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THE SEPARATION OF THE HEME AND APOHEME FORMS OF SOLUBLE GUANYLATE CYCLASE

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Two forms of soluble guanylate cyclase from mammalian tissues can be separated on DEAE Sephacel or Blue Sepharose CL-6B. The two forms, referred to as peak I or peak II, migrate identically during electrophoresis on polyacrylamide gels in the presence or absence of Na-dodecyl-SO_4. Peak I is markedly stimulated by sodium nitroprusside and is the heme-containing form of guanylate cyclase. Peak II is only weakly stimulated by nitroprusside and contains no heme absorbance. In fresh tissue extracts, peak I is the predominant form, but it can be converted to peak II by treatments (pH 5.0, storage at 4°C) that result in the loss of the heme absorbance from the enzyme. Peak II is not formed from peak I by proteolysis.

INTRODUCTION

Guanylate cyclase, the enzyme that catalyzes the formation of cyclic GMP from GTP, is found in both particulate and soluble fractions of most mammalian tissues. Although cyclic GMP concentrations are known to be altered by many agents (1,2) including carcinogens (3) and vasodilators (4), the mechanisms of regulation of cyclic GMP metabolism are not yet understood. The soluble form of guanylate cyclase appears to be regulated by oxidation/reduction-related phenomena, since fatty acid peroxides (5), and NO and compounds which contain and/or may release NO (6) activate the enzyme. The mechanism by which these agents activate soluble guanylate cyclase is not clear. It has been suggested that the enzyme contains more than one oxidizable group by which the enzyme can be activated (1,4,7). Recent observations of Gerzer, et al. (8,9)

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demonstrated that the soluble form of the enzyme purified from bovine lung contains heme. The heme-containing form of the enzyme appears to be highly stimulated by sodium nitroprusside and similar agents (8-12), whereas apparently heme-free guanylate cyclase is only stimulated weakly by such agents (13,14). Braughler et al., however, have suggested that guanylate cyclase does not contain heme (13). Very recently it has been proposed that heme is an inhibitor of guanylate cyclase and that activation of the enzyme occurs through replacement of heme by protoporphyrin IX (10). Thus, whether or not forms other than the heme-containing form of the enzyme are present in tissues and whether or not heme has a functional role in the regulation of guanylate cyclase activity remains controversial.

We report here that the heme-containing enzyme can be easily separated from the apparently heme-free enzyme by chromatographic methods. We also show that fresh tissue extracts contain the heme form of the enzyme.

MATERIALS AND METHODS

Materials. Blue Sepharose CL-6B and DEAE-Sephacel were obtained from Pharmacia. [α-^{32}P]GTP was purchased from New England Nuclear. Unlabeled nucleotides, sodium nitroprusside and bovine serum albumin (essentially fatty acid free) were from Sigma. Other chemicals were obtained from Sigma or Fisher.

Methods. Purification of guanylate cyclase. Soluble guanylate cyclase from bovine lung was purified essentially as previously reported (8). In brief, the extracts from 10 bovine lungs were applied to DEAE-Sephacel, and fractions containing enzyme were then pooled and precipitated with 50% (w/v) $(NH_4)_2SO_4$, followed by chromatography on Blue Sepharose CL-6B and preparative gel electrophoresis. The purified enzyme migrated as a single band on analytical gels under both denaturing and non-denaturing conditions. Basal activity in the presence of Mn^{2+} was 300-500 nmol cyclic GMP formed/min/mg. For the determination of the chromatographic behavior of guanylate cyclase, partially purified enzyme was used as indicated in the text.

Guanylate cyclase assay. Guanylate cyclase activity was determined as previously described (8). The assay mixture generally contained 3 mM Mn^{2+}, 10 mM dithiothreitol, 50 mM triethanolamine/HCl-buffer, pH 7.5, 100 μM [α-^{32}P] GTP (1-3 x 10^5 cpm), 0.6 mg/ml of bovine serum albumin and 20 μl of enzyme. Reactions (100 μl volume) were started by the addition of substrate in the presence or absence of sodium nitroprusside. The cyclic [^{32}P] GMP formed was estimated as described previously (8).

