Supernatant	—	—	0.2	—
2	DE-52 DEAE-cellulose	10 700	6400	0.6	100
3	Phenyl-Sepharose	1020	6100	6.0	95
4	DEAE-Bio-Gel	510	8500	17	133
5	Ultrogel AcA-34	140	5190	37	80
6	GTP-agarose	0.9	720	800	11
7	Electrophoresis	—	—	1700	6

Fig. 1. (a) *Preparative polyacrylamide-gel electrophoresis of guanylate cyclase and (b) polyacrylamide-slab-gel electrophoresis in the presence of SDS of fractions from the preparative electrophoresis*

(a) Enzyme (0.2 mg) from step 6 was subjected to electrophoresis on 8% polyacrylamide gel (Tsai *et al.*, 1978). Samples of fractions (50–200 ng of protein) were assayed with 5 mM-MnCl₂ and activator (10 µg). Total activity per fraction is recorded in nmol/10 min. (b) Proteins in samples of fraction from the preparative electrophoresis shown in (a) were precipitated with 10% (w/v) trichloroacetic acid, dissolved in 1% (w/v) SDS/5% (v/v) 2-mercaptoethanol (3 min, 100°C), transferred to an 8% polyacrylamide slab gel, and subjected to electrophoresis in the presence of SDS (Laemmli, 1970). Standard proteins (STD) are, from the top, phosphorylase *b*, bovine serum albumin and ovalbumin.

the present paper, guanylate cyclase purified up to step 6 (chromatography on GTP-agarose) was used. These preparations usually had specific activities of 0.6–1.2 µmol/min per mg of protein and exhibited one or two contaminating protein bands on electrophoresis in the presence of SDS.

Preparation of deoxyHb and NOHb

Stock solutions of deoxyHb (0.5 mM) were prepared as described by Craven & DeRubertis (1978). Na₂S₂O₄ (final conc. 2 mg/ml) was added to a solution of Hb (75% methaemoglobin) in 50 mM-Tris/HCl buffer, pH 7.6 for 5 min on ice under N₂. The solution was applied to a column of Sephadex PG-10 equilibrated and eluted with the Tris buffer. The fractions containing deoxyHb were immediately flushed with N₂ and stored at 4°C until used

(within 1 week). NOHb was prepared immediately before use by flushing a solution of deoxyHb (0.5 mM) with NO under atmosphere of N₂ for 2–3 min at 0°C. The tube was then capped and after 2–3 min was flushed with N₂.

Guanylate cyclase was assayed in a total volume of 100 µl containing 5 mM-MnCl₂ or 6 mM-MgCl₂, 1 mM-[α-³²P]GTP (3 × 10⁶ c.p.m.), 1–2 mM-dithiothreitol and 50 mM-Tris/HCl buffer, pH 7.6 (Tsai *et al.*, 1978, 1980). Theophylline (6 mM) and cyclic GMP (1 mM) were included in assays of enzyme from steps 1 to 5 of purification. After incubation at 37°C for 10 min, assays were terminated by addition of 5% (w/v) trichloroacetic acid containing 1 mM-GTP and 1 mM-cyclic GMP. Cyclic GMP was isolated for radioassay by using Dowex 1 (Cl⁻ form), and alumina (Nesbitt *et al.*, 1976). To assess effects of NO and NOHb on guanylate cyclase activity, enzyme samples (100–200 µl) were incubated at 0°C with NO under N₂ or with NOHb for 2 min in buffer containing 2 mM-dithiothreitol before addition of other assay components. Although fresh solutions of NOHb were prepared for each experiment, considerable variation in their effectiveness was noted. For the experiment illustrated in Fig. 2, deoxyHb at the indicated concentration was added to the enzyme sample at 0°C; NO (two bubbles) was introduced and assays were initiated immediately with addition of substrate. With this procedure, activation was more reproducible and greater than that observed with the same nominal concentration of preformed NOHb (see, for example, Table 2). The guanylate cyclase activator was prepared as described previously (Tsai *et al.*, 1980, 1981).

