

Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase

(L-arginine/endothelium-derived relaxing factor/nitric oxide/cGMP/calmodulin)

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ABSTRACT The soluble form of guanylyl cyclase-activating-factor (GAF) synthase from rat cerebellum was purified to homogeneity by sequential affinity chromatographic steps on adenosine 2',5'-bisphosphate (2',5'-ADP)-Sephacryl and calmodulin-agarose. Enzyme activity during purification was bioassayed by the L-arginine-, NADPH-, and Ca²⁺/calmodulin-dependent formation of a plasma membrane-permeable nitric oxide-like factor that stimulated soluble guanylyl cyclase in RFL-6 cells. With calmodulin and NADPH as cofactors, purified soluble GAF synthase induced an increase of 1.05 μ mol of cGMP per 10⁶ RFL-6 cells per 3 min per mg of protein. The coproduct of this signal-transduction pathway appeared to be L-citrulline. GAF synthase catalyzed the conversion of 107 nmol of L-arginine into L-citrulline per min per mg of protein. Based on these assays, this represents a purification of GAF synthase of approximately 10,076- and 8925-fold with recoveries of 16% and 19%, respectively. Rechromatography of the purified enzyme on Mono P (isoelectric point = 6.1 \pm 0.3), Mono Q, and Superose 12 or 6 resulted in no further purification or increase in specific activity. A Stokes radius of 7.9 \pm 0.3 nm and a sedimentation coefficient $s_{20,w}$ of 7.8 \pm 0.2 S were used to calculate a molecular mass of about 279 \pm 25 kDa for the native enzyme. SDS/PAGE revealed a single protein band with a molecular mass of about 155 \pm 3 kDa. These data suggest that soluble GAF synthase purified from rat cerebellum is a homodimer of 155-kDa subunits and that enzyme activity is dependent upon the presence of calmodulin.

In various mammalian cell types [e.g., macrophages (1), endothelial cells (2), polymorphonuclear neutrophils (3, 4), brain (5, 6), and neuronal cell lines (7, 8)], L-arginine is oxidized to yield the endothelium-derived relaxing factor with physicochemical and pharmacological properties similar to nitric oxide (NO) or a NO-containing compound (9, 10). Both within the same cell and in specific target cells, this NO-like factor (or group of factors) activates soluble guanylyl cyclase, thereby acting as an intra- and intercellular signal molecule to regulate the intracellular concentration of cGMP (11–14). Earlier studies have demonstrated that L-arginine could activate guanylyl cyclase in brain and neuronal cells (15, 16); however, the mechanisms were not elucidated. This laboratory has proposed (17, 18) that hormonal regulation of cGMP accumulation could be mediated through such a signal transduction pathway. Since the exact chemical structure of this ubiquitous factor (or group of factors) remains to be clarified (19, 20), we suggest the preliminary name of guanylyl cyclase-activating factor (GAF). The formation of GAF from L-arginine is catalyzed by an NADPH-dependent GAF synthase (6, 21–25). GAF synthase and its target enzyme, soluble guanylyl cyclase, represent the protein components

of this signal-transduction pathway (13, 14, 17, 18, 26, 27). In rat cerebellum, GAF synthase activity is due to a cytosolic Ca²⁺/calmodulin-regulated isoform of the enzyme. GAF synthase was found to catalyze the conversion of L-arginine to both L-citrulline and a labile NO-like activator of soluble guanylyl cyclase (i.e., GAF). The enzyme was purified by the sequential use of two affinity chromatography columns, ADP-Sepharose and calmodulin-agarose, from the crude supernatant fraction to homogeneity (8925- to 10,076-fold, assayed by citrulline and soluble guanylyl cyclase stimulation, respectively). Purified GAF synthase is a (homo)dimer of protein subunits of 155 \pm 3 kDa. Some of these observations have been presented in abstract form (28–30).

MATERIALS AND METHODS

Materials. Calmodulin-agarose and Sepharose 4B were obtained from Sigma. All other column materials and standard marker proteins were purchased from Pharmacia. Glycine and Tris (Bio-Rad) and SDS (Boehringer Mannheim) were of electrophoresis grade. All other reagents were of analytical grade. Biotinylated calmodulin, biotinylated alkaline phosphatase, and avidin were kindly provided by M. L. Billingsley (Department of Pharmacology, Pennsylvania State University).

