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

(H-arginine/endothelium-derived relaxing factor/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-Sepharose 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 pmol 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 ± 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 ± 0.3 nm and a sedimentation coefficient of 7.8 ± 0.2 S were used to calculate a molecular mass of about 279 ± 25 kDa for the native enzyme. SDS/PAGE revealed a single protein band with a molecular mass of about 155 ± 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 ± 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 cerebellum (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 μM leupeptin/1 μM pepstatin A/0.1 mM phenylmethylsulfonyl fluoride (PMSF)/12.5 mM 2-mercaptoethanol] at 4°C for 30 s. The homogenate was centrifuged at 105,000 × 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 μM leupeptin/1 μM 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 (2.5 M NaCl), and again with about 4 column volumes of buffer 2. GAF synthase was then eluted with buffer 4 (2.0 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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Ca2+-Dependence. Dialyzed pool from 2',5'-ADP-Sepharose was incubated with preswollen calmodulinagarose 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 5/1.1 NaCl/10% (vol/vol) glycerol/5 mM EGTA instead of CaCl2). Protein-containing fractions were pooled.

Superoxide 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 PMSE/12.5 mM 2-mercaptoethanol) and washed with about 50 column volumes of buffer 5 and about 50 column volumes of buffer 6 (buffer 5/0.3 M NaCl). GAF synthase was then eluted with buffer 7 [buffer 5/1.1 NaCl/10% (vol/vol) glycerol/5 mM EGTA instead of CaCl2]. Protein-containing fractions were pooled.

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 mM L-[2-3H]arginine (5.5 GBq/mmol), 100 mM NaADPH, 30 mM calmodulin, and 2 mM CaCl2 in a final volume of 100 μl. The reaction was stopped by adding 0.5 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-[2-3H]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 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 pmol 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 NaADPH. Lower concentrations of NaADPH resulted only in a partial elution of GAF synthase from 2',5'-ADP-Sepharose or substantial peak-broadening. Assay of samples from the column
fractio
ts 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 M 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 mg 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 chaotrope 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.

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 cm\(^3\)/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 pH 6.1 ± 0.3 (isoelectric point). Under first-order condi-
Table 2. Characterization of soluble GAF synthase

<table>
<thead>
<tr>
<th>Characterization</th>
<th>Value(s)</th>
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</thead>
<tbody>
<tr>
<td>Stokes radius, nm</td>
<td>7.9 ± 0.3*</td>
</tr>
<tr>
<td>s2o, S</td>
<td>7.8 ± 0.2†</td>
</tr>
<tr>
<td>Native molecular mass, kDa</td>
<td>279 ± 25‡</td>
</tr>
<tr>
<td>Denatured molecular mass, kDa</td>
<td>155 ± 3</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>Kinetic data</td>
<td></td>
</tr>
<tr>
<td>Vmax, nmol of citrulline per min per mg</td>
<td>107</td>
</tr>
<tr>
<td>Km (L-arginine), µM</td>
<td>2.2</td>
</tr>
<tr>
<td>IC50 (N⁵-nitro-L-arginine), µM</td>
<td>0.9‡</td>
</tr>
<tr>
<td>IC50 (N⁵-methyl-L-arginine), µM</td>
<td>1.6‡</td>
</tr>
<tr>
<td>K0.5 (Ca²⁺), µM</td>
<td>ND</td>
</tr>
<tr>
<td>K0.5 (Ca²⁺), µM</td>
<td>0.35</td>
</tr>
<tr>
<td>K0.5 (calmodulin), nM</td>
<td>3.5</td>
</tr>
<tr>
<td>IC50 (trifluoperazine), µM</td>
<td>4.0†</td>
</tr>
<tr>
<td>IC50 (calmidazolium), µM</td>
<td>2.2‡</td>
</tr>
</tbody>
</table>

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 µM L-arginine.

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 Ca²⁺/calmodulin-regulated form (type I; refs. 8, 25, and 31), an inducible soluble tetrahydrobiopterin/FAD-dependent but Ca²⁺/calmodulin-independent form (type II; refs. 21 and 44), and a particular 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 N⁵-oxo derivative of L-arginine (47), hydroxylamine (H₂NOH; 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 Ca²⁺/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 cochromatographed 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 metabolically inert citrulline coupled with the formation of GAF synthase using 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 nM) and, more importantly, low protein (<30 µg/ml) concentrations have destabilizing effects on the purified enzyme, even if stored below −70°C in the presence.
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 facilitate the isolation of intact cell systems and allow the definitive chemical identification of GAF synthase activity. 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 synthase 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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