Results

At all stages of purification, activity was greater in assays with Mn²⁺ rather than Mg²⁺, as has been observed previously (Craven & DeRubertis, 1978; Tsai *et al.*, 1978, 1980; Braugher *et al.*, 1979a,b; Gerzer *et al.*, 1981a,b; Ignarro *et al.*, 1982a,b). At several steps in purification, the enzyme exhibited the same Stokes radius (4.7 nm) and *s*_{20,w} (6.4 S), indicating that the apparent size of the native enzyme (*M*_r 133 000) was not affected by the presence of other proteins or the procedures employed (results not shown).

NO did not enhance activity of the enzyme purified through preparative electrophoresis (step 7) (Table 2), and at higher concentrations it was inhibitory, more so in assays with Mn²⁺ than with Mg²⁺. NO plus haem did not alter activity (results not shown), but activity was markedly enhanced by NOHb; the percentage activation was greater with Mg²⁺ than with Mn²⁺ (Table 2). Hb alone increased activity relatively little. Activator produced the same

Table 2. Activation of purified guanylate cyclase by NO, NOHb, deoxyHb or activator

Samples of purified guanylate cyclase from preparative electrophoresis (0.12 μ g of protein) were assayed with 5 mM-MnCl₂ or 6 mM-MgCl₂ and other additions as indicated. For full details see the text.

Additions	Guanylate cyclase activity (nmol/min per mg of protein)	
	MgCl ₂	MnCl ₂
None	49	390
NO (100 μ l)	40	380
NO (200 μ l)	38	210
DeoxyHb (25 μ M)	99	620
NOHb (10 μ M)	1500	2000
NOHb (25 μ M)	2600	—
Activator (10 μ g)	240	1500
Activator plus NO (100 μ l)	200	980

percentage increase with Mg²⁺ and Mn²⁺; it did not support NO activation (Table 2).

After elution from DE-52 DEAE-cellulose (step 2), cyclase activity was markedly enhanced by NO; responsiveness to NO decreased with succeeding steps of purification, and was minimal or absent after step 6 and step 7 (Table 2). As reported by others (Gerzer *et al.*, 1981b; Ignarro *et al.*, 1982b), the cyclase from bovine lung remained responsive to NO after binding to Affi Blue Sepharose and elution with GTP. As with the rat liver enzyme (Table 2), the bovine lung cyclase was activated little, if at all, by NO after chromatography on GTP-agarose (results not shown).

With the liver enzyme purified through chromatography on GTP-agarose (step 6), 2.5–25 μ M-deoxyHb increased activity by 200% in assays with Mn²⁺ or Mg²⁺ (Fig. 2). NOHb (1 μ M) increased activity with Mg²⁺ 120-fold and only about one-tenth that with Mn²⁺. Higher concentrations of NOHb were less effective (Fig. 2), as were lower concentrations (results not shown). Another protein that contains haem, cytochrome *c*, and an iron-containing protein, ferredoxin, also increased basal guanylate cyclase activity; unlike Hb, however, they did not support NO activation (results not shown).

The concentration of Mn²⁺ required to produce a half-maximal activity (1.2 mM) was consistently less than the concentration of Mg²⁺ that supported half-maximal activity (3 μ M). Neither NOHb (Fig. 3) nor activator (results not shown) altered the dependence on Mg²⁺ or Mn²⁺. Maximal activity (with 1 mM-GTP) was observed with 5 mM of either (Fig. 3).

In assays with Mn²⁺ with or without activator, activity was maximal at pH 7.2–7.6 and declined with increasing pH (results not shown), as we observed with less-purified preparations of guanylate

Fig. 2. Effects of NOHb and Hb on guanylate cyclase activity

Samples of guanylate cyclase (0.13 μ g of protein) from step 6 were incubated at 0°C with the indicated concentrations of NOHb (▲ and Δ) or deoxyHb (● and ○) for 2 min before assay with 5 mM-MnCl₂ (● and ▲) or 6 mM-MgCl₂ (○ and Δ). For full details see the text.

cyclase (Tsai *et al.*, 1978). A similar pH optimum was observed in assays with Mn²⁺ or Mg²⁺ and NOHb; basal activity with Mg²⁺, however, did not decrease with increasing pH from 7.6 to 8.5 in Tris buffer (results not shown) as noted also by Braugher (1980).