Crude Supernatant Fraction. Frozen rat cerebella (Pel-Freez) were thawed and homogenized with a Polytron (Brinkmann) in ice-cold buffer 1 [50 mM Tris-HCl, pH 7.6/0.5 mM EDTA/0.5 mM EGTA/1 μ M leupeptin/1 μ M pepstatin A/0.1 mM phenylmethylsulfonyl fluoride (PMSF)/12.5 mM 2-mercaptoethanol] at 4 ml of buffer per g of tissue (wet weight). All subsequent procedures were conducted at 4°C. The homogenate was centrifuged at 105,000 \times g for 60 min. The resulting supernatant fraction was dialyzed for two 3-hr periods against 3 liters of buffer 2 (10 mM Tris-HCl, pH 7.6/0.1 mM EDTA/0.1 mM EGTA/1 μ M leupeptin/1 μ M pepstatin A/0.1 mM PMSF/12.5 mM 2-mercaptoethanol).

Adenosine 2',5'-Bisphosphate (2',5'-ADP)-Sepharose. The dialyzed crude supernatant fraction (300 ml) was incubated with 1 ml of preswollen 2',5'-ADP-Sepharose (Pharmacia) with gentle agitation (30 min, 4°C) and then transferred to a fritted chromatography column. The column was packed and washed with about 5 column volumes of buffer 2, about 3 column volumes of buffer 3 (buffer 2/0.5 M NaCl), and again with about 4 column volumes of buffer 2. GAF synthase was then eluted with buffer 4 (buffer 2/10 mM NADPH). Protein-containing fractions (tested for the absence of endoprotease activity; fibrin method; Boehringer Mannheim) were pooled

Abbreviations: GAF, guanylyl cyclase-activating factor; PMSF, phenylmethylsulfonyl fluoride; 2',5'-ADP, adenosine 2',5'-bisphosphate.

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and dialyzed for two 3-hr periods against 3 liters of buffer 5 (50 mM Tris-HCl, pH 7.6/1 mM CaCl₂/1 μM leupeptin/1 μM pepstatin A/0.2 mM PMSF/12.5 mM 2-mercaptoethanol).

Calmodulin-Agarose. The dialyzed pool from the 2',5'-ADP-Sepharose was incubated with preswollen calmodulin-agarose and noncoupled Sepharose 4B, 1:8 (vol/vol) (equivalent to 150 μg of calmodulin per ml of packed gel; both from Sigma) with gentle agitation (30 min, 4°C) and then transferred to a fritted chromatography column. The column was packed and washed with about 80 column volumes of buffer 5 and about 80 column volumes of buffer 6 (buffer 5/0.3 M NaCl). GAF synthase was then eluted with buffer 7 [buffer 5/1 M NaCl/10% (vol/vol) glycerol/5 mM EGTA instead of CaCl₂]. Protein-containing fractions were pooled.

Superose 12 or 6 Gel-Permeation Chromatography. For rechromatography and physical characterization of purified GAF synthase, the pool from the calmodulin-agarose column was dialyzed for two 3-hr periods against 3 liters of buffer 8 (20 mM Tris-HCl, pH 7.6/0.1 mM EDTA/0.1 mM EGTA/1 μM leupeptin/1 μM pepstatin A/0.1 mM PMSF/12.5 mM 2-mercaptoethanol) or buffer 9 (buffer 8/0.5 M NaCl). A 100-μl sample of the dialyzed pool was applied to a 24-ml FPLC Superose 12 or 6 gel-permeation column (HR 10/30; Pharmacia) and GAF synthase was eluted with buffer 8 or buffer 10. Fractions (250 μl) were immediately assayed for GAF synthase activity and/or protein.

Velocity Sedimentation. The purified GAF synthase (50 μg in 100 μl) was layered onto preformed 12.5-ml 12–26% sucrose gradients containing 5% glycerol in buffer 9. Standards were diluted and applied to identical gradients. All tubes were centrifuged at 150,000 × *g* in a Beckman SW 40 rotor for 24 hr at 5°C. Fractions (400 μl) were collected from the bottom of the tubes and assayed for protein as described. Standards utilized were porcine thyroglobulin (*s*_{20,w} = 19.44 S), horse spleen apoferritin (*s*_{20,w} = 17.6), bovine liver catalase (*s*_{20,w} = 11.3 S), yeast alcohol dehydrogenase (*s*_{20,w} = 7.4 S), and bovine serum albumin (*s*_{20,w} = 4.6 S).