Other methods. Protein was determined according to Lowry et al. (15). Analytical gel electrophoresis was performed essentially as described by Weber and Osborn (8,16).

RESULTS AND DISCUSSION

Freshly prepared extracts from bovine lung contained one peak (peak I) of guanylate cyclase activity (Fig. 1A), which was eluted at a conductivity of 3 to 6 mmho and was highly stimulated by sodium nitroprusside. After dialysis

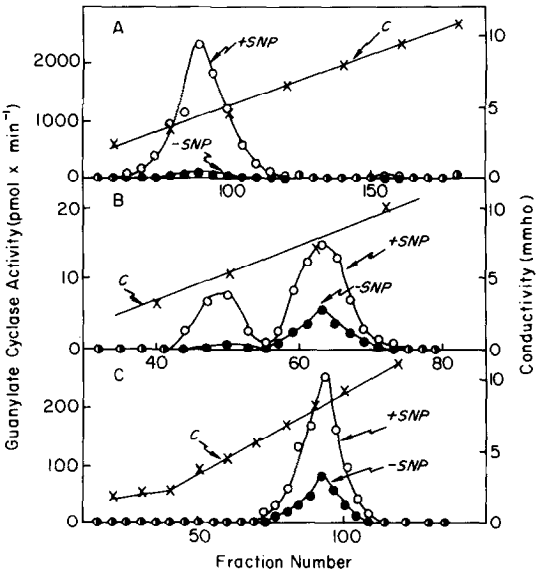

Fig. 1. Chromatography of soluble guanylate cyclase on DEAE Sephacel. Columns were equilibrated with extract buffer (20 mM triethanolamine/HCl (pH 7.5), 2 mM EDTA, 0.2 mM benzamidine and 50 mM 2-mercaptoethanol) and guanylate cyclase was eluted with a linear gradient of NaCl. (A) Elution profile of freshly prepared extracts from bovine lungs. (B) Chromatography of the peak I-containing fractions after dialysis and storage in 50% glycerol (v/v) at -20°C for four months. (C) Chromatography of the peak II-form of guanylate cyclase obtained from Blue Sepharose. Enzyme activity was determined in the absence (-●-, -SNP) or presence (-o-, +SNP) of 0.1 mM sodium nitroprusside; -x-, conductivity.

of peak fractions, enzyme activity again chromatographed in the same position on DEAE-Sephacel and continued to be highly stimulated by sodium nitroprusside. However, when dialyzed fractions were stored at 4°C for several days, at -20°C in 50% glycerol for several months, or were subjected to pH 5, an additional peak of activity was eluted from the DEAE Sephacel column (Fig. 1B). This form (peak II) of guanylate cyclase eluted at 7 to 9 mmho and was stimulated only 2- to 4-fold by sodium nitroprusside. When the pooled peak II fractions were again chromatographed on DEAE Sephacel, they eluted at 7 to 9 mmho and continued to be only slightly stimulated by sodium nitroprusside (not shown). These data suggest that only the first peak of activity is present in freshly prepared extracts and that the second peak of activity is formed only after storage or after pH 5-treatment.

Figure 2 shows the elution profile of guanylate cyclase from Blue Sepharose. Two peaks were eluted from the Blue Sepharose column when either