In assays with Mg²⁺ (4.8 mM in excess of GTP), the enzyme exhibited a single *K_m* for GTP (0.35 mM), which was not consistently altered by NOHb or activator (Fig. 4). Variations in pH between 6.8 and 8.3 altered *V_{max}*, but not the *K_m* for GTP (results not shown). Data from assays with Mn²⁺ (2 mM in excess of GTP) yielded plots of 1/*v* versus 1/[S] that were non-linear (Fig. 5), with apparent *K_m* values of 50–60 μ M and 1.5 mM for

Fig. 3. Effects of MgCl_2 and MnCl_2 on guanylate cyclase activity

Samples of guanylate cyclase (step 6, $0.27 \mu\text{g}$ of protein) were assayed with 1 mM -GTP and the indicated concentration of MgCl_2 (○ and △) or MnCl_2 (● and ▲) without (○ and ●) or with (△ and ▲) $3 \mu\text{M}$ -NOHb. For full details see the text. Maximal activities (100%) with MgCl_2 without and with NOHb were 26 and $300 \text{ nmol/min per mg}$ of protein respectively, and with MnCl_2 were 50 and $700 \text{ nmol/min per mg}$ of protein respectively.

Fig. 4. Effect of substrate concentration on guanylate cyclase activity with MgCl_2

Samples of guanylate cyclase from step 6 ($0.13 \mu\text{g}$ of protein) were assayed with the indicated concentrations of MgGTP plus 4.8 mM - MgCl_2 with $7 \mu\text{g}$ of activator (○), $0.7 \mu\text{M}$ -NOHb (△) or no additions (●). For full details see the text.

Fig. 5. Effect of substrate concentration on guanylate cyclase activity with MnCl_2

Samples of guanylate cyclase from step 6 ($0.26 \mu\text{g}$ of protein) were assayed with the indicated concentrations of MnGTP plus 2 mM - MnCl_2 with $10 \mu\text{g}$ of activator (○), $5 \mu\text{M}$ -NOHb (△) or no additions (●). For full details see the text.

Table 3. Inhibition of guanylate cyclase activity by vanadate, tungstate and borate

Samples of guanylate cyclase (step 6, $0.27 \mu\text{g}$ of protein) were assayed with MgCl_2 or MnCl_2 , $1.8 \mu\text{M}$ -NOHb, activator ($10 \mu\text{g}$), $50 \mu\text{M}$ - Na_3VO_4 , 1 mM - Na_2WO_4 and/or 2 mM - $\text{Na}_2\text{B}_4\text{O}_7$, as indicated. For full details see the text. Activities relative to control values (=100) are given in parentheses.

Assay with	Guanylate cyclase activity (nmol/min per mg of protein)			
	Control	Na_3VO_4	Na_2WO_4	$\text{Na}_2\text{B}_4\text{O}_7$
MgCl_2 (6 mM)	14 (100)	6 (43)	2 (14)	12 (86)
Plus NOHb	270 (100)	130 (48)	15 (6)	220 (82)
Plus activator	110 (100)	56 (51)	19 (17)	97 (88)
MnCl_2 (5 mM)	120 (100)	52 (43)	6 (5)	67 (56)
Plus NOHb	510 (100)	310 (61)	27 (5)	190 (37)
Plus activator	820 (100)	540 (66)	570 (69)	410 (50)

plots were also non-linear when assays were performed at pH 6.5, 7.3 or 8.5 (results not shown).

The purified guanylate cyclase was inhibited 50% by $50 \mu\text{M}$ - Na_3VO_4 in assays with Mg^{2+} or Mn^{2+} with or without NOHb or activator (Table 3). Reduction of vanadate from V^{V} to V^{IV} abolished the inhibitory effect (results not shown). Na_2WO_4 (1 mM) caused virtually complete inhibition under all conditions except in the presence of Mn^{2+} and activator (Table 3). In assays with Mn^{2+} , with or without NOHb or activator, 2 mM - $\text{Na}_2\text{B}_4\text{O}_7$ was inhibitory, but it had relatively little effect in assays with Mg^{2+} (Table 3).

GTP, which were not significantly altered by NOHb or activator (Fig. 5) or by higher concentrations of excess Mn^{2+} (results not shown). Double-reciprocal

Table 4. *Effects of inhibitors on guanylate cyclase activity*

Samples of guanylate cyclase (step 6, Expt. I, 0.1 μ g of protein; Expt. II, 0.12 μ g of protein) were assayed with $MgCl_2$ or $MnCl_2$, 1 μ M-protoporphyrin IX, 1 μ M-NOHb, activator (3 μ g) and the addition indicated in Expt. I, 0.5 mM-FMN, 0.5 mM-NAD⁺ or 0.5 mM-NADH; in Expt. II, 1 mM-NaCN, 1 mM-K₃Fe(CN)₆, 0.6 mM-rotenone or 1 mM-Methylene Blue.