Chromatofocusing. GAF synthase was dialyzed against buffer 8, applied to a 5-ml FPLC Mono P chromatofocusing column (Pharmacia), preequilibrated with 25 mM Bistris [[bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane]-HCl (pH 7.1), and eluted with Polybuffer 74-HCl (Pharmacia; pH 5.0). Fractions were immediately assayed for GAF synthase activity and pH.

Assay of GAF Synthase Activity. GAF synthase was bioassayed with cultured rat lung fibroblasts (RFL-6; American Type Culture Collection) rich in cytosolic soluble guanylyl cyclase, as described (7, 31–33). Briefly, confluent RFL-6 monolayers, cultured in 6- or 12-well plates, were washed twice with 2 ml of Locke's salt solution (154 mM NaCl/5.6 mM KCl/2 mM CaCl₂/1.0 mM MgCl₂/3.6 mM NaHCO₃/5.6 mM glucose/10.0 mM Hepes, pH 7.4) and then incubated for 30 min in 0.5 ml of Locke's salt solution containing 0.6 mM 3-isobutyl-1-methylxanthine. After this preincubation, enzyme preparations or column fractions were added and incubated in the presence of manganese-containing superoxide dismutase (20 units/ml), up to 100 μM L-arginine-HCl, up to 100 μM NADPH, and up to 30 nM calmodulin, in a final volume of 1.0 ml for 3 min. Cells were then washed rapidly with 3 ml of ice-cold Dulbecco's phosphate-buffered saline (Sigma); then 1 ml of 50 mM sodium acetate (pH 4.0) was added and the cells were frozen in liquid nitrogen. The cell-associated cGMP content was determined by radioimmunoassay (34). In some experiments, N^G-nitro-L-arginine, N^G-methyl-L-arginine (Calbiochem), trifluoperazine, or calmidazolium was included in the incubation mixture. Experiments with defined Ca²⁺ concentrations were performed in the presence of 1 mM EGTA as described (8).

Crude supernatant fractions contained hemoglobin that quenched the detection of GAF due to inhibition of guanylyl

cyclase activation (35). Therefore, to determine the specific GAF synthase activity before purification, crude supernatant fractions were incubated batchwise with phosphocellulose (pH 6.5; ref. 31). After this treatment, no hemoglobin was detected in the supernatant fraction (absorption at 420 nm) and GAF synthase activity was assayed.

Assay of Citrulline Formation. To determine the GAF synthase-catalyzed conversion of L-arginine into L-citrulline (25), samples of crude supernatant fractions or purified protein pools (50 μl) were incubated for 10 min at 37°C in the presence of 3 μM L-[2,3-³H]arginine (5.5 GBq/mmol), 100 μM NADPH, 30 nM calmodulin, and 2 mM CaCl₂ in a final volume of 100 μl. The reaction was stopped by adding 400 μl of a stop buffer (10 mM EGTA/100 mM Hepes, pH 5.5/1 mM L-citrulline). The incubate was then applied to a 1-ml Dowex AG 50WX-8 column (Na⁺-form; mesh size, 100–200) preequilibrated with the stop buffer. L-[2,3-³H]Citrulline was eluted twice with 0.75 ml of water and radioactivity was determined by liquid scintillation counting.

Protein Determination. Protein concentrations were determined according to Bradford (36) using bovine serum albumin as a standard.

SDS/PAGE and Calmodulin Affinity Labeling. The purity of enzyme preparations was monitored with SDS/PAGE using 7.5% gels, as described by Laemmli (37). Gels were stained with silver nitrate by the method of Morrissey (38). Molecular mass standards (Pharmacia) included myosin, α₂-macroglobulin, β-galactosidase, phosphorylase *b*, transferrin, bovine serum albumin, glutamic dehydrogenase, and ovalbumin. After SDS/PAGE, proteins were also transferred semi-dry to nitrocellulose membranes (39) and then subsequently incubated with biotinylated calmodulin and avidin/alkaline phosphatase, as described by Billingsley *et al.* (40).

RESULTS

Crude Supernatant Fraction. In rat cerebellum crude supernatant fraction, the detection of GAF synthase activity by bioassay was quenched by the presence of contaminating hemoglobin, which inactivates GAF, presumably, by binding and/or oxidizing NO or an NO-containing compound (35). Therefore, to determine the starting specific activity in crude samples, preparations were treated with phosphocellulose. This procedure, however, was not required for subsequent purification of the enzyme.