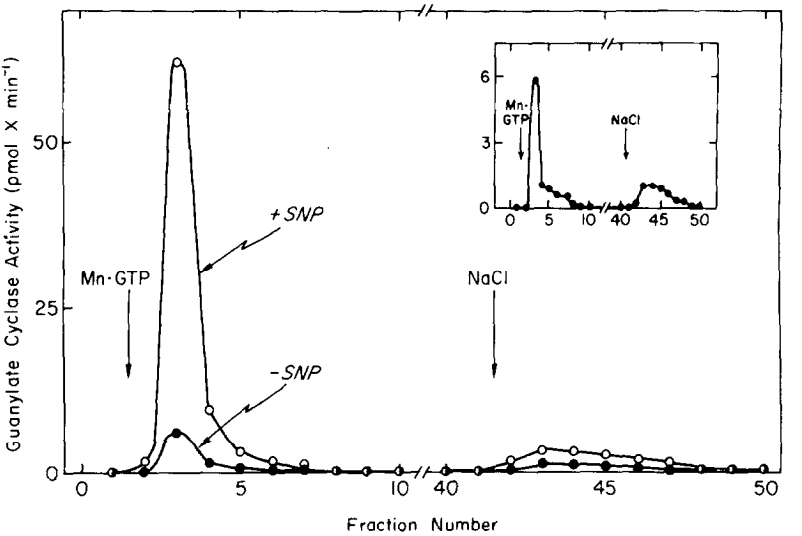

Fig. 2. Chromatography of soluble guanylate cyclase on Blue Sepharose. Guanylate cyclase purified on DEAE Sephacel and by $(NH_4)_2SO_4$-precipitation was applied to Blue Sepharose as previously described (8). The peak I-form of guanylate cyclase was eluted with 0.5 mM Mn·GTP at pH 7.5 and the peak II-form by the subsequent addition of 500 mM NaCl to the elution buffer. Guanylate cyclase activity was determined in the absence (-●-,-SNP) or presence (-o-, +SNP) of 0.1 mM sodium nitroprusside. The inset shows the elution profile of basal guanylate cyclase activity.

fresh extracts or the peak I-enzyme from DEAE Sephacel was applied. The first peak of activity was eluted with Mn·GTP, was stimulated more than 10-fold by sodium nitroprusside and was the heme-containing form of guanylate cyclase (11). A second peak of activity was eluted with salt (500 mM NaCl in the presence or absence of Mn·GTP) and was stimulated 2- to 4-fold by sodium nitroprusside. Most of the activity (>70°/o) was also eluted as peak I when freshly prepared extracts from different tissues were passed over the Blue Sepharose (Table 1). However, when enzyme was stored at 4°C for several days or treated at pH 5, a shift of activity from peak I to peak II was observed. Elution of a peak I and peak II form of the enzyme also was observed with extracts from rat lung and human platelets (Table I). As shown for human platelets, a shift of activity from peak I towards peak II also could be induced in tissues other than bovine lung.

When peak I from Blue Sepharose was immediately chromatographed on DEAE Sephacel, the enzyme was eluted as peak I, and chromatography of the Blue Sepharose peak II-enzyme on DEAE Sephacel yielded only peak II-enzyme

Table I. Chromatography of Soluble Guanylate Cyclase on Blue Sepharose CL-6B.

Enzyme	Pretreatment	% Eluted As Peak I (basal activity)	Peak II
bovine lung	fresh extracts	80	20
"	DEAE Sephacel Peak I	55	45
"	DEAE Sephacel 5 min pH 5	33	67
"	DEAE Sephacel 60 min pH 5	19	81
"	DEAE Sephacel 5 days 4°C	40	60
"	DEAE Sephacel 5 days 4°C + 5 min pH 5	5	95
human platelets	fresh extracts	80	20
"	fresh extracts 5 min pH 5	45	55
rat lung	fresh extracts	70	30

Guanylate cyclase fractions (either fresh extracts or the DEAE-peak I-enzyme) were chromatographed on Blue Sepharose with a buffer containing 20 mM triethanolamine/HCl, pH 7.5, 0.2 mM EDTA, 50 mM 2-mercaptoethanol and 0.2 mM benzamidine. The peak I-form of guanylate cyclase was eluted by the addition of 0.5 mM Mn·GTP and the peak II-form by the subsequent addition of 500 mM NaCl to the buffer. Stimulation of the eluted peak I- and peak II-form by sodium nitroprusside was >10 and <4-fold, respectively.

regardless of whether or not fresh extracts or the DEAE peak I-enzyme had been used for the Blue Sepharose chromatography (Fig. 1C). The above data indicate that two peaks of guanylate cyclase activity can be reproducibly separated from each other on Blue Sepharose and that peak II-enzyme is generated during the purification procedure.