Addition	Guanylate cyclase activity (nmol/min per mg of protein)				
	$MgCl_2$			$MnCl_2$	
	Control	+ Protoporphyrin IX	+ NOHb	Control	+ Activator
Expt. I					
None	17	140	750	59	320
FMN	25	103	490	62	310
NAD ⁺	26	111	104	68	340
NADH	19	104	340	71	320
Expt. 2					
None	25	370	1150	220	720
NaCN	23	370	1330	180	740
K ₃ Fe(CN) ₆	7	150	520	150	510
Rotenone	11	17	600	150	510
Methylene Blue	25	33	67	220	320

Fig. 6. *Inhibition of guanylate cyclase activity with Methylene Blue*

Samples of guanylate (step 6, 0.12 μ g of protein) were assayed with the indicated concentrations of Methylene Blue and 1 mM-GTP in the presence of 6 mM- $MgCl_2$ or 5 mM- $MnCl_2$. For full details see the text. Control activities (100%) with $MgCl_2$ with no addition (■), 1 μ M-protoporphyrin (Δ) and 1 μ M-NOHb ($\square$) were 27, 128 and 545 nmol/min per mg of protein respectively, and with $MnCl_2$ without ($\circ$) and with 10 μ g of activator ($\bullet$) were 78 and 726 nmol/min per mg of protein respectively.

Fig. 7. *Inactivation of rat liver guanylate cyclase by diamide*

Guanylate cyclase from step 6 (180 μ g/ml) was incubated with 0.24 mM- ($\circ$), 0.3 mM- (Δ), 0.36 mM- ($\blacksquare$), 0.42 mM- ($\bullet$) or 0.48 mM- ($\square$) diamide at 22°C. At the indicated times, samples (5 μ l, 0.9 μ g of protein) were assayed. For full details see the text.

Concentrations of inhibitors used in Table 3 were selected on the basis of experiments in which many concentrations of each were tested with enzyme from step 5 (results not shown).

In assays with Mg^{2+} or Mn^{2+} or with Mn^{2+} plus activator, activity was unaffected by 0.5 mM-FMN, -NAD⁺ or -NADH. In assays with Mg^{2+} , these

compounds inhibited activation by NOHb by approx. 50% and that by protoporphyrin IX by approx. 20% (Table 4). In assays with Mg^{2+} , with or without NOHb and protoporphyrin IX, and with Mn^{2+} , with or without activator, NaCN (1 mM) had little effect on activity (Table 4). $K_3Fe(CN)_6$ (1 mM) inhibited activity in all assays with Mg^{2+} to a greater extent than with Mn^{2+} (Table 4). Rotenone (0.6 mM) inhibited activity under all conditions and virtually abolished activation by protoporphyrin IX. Methylene Blue (1 mM) had little effect on basal activity (Table 4) or increased it somewhat in the presence of Mn^{2+} (Fig. 6), but it markedly inhibited activation by either NOHb or protoporphyrin IX in assays with Mg^{2+} as well as effects of activator in assays with Mn^{2+} (Table 4). The concentration-dependence of the effects of Methylene Blue is shown in Fig. 6. Methylene Blue also inhibited the effects of cytochrome *c* and ferredoxin on cyclase activity (results not shown).

Activity of the enzyme purified through chromatography on GTP-agarose was inhibited by diamide or oxidized glutathione in a reversible fashion, as reported previously (Tsai *et al.*, 1981). Inactivation with diamide at 22°C obeyed pseudo-first-order kinetics (Fig. 7). A plot of $\log(1000/t_{0.5})$ versus $\log$ (diamide concentration) yielded a straight line with a slope of 1.1. Similar results were obtained with oxidized glutathione. Treatment with diamide or *p*-chloromercuribenzoate inhibited activation by NOHb (S.-C. Tsai, R. Adamik, V. C. Manganiello & M. Vaughan, unpublished work).