Crude supernatant fractions apparently contained residual low molecular mass components that interfered with the first affinity column step (2',5'-ADP-Sepharose). For the purification of GAF synthase, the crude supernatant fraction was dialyzed against the 2',5'-ADP-Sepharose equilibration buffer.

Purification and Stability of GAF Synthase. The purification of GAF synthase is shown in Table 1. The data reported here are representative of five experiments utilizing the same column materials. Purified GAF synthase had a specific activity that induced the synthesis of 1.05 μmol of cGMP per mg of protein per 3 min in RFL-6 cells and catalyzed the formation of 107 nmol of citrulline per mg of protein per min from radiolabeled L-arginine. This represents a 10,076-fold or 8925-fold purification from the crude supernatant fraction with a 16 and 19% recovery, respectively. Based on these data, soluble GAF synthase represents ≈0.01% of the crude supernatant fraction proteins of rat cerebellum.

The elution profiles of GAF synthase activity and proteins from the 2',5'-ADP-Sepharose are shown in Fig. 1. Although most of the protein in the crude supernatant fraction passed through the column, GAF synthase bound to the affinity matrix and was specifically eluted with 10 mM NADPH. Lower concentrations of NADPH resulted only in a partial elution of GAF synthase from 2',5'-ADP-Sepharose or substantial peak-broadening. Assay of samples from the column

Table 1. Purification of soluble GAF synthase

Fraction	Protein, μg	Soluble guanylyl cyclase activation, cGMP content per 10^6 RFL-6 cells				Citrulline formation			
		Total activity, nmol/ 3 min	Specific activity, nmol per 3 min per mg	Yield, %	Purification factor	Total activity, nmol/ min	Specific activity, nmol per min per mg	Yield, %	Purification factor
Crude supernatant (105,000 \times g)*	270,000	28.1 [†]	0.104 [†]	100	1	3.24	0.012	100	1
2',5'-ADP-Sepharose (NADPH eluate)	294	14.0	47.6	50	458	1.96	6.67	60	555
Calmodulin-agarose (EGTA eluate)	4.2	4.40	1047	16	10,076	0.6	107	19	8925

*This preparation was performed with 75 frozen rat cerebella.

[†]Determined after removal of hemoglobin by batch-wise incubation with phosphocellulose (pH 6.5; ref. 32).

fractions confirmed the presence of GAF synthase in the protein peak eluted from the 2',5'-ADP-Sepharose. However, protein-containing fractions were immediately pooled, dialyzed, and further purified. The enzyme activity in the pool of 2',5'-ADP-Sepharose, as determined by bioassay, was completely Ca^{2+} /calmodulin-dependent and was, thus, defined as being free of GAF synthase-regulating Ca^{2+} -binding protein(s). As this protein pool contained 0.1 mM EDTA and 0.1 mM EGTA, which interfered with the enzyme binding to the second affinity column (i.e., calmodulin-agarose), the 2',5'-ADP-Sepharose pool was dialyzed against the EDTA- and EGTA-free but Ca^{2+} -containing equilibration buffer of the calmodulin-agarose.

For the subsequent binding of GAF synthase to calmodulin-agarose, the optimal ratio between commercially available (Sigma) preswollen calmodulin-agarose and nonlabeled Sepharose 4B was found to be 1:8 (vol/vol) resulting in a final concentration of about 8.6 nmol of calmodulin per ml of packed gel. This allowed the preparation of a high-affinity column for which specific elution conditions (5 mM EGTA/10% glycerol/1 M NaCl) for GAF synthase were developed. The elution profiles of GAF synthase activity and proteins from the calmodulin-agarose are shown in Fig. 2. Lower concentrations of EGTA, omitting glycerol, or choosing a higher calmodulin concentration per ml of gel led to no or only partial elution of GAF synthase, substantial peak-broadening, or required chaotropic elution conditions to recover GAF synthase. Protein-containing fractions were immediately pooled. Assay of samples of the column fractions confirmed the presence of GAF synthase in the protein peak eluted from the calmodulin-agarose.

