SOLUBLE GUANYLATE CYCLASE PURIFIED FROM BOVINE LUNG CONTAINS HEME AND COPPER

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1. Introduction

The activity of soluble guanylate cyclase appears to be affected by redox mechanisms (reviews [1,2]); stimulation by various oxidants, oxygen radicals, unsaturated fatty acids and their peroxides has been reported; in addition, nitric oxide, sodium nitroprusside and other drugs can activate soluble guanylate cyclase in crude extracts. Stimulation by such agents is largely decreased after purification of soluble guanylate cyclase [3–5]. Heme [3] and copper [6] may play a role in the regulation of soluble guanylate cyclase activity. Neither compound, however, has so far been detected in purified preparations of soluble guanylate cyclase [3,4,7–9].

Purification of soluble guanylate cyclase from bovine lung can be achieved without major loss in stimulation by sodium nitroprusside and related agents [10]. In addition, it was observed that in the last purification step a chromophore with an absorption maximum at ~430 nm copurified with the enzyme activity [11]. We report here that this chromophore is a heme, which has been identified as ferroprotoporphyrin IX. In addition, copper was detected in the purified preparations. Both compounds may be involved in the regulation of guanylate cyclase activity.

2. Experimental

Soluble guanylate cyclase was purified from bovine lung as in [10]. Visible absorption spectroscopy was performed using a Beckman ACTA M VI spectrophotometer in cuvettes of 1 cm pathlength. The pyridine hemochrome spectrum of guanylate cyclase was determined according to [12]. Cu and Fe were determined by atomic absorption spectroscopy, using a Perkin-Elmer instrument model 5000. Determinations of Fe and Cu were performed in enzyme preparations dialyzed against a 20 mM triethanolamine–HCl buffer (pH 7.5) containing 0.2 mM EDTA and 50 mM 2-mercaptoethanol. Fe and Cu in the buffer were <10 and 1 ng/ml, respectively. Values obtained in purified enzyme preparations were corrected for these blanks. Guanylate cyclase activity was determined with 3 mM Mg^{2+} as in [10], with omissions of cyclic GMP and EGTA in the assay. All values represent means of duplicate determinations from representative experiments. Protein was determined as in [13].

3. Results

The visible spectrum of the purified enzyme is shown in fig.1. The native enzyme exhibited absorbance maxima at 433 nm and 565 nm and a shoulder at ~550 nm. Addition of sodium dithionite had no effect on these maxima. CO as well NO shifted the absorbance bands (see fig.1; cf. table 1). Effects seen with NO were also observed in the presence of sodium nitroprusside or 3-morpholino-sydnonimine (not shown). The occurrance of three absorbance bands in the native enzyme preparations, the positions of the absorbance maxima, their relative intensities and the spectral changes observed upon the additions of CO or NO are typical for the α-, β- and Soret-bands of a hemoprotein [12]. Determination of the pyridine hemochrome revealed the α-, β- and Soret-bands of the ferroprotoporphyrin IX pyridine hemochrome (see fig.1).

Addition of $K_3[Fe(CN)_6]$ did not change the positions of the absorbance maxima but reduced the absorption (fig.2). Oxygen exposure shifted the Soret-band to 429 nm (see fig.2; cf. table 1) and decreased the