Discussion

From estimates of the Stokes radius (4.7 nm) and $s_{20,w}$ (6.4 S) at several stages during purification, the calculated M_r value of the native enzyme is 133 000. Electrophoresis of the purified enzyme under denaturing conditions indicates that the enzyme is a homodimer with a subunit M_r of 69 000. These estimates of apparent molecular size and subunit composition are in basic agreement with those published for the enzymes purified from rat liver and bovine lung (Braugher *et al.*, 1979a,b; Ignarro *et al.*, 1982a,b). No previous workers, however, have attempted to correlate peptide patterns (electrophoresis in the presence of SDS) with activity in individual and successive fractions isolated during those final step(s) that yield the purified enzymes. Such analyses might be important in understanding differences in responsiveness to NO, protoporphyrin IX and NOHb in preparations from different sources.

Gerzer *et al.* (1981a,b) reported that purified preparations of guanylate cyclase from bovine lung contained 1 mol of haem (ferroprotoporphyrin IX) and 1 mol of copper per mol of native enzyme. The

lung enzyme purified by Ignarro *et al.* (1982a,b) apparently also contained haem, and it seems probable that haem was present in the purified liver guanylate cyclase described by Braugher *et al.* (1979a). These preparations were markedly activated by nitroprusside or NO, whereas the purified enzyme described in the present paper was activated little, if at all, by NO. The precise mechanisms responsible for loss in responsiveness in our preparations and those of others (Gerzer *et al.*, 1981a; Ignarro *et al.*, 1982a,b) remain to be defined. By using procedures similar to those of Gerzer *et al.*, (1981a) and Ignarro *et al.* (1982a,b), NO-responsive preparations were isolated from bovine lung. Chromatography of the lung preparations as well as rat liver preparations described in the present paper on agarose-GTP was sufficient to decrease responsiveness to NO markedly. When Hb was added to the purified liver cyclase, NO increased activity to values (2–8 μ mol/min per mg of protein) similar to those reported for the lung enzyme (Gerzer *et al.*, 1981b; Ignarro *et al.*, 1982b). Craven & DeRubertis (1978) first reported that haem or haemoproteins could restore responsiveness of liver guanylate cyclase to NO, nitroprusside and related compounds. Although the specific activities of our guanylate cyclase preparations were more than 10-fold higher, there were many similarities in the responses to Hb, NO and NOHb as well as the effects of Mg^{2+} versus Mn^{2+} . Not all haem-containing proteins, however, are capable of supporting activation of guanylate cyclase by NO; for example, cytochrome *c* did not restore responsiveness to NO. Essentially all of the observations on enzyme preparations such as those described in the present paper that presumably lacked haem (Ignarro *et al.*, 1982a,b) or contained haem (Gerzer *et al.*, 1981a,b; Ignarro *et al.*, 1982a,b) are consistent with the conclusion that haem is required for activation by NO and related compounds. The proximate activating molecule is in all probability NO-haem.

In the presence of NOHb, cyclase activity was at least as great with Mg^{2+} as it was with Mn^{2+} ; similar findings have been reported by Craven & DeRubertis (1978) with partially purified liver enzyme and by Ignarro *et al.* (1982a) with a purified lung guanylate cyclase. In the absence of activator, guanylate cyclase activity was higher with Mn^{2+} than with Mg^{2+} , as has been observed repeatedly with the enzymes at all stages of purification (Craven & DeRubertis, 1978; Tsai *et al.*, 1978, 1980; Braugher *et al.*, 1979a,b; Gerzer *et al.*, 1981a,b; Ignarro *et al.*, 1982a,b). In assays with Mg^{2+} , with or without NOHb or activator, the enzyme described in the present paper exhibited a K_m for GTP of approx. 0.35 mM, whereas with Mn^{2+} Lineweaver-Burk plots were non-linear (apparent K_m values approx. 60 μ M and 1.5 mM). Similar

differences between assays with Mn^{2+} and Mg^{2+} were also noted for the lung enzyme (Ignarro *et al.*, 1982a,b). Neither activator nor NOHb altered metal-ion- or pH-dependence of enzyme activity or K_m values for GTP (in assays with Mn^{2+} or Mg^{2+}). There seems to be increasing agreement that Mg^{2+} is probably the physiologically relevant bivalent cation and that Mn^{2+} , in addition to substitution for Mg^{2+} in the catalytic process, can activate the guanylate cyclase (Gerzer *et al.*, 1981a; Ignarro *et al.*, 1982a; Brauhler, 1980).