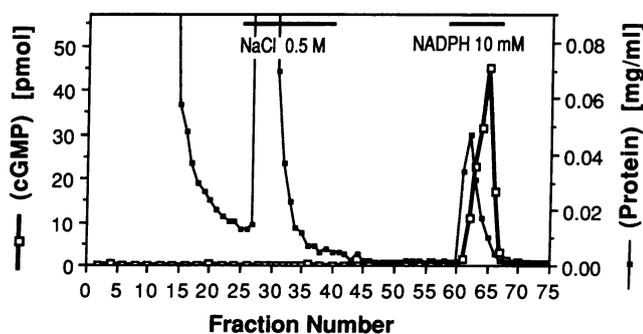


FIG. 1. 2',5'-ADP affinity chromatography of soluble GAF synthase. A crude supernatant fraction (70 ml) was bound to 2',5'-ADP-Sepharose 4B (30 min, 4°C) and then a column (22 \times 7 mm, about 2.4 ml) was packed. After washing the column, soluble GAF synthase was eluted with 10 mM NADPH. Fractions of 2 ml were collected and 100 μl per fraction was assayed for GAF synthase activity. \square , GAF synthase activity (pmol of cGMP per 10^6 RFL-6 cells per 3 min); \blacksquare , protein concentration in each column fraction.

Further chromatography of GAF synthase on Mono Q or Superose 12 or 6 resulted in no further purification or increase of specific activity.

Physical Characterization and Kinetic Data. With gel-permeation chromatography on Superose 12 or 6, the purified enzyme was eluted as a single protein peak. The Stokes radius R_s (41) was 7.9 ± 0.3 nm (Superose 6; Table 2). Superose 12 gave underestimations of the Stokes radius. This was most likely due to the higher degree of cross-linking and, thus, to increased hydrophobic interactions between GAF synthase and Superose 12 resulting in a higher elution volume. Native gel electrophoresis confirmed the presence of a single protein (data not shown). Samples of the crude supernatant fraction, fractions from the chromatography columns, and the purified enzyme were denatured with SDS and 2-mercaptoethanol and analyzed by SDS/PAGE (Fig. 3) using 7.5% gels. Silver staining of the gels revealed a single protein band in the purified preparation with a molecular mass of 155 ± 3 kDa. The sedimentation coefficient of native GAF synthase $S_{20,w}$ was measured relative to internal standards by velocity sedimentation through a 12–26% (wt/wt) sucrose gradient and was 7.8 ± 0.2 S. Thus, by assuming a partial specific volume of $0.725 \text{ cm}^3/\text{g}$ for GAF synthase, the frictional ratio (f/f_0) of the native enzyme was 1.83 and the calculated (41) molecular mass was 279 ± 25 kDa, in accordance with a dimer of two 155-kDa subunits.

After transfer of proteins to nitrocellulose membranes, the 155-kDa monomer was Ca^{2+} -dependently affinity-labeled with biotinylated calmodulin (data not shown). When chromatofocused on a Mono P column, GAF synthase was eluted at $\text{pH } 6.1 \pm 0.3$ (isoelectric point). Under first-order condi-

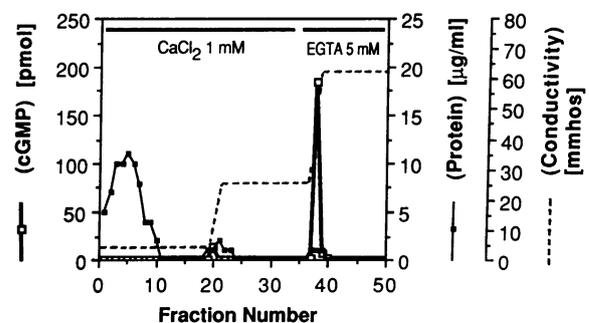


FIG. 2. Calmodulin affinity chromatography of soluble GAF synthase. The NADPH eluate of the 2',5'-ADP-Sepharose, pooled according to protein concentration (20.0 ml), was bound to calmodulin-agarose (30 min, 4°C) and then a column (9 \times 6 mm, about 0.4 ml) was packed. After washing the column, soluble GAF synthase was eluted with 5 mM EGTA/1 M NaCl. Fractions of 2 ml were collected and 100 μl per fraction was assayed for GAF synthase activity. \square , GAF synthase activity (pmol of cGMP per 10^6 RFL-6 cells per 3 min); \blacksquare , protein concentration in each fraction.