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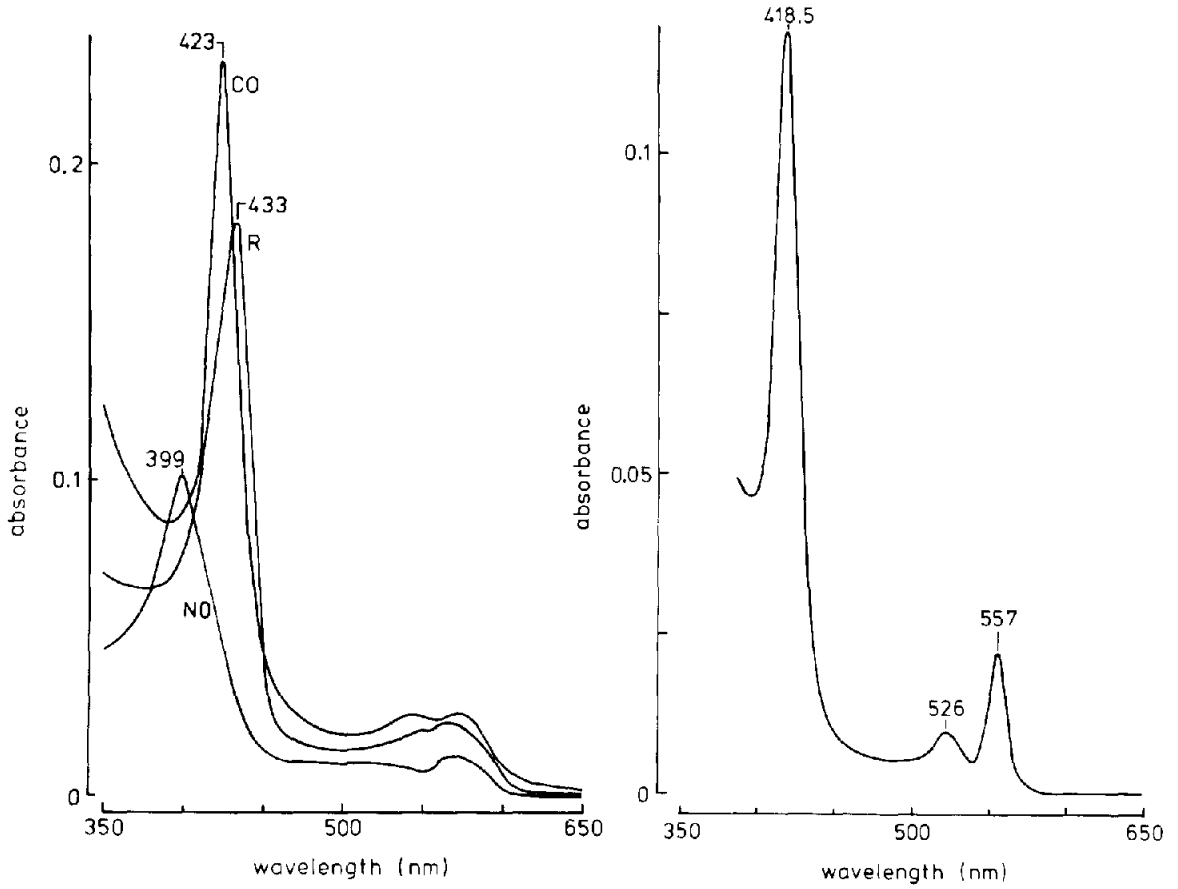

Fig.1. Visible absorption spectra of purified guanylate cyclase. Spectra of the enzyme (2.3 μM) were recorded in 20 mM triethanolamine–HCl buffer (pH 7.5) with 50% (v/v) glycerol, 20 mM 2-mercaptoethanol and 0.2 mM EDTA. Left: Spectra were recorded in the absence (R) or presence of saturating concentrations of carbon monoxide (CO) or nitric oxide (NO). Right: The pyridine hemochrome spectrum was recorded immediately after the additions of 50 μl pyridine and 25 μl 1 N NaOH. Addition of sodium dithionite had no effect on the spectrum.

absorbance; in addition, the β-band increased relative to the α-band (compare table 1). Reduction of the absorbance maxima was also observed when the enzyme was exposed to pH 5.0 (see fig.2). These results suggest that the heme present in purified guanylate cyclase is easily destroyed or removed by oxidation, oxygenation or pH 5-treatment. In contrast, the addition of NaCN had no effect on the absorbance maxima at ≤50 mM but caused a shift similar to that observed with oxygenation at higher levels (see fig.3). This suggests that either NaCN (at ≤50 mM) did not interact with the heme present in the enzyme preparation or that an interaction with the heme did not induce detectable changes in the visible spectrum. A summary of the spectroscopic properties of purified guanylate cyclase is given in table 1.

Metal determinations in several purified guanylate cyclase preparations revealed the presence of both iron and copper. Iron was found at 0.88 ± 0.10 (mean ± SEM) mol/mol guanylate cyclase (4 preparations) and copper at 1.08 ± 0.16 mol/mol (5 preps.).

Although NaCN did not affect the visible absorbance spectrum of purified guanylate cyclase, it reduced the stimulation of the enzyme by sodium nitroprusside in a concentration dependent manner (fig.3). The enzyme stimulated by 50 μM sodium nitroprusside was inhibited halfmaximally by ~1 mM NaCN. This inhibition was not affected by the addition of exogenous heme (at ≤100 μM). However, the inhibition was effectively prevented by the addition of $CuCl_2$ (see fig.3). SeO_2 and $NiCl_2$ (≤0.1 mM) did not prevent the inhibitory effect of NaCN (1 mM) but reduced this inhibition to