It has been reported previously that pH 5-treatment or oxidation of soluble guanylate cyclase decreases the heme-absorbance (433 nm) of purified enzyme preparations (9). These previous data suggested that the presence or absence of heme could account for the difference between the peak I-and II forms of guanylate cyclase. Fig. 3 shows the visible absorbance spectrum of guanylate cyclase before and after treatment at pH 5 in comparison with the spectrum of free heme. Whereas the spectrum of the enzyme before the pH

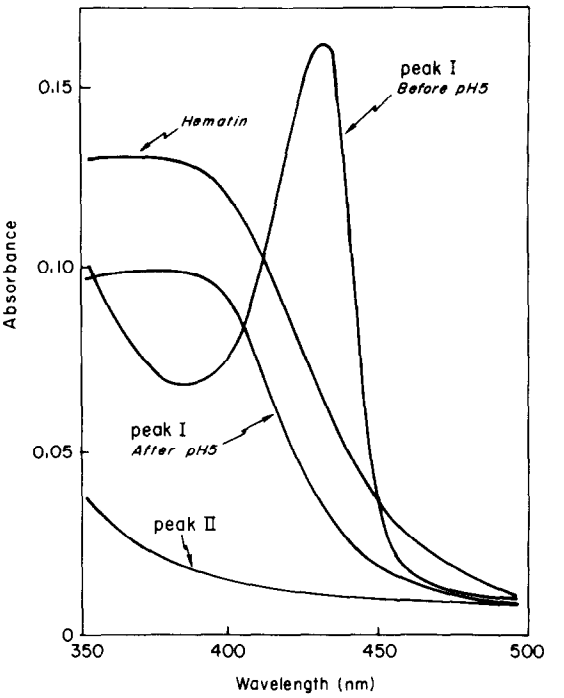

Fig. 3. The visible absorbance spectrum of the peak I and II-forms of guanylate cyclase. The absorbance spectrum of peak I from Blue Sepharose was determined before and after a pH 5-treatment (9) for 60 min in a buffer containing 20 mM triethanolamine/HCl, pH 7.5, 50 μM 2-mercaptoethanol, 0.2 mM EDTA and 0.2 mM benzamidine. The spectrum was then repeated after subsequent chromatography of the enzyme on DEAE-Sephacel (peak II). For comparison, the spectrum of 10 μM hematin in the above buffer is included. Basal guanylate cyclase activity was stimulated 15 and 2-fold by sodium nitroprusside before and after the pH 5-treatment with or without chromatography, respectively.

5-treatment was identical to the spectrum reported previously for the soluble form of guanylate cyclase purified to apparent homogenity (9), the heme-spectrum after the pH 5-treatment was identical to the spectrum of free heme. This absorbance maximum of the unbound heme could be removed from the enzyme by subsequent chromatography of the pH 5-treated enzyme on either DEAE-Sephacel (Fig 3) or Blue Sepharose. Peak II-enzyme obtained without the pH 5-treatment also did not contain detectable heme-absorbance (not shown). The fractions from Blue Sepharose, as shown here, had an absorbance maximum identical to the absorbance maximum present in purified guanylate cyclase (9). A heme-protein other than soluble guanylate cyclase could not be detected in subsequent chromatography as determined in this study (not shown) and also reported earlier (11). This suggested that soluble guanylate cyclase