Table 2. Characterization of soluble GAF synthase

	Value(s)
Physical characterization	
Stokes radius, nm	7.9 ± 0.3*
$s_{20,w}$, S	7.8 ± 0.2†
f/f_0	1.83‡
Native molecular mass, kDa	279 ± 25‡
Denatured molecular mass, kDa	155 ± 3
Isoelectric point	6.1 ± 0.3
Kinetic data	
V_{max} , nmol of citrulline per min per mg	107
K_m (L-arginine), μ M	2.2
IC ₅₀ (N^G -nitro-L-arginine), μ M	0.9§
IC ₅₀ (N^G -methyl-L-arginine), μ M	1.6§
$K_{0.5}$ (Mg^{2+}), μ M	ND
$K_{0.5}$ (Ca^{2+}), μ M	0.35
$K_{0.5}$ (calmodulin), nM	3.5
IC ₅₀ (trifluoperazine), μ M	4.0¶
IC ₅₀ (calmidazolium), μ M	2.2¶

ND, no dependence.

*Measured relative to internal standards by gel permeation chromatography (Superose 6).

†Measured relative to internal standards by velocity sedimentation through a 12–26% sucrose gradient.

‡Calculated (41) from the Stokes radius and sedimentation coefficient by assuming a partial specific volume of 0.725 cm³/g.

§Assayed in the presence of 10 μ M L-arginine.

¶Assayed in the presence of 1 mM Ca^{2+} /30 nM calmodulin.

tions, GAF synthase activity was proportional to the amount of protein, and product accumulation (L-[³H]citrulline) was linear with time (up to 30 min; data not shown). Kinetic data for purified GAF synthase (V_{max} ; K_m for L-arginine and NADPH; $K_{0.5}$ for calmodulin and Ca^{2+}) as well as the IC₅₀ for two calmodulin inhibitors (trifluoperazine and calmidazolium) and two inhibitory L-arginine analogs (N^G -nitro-L-arginine and N^G -methyl-L-arginine) are shown in Table 2.

DISCUSSION

The NO or nitrovasodilator signal transduction pathway regulates the concentration of intracellular cGMP in numerous cells and tissues (11–14, 17, 18, 26, 27, 42, 43). NO-containing compounds act by directly stimulating soluble guanylyl cyclase (12). The key enzyme of the NO signal transduction

pathway, the L-arginine-converting GAF synthase, appears to exist in at least three isoforms: a constitutive soluble Ca^{2+} /calmodulin-regulated form (type I; refs. 8, 25, and 31), an inducible soluble tetrahydrobiopterin/FAD-dependent but Ca^{2+} /calmodulin-independent form (type II; refs. 21 and 44), and a particulate form (type III; ref. 45). All isoforms have in common the ability to catalyze the NADPH-dependent oxidation of one or both of the terminal guanidino nitrogens of L-arginine (1, 2) yielding L-citrulline as a coproduct. The endothelium-derived GAF (endothelium-derived relaxing factor) has a half-life of about 30 sec (46). In various cells and tissues, it has been proposed that GAF or endothelium-derived relaxing factor is chemically identical with NO (1, 9, 10), an N^G -oxo derivative of L-arginine (47), hydroxylamine (H_2NOH ; ref. 19), or an S-nitroso thiol (20). The complexity of free-radical side-reactions, the low concentrations, and the lability of GAF in cultured cells or crude cell-free preparations have so far hindered the definite identification of GAF and the clarification of whether indeed different forms, i.e., a group of L-arginine-derived GAFs, are synthesized.

In this report we describe a rapid two-step affinity chromatographic purification scheme, physical characterization, and kinetic data for the soluble Ca^{2+} /calmodulin-regulated isoform of GAF synthase (type I isoform) from rat cerebellum. The purity of the enzyme preparation was confirmed with a variety of procedures including SDS/PAGE and additional chromatography with Superose 12 or 6 or Mono Q and chromatofocusing.

By comparison of the molecular masses determined by gel-permeation chromatography and denaturing SDS/PAGE, soluble GAF synthase appears to exist as a dimer (about 280 kDa) of two presumably identical subunits (about 155-kDa). Affinity labeling of the GAF synthase monomer with biotinylated calmodulin indicated that calmodulin regulates this GAF synthase isozyme by a high-affinity interaction. Calmodulin-dependent GAF- and citrulline-forming activities cochromatograph with the reported purification scheme. Thus, under optimal conditions, citrulline formation and GAF biosynthesis appear to be coupled.