Ignarro *et al.* (1982a) found that haem-containing lung guanylate cyclase was activated 30–40-fold by protoporphyrin IX (to the same extent as by NO or NO-haem in assays with Mg^{2+}). More recently, they reported activation by protoporphyrin IX of lung guanylate cyclase preparations deficient in haem (Ignarro *et al.*, 1982b). Protoporphyrin IX, which increased activity of the enzyme described in the present paper, did not support activation by NO. Whether the striking activation by protoporphyrin IX observed by Ignarro *et al.* (1982a,b) was due to the presence of some component lacking in our guanylate cyclase preparation remains to be determined.

We (Tsai *et al.*, 1980) and others (Brandwein *et al.*, 1981) have reported that the soluble liver guanylate cyclase can be reversibly inactivated by certain thiol-reactive reagents and disulphide compounds. Inactivation of the enzyme by diamide, as shown in the present work, apparently results from the oxidation of a single class of thiol groups. The purified guanylate cyclase was also inhibited by approx. 50% by $50\mu M$ - Na_3VO_4 in assays with Mg^{2+} or Mn^{2+} with and without NOHb or activator. In view of the proposed role of redox reactions in the regulation of guanylate cyclase, it is of interest that reduction of V^V to V^{IV} abolished the inhibitory effect of vanadate. The concentration of VO_4^{3-} required to inhibit guanylate cyclase is, however, much greater than that required to inhibit the $Na^+ + K^+$ -dependent ATPase (Cantley *et al.*, 1977) and, in light of the reported vanadate content of tissues, the effect may not be of physiological significance. Na_2WO_4 , at considerably higher concentrations, was also inhibitory under all assay conditions; however, $2mM$ - $Na_2B_4O_7$, which caused approx. 50% inhibition in assays with Mn^{2+} , had relatively little effect in assays with Mg^{2+} . It is possible that $B_4O_7^{2-}$ inhibits the postulated activating effect of Mn^{2+} more than it does the catalytic activity of the guanylate cyclase.

FMN, NAD^+ and NADH selectively inhibited activation by NOHb and protoporphyrin IX in assays with Mg^{2+} without altering effects of activator in assays with Mn^{2+} . Ohlstein *et al.* (1982) have also reported that FMN inhibited activation by NO-haem and protoporphyrin IX without altering basal activity. Rotenone inhibited activation by

protoporphyrin IX to a greater extent than that by NOHb or activator. Whether these compounds inhibited activity solely by virtue of their redox properties is unknown. Methylene Blue markedly decreased activation by protoporphyrin IX, NOHb or activator as well as by the proteins Hb, cytochrome *c* and ferredoxin without inhibiting basal activity. In some experiments, Methylene Blue actually increased basal activity. In increasing basal activity, Methylene Blue may mimic (perhaps by interaction with hydrophobic domains in the cyclase) some of the effects of haemoglobin, cytochrome *c* and activator. Inhibition of activation by protoporphyrin IX and especially by NOHb may be related to the redox properties of Methylene Blue.

Though apparently deficient in haem, the enzyme preparation described in the present paper responds dramatically to NOHb (50–100-fold activation). Its activity can also be effectively regulated (with or without added haem) by the bivalent cations Mn^{2+} and Mg^{2+} , by several kinds of molecules (i.e., activator; proteins such as Hb, cytochrome *c* and ferredoxin; compounds such as Methylene Blue) and a number of compounds that show some selectivity in apparently inhibiting the effects of different activators. In utilizing Hb for activation by NO, the cyclase may be analogous to prostaglandin endoperoxide synthase, which requires haem for activity but when isolated in the haem-deficient state can be activated by certain haem-containing proteins (Ueno *et al.*, 1982). The earlier work of Craven & DeRubertis (1978) and the more recent observations by Ignarro *et al.* (1982b) suggest that haem-deficient preparations of guanylate cyclase can bind haem and be activated by NO. A purified guanylate cyclase, such as that described in the present paper, should be particularly useful for defining the nature of the interaction with haem and elucidating the mechanism of haem-dependent processes (i.e., NO activation) as well as haem-independent processes (i.e., activation by protoporphyrin IX and other effectors).

We thank Mrs. D. Marie Sherwood for expert secretarial assistance.

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