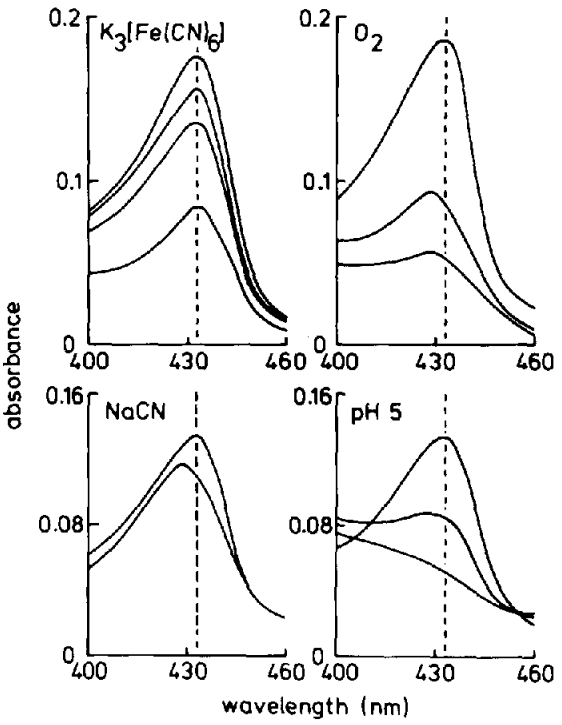

Fig.2. Effects of various treatments on the Soret-band of reduced guanylate cyclase. The upper line in each panel represents the spectrum of the untreated enzyme. Enzyme was 2.3 μM and 1.75 μM in the upper and lower panels, respectively. The spectra recorded after the addition of a solution were corrected for dilution. The dotted lines indicate the wavelength of 433 nm. Upper left: Effect of $K_3[Fe(CN)_6]$ (0.5, 1 or 2 mM). Upper right: Effect of oxygen. The cuvette containing the guanylate cyclase was gassed with oxygen (5 ml/min) for 30 s or 90 s. Lower left: Effect of NaCN. At ≤50 mM, NaCN did not change the absorbance; the lower line represents the spectrum recorded in the presence of 70 mM NaCN. Lower right: Effect of pH 5-treatment. Buffer containing guanylate cyclase was adjusted to pH 5.0 by addition of HCl. After 30 s or 45 s of standing at 4°C, the pH was readjusted to 7.5 by the addition of NaOH, and the spectrum was recorded immediately.

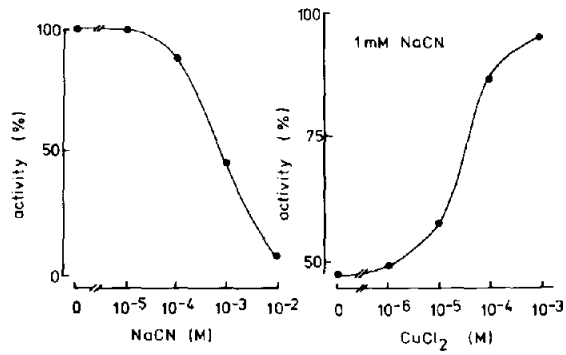

Fig.3. Influence of NaCN on the activity of sodium nitroprusside-activated guanylate cyclase. The assay was performed for 10 min at 37°C; sodium nitroprusside was 50 μM. Left: Effect of NaCN. Right: Effect of $CuCl_2$ in the presence of 1 mM NaCN. Guanylate cyclase activity was 0.11 and 4.5 μmol . mg^{-1} . min^{-1} without and with sodium nitroprusside, respectively.

Table 1
Summary of spectral data of soluble guanylate cyclase purified from bovine lung

Enzyme		Absorption bands		
		α	β	Soret
Reduced	λ	~565	(~550)	433
	ε	10		80
NO	λ	~570		399
	ε	5.5		45
CO	λ	~570	~540	423
	ε	11	11	102
O_2	λ		~550	429
	ε		(↓)	(↓)
$K_3[Fe(CN)_6]$	λ	~565	(~550)	433
	ε	(↓)	(↓)	(↓)
Pyridine hemochrome (reduced)	λ	557	526	418.5
	ε	14	6.3	80

ε, millimolar absorption coefficient; λ, wavelength in nm; (↓) decreased specific absorption

25% and 30%, respectively, when added at 1 mM. Other divalent metal ions such as Zn^{2+}, Hg^{2+}, Ba^{2+}, Rb^{2+}, Ag^{2+}, Cd^{2+}, Co^{2+} and Fe^{2+} were ineffective or were inhibitory by themselves.

4. Discussion

The chromophore present in preparations of pure soluble guanylate cyclase from bovine lung has been identified as ferroprotoporphyrin IX. It is unlikely that this chromophore is a contamination of the pure enzyme since the heme was present at stoichiometric concentrations after a >7000-fold purification of the enzyme, involving 4 different purification steps. In addition, the pure enzyme contained ~1 mol of each

iron and copper/mol native enzyme. The reproducible detection of heme, iron and copper in several purified guanylate cyclase preparations suggests that these factors are associated with the enzyme.

The mM absorption coefficient of the Soret-band observed in quanylate cyclase preparations ($\epsilon \cong 80$) was higher than that reported for 2 mol/heme present/mol prostaglandin endoperoxidase ($\epsilon \cong 61$) [14] but lower than that reported for other hemoproteins [12]. In connection with the presence of 1 mol iron/mol native enzyme, the observed absorption is compatible with a stoichiometry of 1 mol heme/mol native guanylate cyclase.

So far, the precise functions of heme and copper in the regulation of guanylate cyclase activity have not been elucidated. Either component represents a possible prosthetic group for interactions with various compounds such as oxygen, fatty acids and nitric oxide, which have been shown to affect cyclic GMP formation. The functions of these two enzyme constituents and their interactions with sulfhydryl groups, which are apparently also involved in the regulation of guanylate cyclase activity, need to be resolved in further investigations.

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