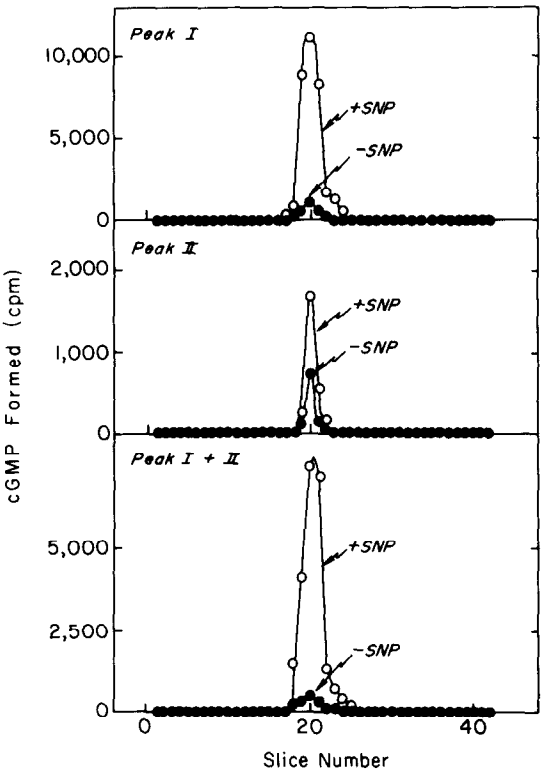

Fig. 4. Analytical polyacrylamide gel electrophoresis of the peak I- and II-forms of guanylate cyclase. Purified peak I or peak II or peak I and peak II together were chromatographed on a 10% polyacrylamide slab gel. Peak II was prepared from pure peak I by pH 5-treatment and subsequent chromatography on DEAE-Sephacel and was concentrated by vacuum dialysis using Millipore CX30 immersible membranes. The gel was electrophoresed for 5 hr at 1 W before application of samples and for 12 hr at 1 W thereafter. Two mm slices from the respective slab were allowed to stand at 4°C for 12 hr in a solution containing 20 mM triethanolamine/HCl-buffer, pH 7.5, 1 mg/ml bovine serum albumin and 1 mM dithiothreitol. Guanylate cyclase activity of the solution was then determined using 50 µl aliquots in the absence (-●-, -SNP) or presence (-○-, +SNP) of 50 µM sodium nitroprusside. The assay mixture contained 50 µM [α-^{32}P]GTP and 3 mM $MnCl_2$.

was the only heme-protein present in the peak I-fraction from the Blue Sepharose.

The above results indicate that pH 5-treatment removes the heme from guanylate cyclase and that the peak II-form of the enzyme is the apo-heme enzyme. These results, however, did not exclude other possible differences between the two enzyme forms, e.g., proteolytic modifications. Homogeneous peak I and peak II enzyme migrated identically on analytical gels under non-denaturing conditions (Fig. 4). After electrophoresis and elution from

gel slices the peak I-form of the enzyme was stimulated 10-fold by sodium nitroprusside, whereas the peak II-form again was stimulated only 2-fold.

Under denaturing conditions in the presence of Na-dodecyl-SO_4, the peak I- and II-forms of the enzyme also migrated identically (not shown). Proteolysis, therefore, did not appear to account for the differences in chromatographic behavior of peak I and II.

Our findings indicate that we have described the basic properties of two forms of mammalian soluble guanylate cyclase. These two forms can be easily separated by chromatographic methods and appear to be the holo-enzyme and apo-heme form of soluble guanylate cyclase. Freshly prepared guanylate cyclase migrated initially as the heme-containing form of the enzyme. The apo-heme form arose during the purification, by storage of the enzyme, or by decreasing the pH of the solution. These findings suggest that heme-free enzyme was not present in intact tissues. No evidence was found for the presence of another form of soluble guanylate cyclase such as protoporphyrin IX-containing enzyme (10).

Removal of heme from the enzyme markedly decreased (from 15-fold to 2-fold) but did not eliminate stimulation of the enzyme by sodium nitroprusside. This finding confirms earlier indirect evidence suggesting that soluble guanylate cyclase has at least two different oxidizable sites (1,4,7). One of these sites contains heme, which apparently is required for optimal stimulation of enzyme activity, but is easily lost. The second site may, as proposed, contain sulfhydryl-groups (4,7) or another oxidizable component.

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