Bredt and Snyder (25) have described the purification of a monomeric protein with a molecular mass of about 150 kDa from rat cerebellum, catalyzing the conversion of L-arginine into a metabolite cochromatographing with L-citrulline on thin layer chromatography. However, the authors did not present data showing that this protein forms NO-containing compounds or compounds with the bioactivity of NO. The purification scheme for this 150-kDa protein, as suggested by Bredt and Snyder (25) using DEAE-anion-exchange resin followed by 2',5'-ADP-Sepharose affinity chromatography, was not reproducible in our hands; i.e., it resulted in the enrichment of at least four major protein components as analyzed by SDS/PAGE. Thus, we were unable to compare the 150-kDa protein reported by these authors with GAF synthase, described in the present report. However, similar affinities to calmodulin, NADPH, 2',5'-ADP-Sepharose, and L-arginine suggest that the 150-kDa protein reported by Bredt and Snyder (25) and the GAF synthase monomer reported here are identical.

GAF synthase is highly unstable especially after purification. When we used different and more time-consuming purification procedures in the presence of standard protease inhibitors [some procedures analogous to Bredt and Snyder (25)], purified preparations frequently contained proteins of about 40, 64, and 90 kDa. Additional experiments are needed to determine if these fragments are actually derived from the 155-kDa subunit and whether a specific protease is responsible for this protein modification of GAF synthase.

High NADPH (10 mM) and, more important, low protein (<30 μ g/ml) concentrations have destabilizing effects on the purified enzyme, even if stored below $-70^\circ C$ in the presence

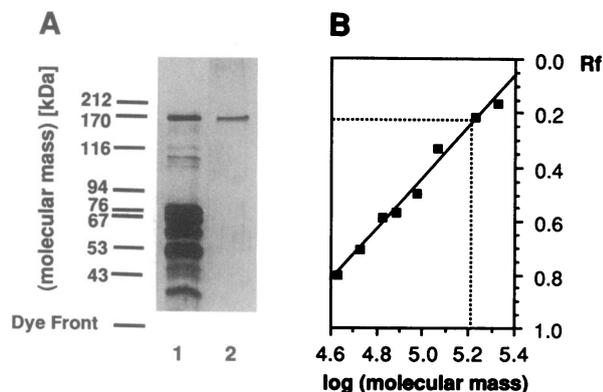


Fig. 3. SDS/PAGE. (A) Silver-stained 7.5% gel from SDS/PAGE. The mobility of the following molecular mass marker proteins is indicated: myosin (212 kDa), α_2 -macroglobulin (170 kDa), β -galactosidase (116 kDa), phosphorylase *b* (94 kDa), transferrin (76 kDa), bovine serum albumin (67 kDa), glutamic dehydrogenase (53 kDa), and ovalbumin (43 kDa). Lanes: 1, 3 μ g of the NADPH eluate from 2',5'-ADP-Sepharose column; 2, 400 ng of the EGTA eluate of the calmodulin-agarose column. (B) Apparent molecular mass of the single protein band in the purified soluble GAF synthase preparation is designated by the dotted line and is 155 ± 3 kDa.

of 10% glycerol. Addition of bovine serum albumin to purified enzyme preparations increases enzyme stability. Furthermore, purified GAF synthase appears to lose activity due to degradation or dissociation of a yet to be identified prosthetic group or cofactor; this process seems to be facilitated by high potassium but not sodium ion concentrations. Reconstitution of pure GAF synthase with tetrahydrobiopterin and a regenerating system (48) of high thiol concentrations and tetrahydrobiopterin reductase increased its activity. A cofactor role for tetrahydrobiopterin has been described for the type II enzyme (e.g., from macrophages, ref. 21). However, the requirement of another unstable and yet to be identified cofactor for type I GAF synthase is likely.

The optimized purification scheme for GAF synthase described in the present study used two affinity chromatographic steps and immediate pooling of column fractions according to the protein concentration. This scheme resulted in a quick and highly reproducible enzyme purification. When working with low volumes, the purification could be further accelerated by replacing the dialysis steps with buffer-exchange procedures on G-25 gel-filtration columns (Pharmacia). This protocol should make it possible to rapidly purify and compare type I GAF synthase from brain and other tissues and with other types (II and III) of the enzyme.

By eliminating or reducing the formation and release of free oxygen radicals and other factors present in intact cell systems, the definitive chemical identification of GAF should now be possible. It will also be of great interest to study the histological and subcellular localization of soluble guanylyl cyclase and soluble GAF synthase by means of immunocytochemical techniques. Furthermore, the molecular cloning of this isoenzyme should facilitate the characterization of the type II and III isoforms.

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