The Inhibitory Guanine Nucleotide-binding Protein (Ni) Purified from Bovine Brain Is a High Affinity GTPase*

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Using modifications of the methods of Bokoch et al. (Bokoch, G. M., Katada, T., Northup, J. K., Ui, M., and Gilman, A. G. (1984) J. Biol. Chem. 259, 3560-3567) and Codina et al. (Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, C. R., Iyengar, R., and Birnbaumer, L. (1984) J. Biol. Chem. 259, 5871-5886), we have purified a pertussis toxin substrate with the expected characteristics of the inhibitory guanine nucleotide-binding protein (Ni) essentially to homogeneity. The purified protein consists of 3 subunits of M₄, 40,000, 35,000, and <10,000. The M₄, 40,000 band is found, upon close examination, to consist of a poorly resolved doublet. Starting with the membranes from 1,320 g of bovine forebrain we purified the protein some 100-fold with ~20% yield to obtain 13 mg of a >95% pure protein. Chromatography on octyl-Sepharose provided efficient separation of Ni from Nₙ (the stimulatory guanine nucleotide-binding protein). Analytical ultracentrifugation indicates an M₄ of 82,000 and a sedimentation coefficient s₂₀,₅₀ of 5.1. The protein is able to restore opiate-mediated inhibition of adenylate cyclase to membranes prepared from NG 108-15 cells which had been treated with pertussis toxin.

Bovine brain Ni has the enzymatic properties of a low Kₘ GTPase with a turnover number of 0.3 and affinities for nucleotides in the order GppNHp ≥ GTP ≥ GDP ≥ ATP, CTP, UTP, and GMP. Na⁺ specifically stimulates the GTPase and low concentrations of Mg²⁺ (<50 µM) are inhibitory. Some Mg²⁺ is apparently necessary because EDTA, but not EGTA, abolishes the GTPase activity.

Guanine nucleotides play an obligatory role in the modulation of the activity of adenylate cyclase by both stimulatory and inhibitory agents (1). Separate regulatory GTP-binding proteins controlling stimulation and inhibition of adenyl cyclase were proposed (1). That involved in stimulation (Nₙ or G/F) has been extensively characterized (2-5), largely because of the availability of a mutant S49 murine lymphoma cell line (6) functionally lacking this activity (7). The protein involved in the coupling of inhibitory receptors to adenylate cyclase (Ni) proved more refractory to study until the discovery of a toxin isolated from Bordetella pertussis which was able to selectively transfer the ADP ribosyl moiety from NAD to this substrate (8, 9). Ni has recently been purified from rabbit liver (10) and from human erythrocytes (5) and appears to consist of a heterotrimer with an α subunit of M₄ near 40,000, a β subunit of M₄, 35,000 and a M₄, <10,000 γ subunit.

In this report we describe the purification of Ni, from bovine brain, a particularly rich source. We examine some physical properties of this protein and its enzymatic activity as a high affinity GTPase.

EXPERIMENTAL PROCEDURES

RESULTS

Reconstitution—As described in the Miniprint Supplement, we purified bovine brain Ni by enrichment of the pertussis toxin substrate. To confirm that this protein really is Ni, it was important to show that it has the distinctive functional property of Ni; namely, that it can couple occupancy of inhibitory receptor by an agonist to attenuation of adenylate cyclase activity. The adenylate cyclase of membranes prepared from pertussis toxin-treated NG 108-15 cells is relatively insensitive to the attenuating effects of opiates and other inhibitory hormones, because of ADP-ribosylation of Ni (8, 9). Incubation of such membranes with purified Ni restores opiate inhibition of adenylate cyclase to levels near those of untreated membranes (Fig. 1). The amounts of Ni required for restoration of inhibitory receptor function are small, half-maximal effects being achieved with approximately 10 nM. Thus, the affinity with which Ni interacts with opiate receptors in membranes is at least 10⁶ M⁻¹.

ADP-ribosylation—Purified Ni can be ADP-ribosylated by pertussis toxin with the time course shown in Fig. 2. With the reasonable assumption that only one ADP ribosyl moiety is incorporated per Ni molecule (27, 28), the preparation appears to be homogeneous. Interestingly, ADP-ribosylation of Ni by pertussis toxin shows a characteristic delay under those conditions. The lag is not observed if ADP-ribosylation is carried out at low ionic strength (data not shown).

Ni, as a GTPase—Opiates and other inhibitory hormones attenuate adenylate cyclase activity by stimulating an associated, low Kₘ GTPase (13). It has often been assumed that this GTPase is a property of Ni (31). Purified bovine brain Ni catalyzes the hydrolysis of GTP with high affinity (Fig. 3). The maximal velocity of the reaction, approximately 4 nmol of GTP hydrolyzed per min/mg of protein is 40 times that observed with membranes of NG 108-15 cells or of rat brain. An Eadie-Hofstee plot of these data is nonlinear (see inset to Fig. 3). Such nonlinearity may reflect either negative cooperativity or the presence of two (or more) species of molecule.

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1 Portions of this paper (including "Experimental Procedures," Tables 1 and 2, and Figs. 1–5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2910, cite the authors, and include a check or money order for $7.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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exerting GTPase activity. It is not yet possible to distinguish between these alternatives with assurance, although assays in the presence of 50 mM Na⁺ result in data which give linear Eadie-Hofstee plots (data not shown). Most of the GTPase activity can be described by a model assuming a $K_m$ for GTP near 100 nM, a number indistinguishable from the $K_m$ deduced earlier for the membrane-bound enzyme (13). GTP hydrolysis by Nᵢ is linear with protein concentration over a wide range (data not shown). That the enzyme is truly a GTPase is shown by the competition data summarized in Fig. 4. In the experiment shown, the abilities of the indicated concentrations of nucleoside triphosphates to inhibit the release of $^{32}$P from $[^{32}$P]GTP were measured. Only GppNHp and GDP inhibit this process strongly at low concentrations. The other nucleoside triphosphates tested (ATP, UTP, and CTP) inhibit only at much higher concentrations. GMP is not inhibitory at the concentrations tested.

A distinguishing feature of the low $K_m$ GTPase in membranes is a specific requirement for Na⁺ for inhibitory receptor coupling (32, 33). In membranes of NG 108-15 cells, Na⁺ in the presence of Mg²⁺ lowers GTPase activity, and inhibitory hormones such as opiates reverse this inhibition (33). Purified Nᵢ behaves differently. Na⁺ stimulates GTPase activity by approximately 50% in the absence but not the presence of Mg²⁺ (Fig. 5). The effect of Na⁺ cannot be attributed to ionic...
The effects of Na⁺ and Mg²⁺ on the hydrolysis of GTP by Ni. Ni, 0.14 μg/assay, freed from detergent and ethylene glycol on a PD 10 column as described under “Experimental Procedures” was assayed for GTPase activity in the presence of increasing concentrations of NaCl and in the absence (——) or presence (○—○) of 5 mM MgCl₂. Each assay also contained 1 μM [γ-³²P]GTP (80,000 cpm) and 20 mM Tris-chloride, pH 7.5. Data points are the mean ± S.E. of triplicate determinations.

**TABLE I**

| Condition | Pi released (nmol/min/mg protein) |
|-----------|----------------------------------|
| Control   | 3.55 ± 0.25                      |
| EDTA      | 0.06 ± 0.04                      |
| EGTA      | 3.28 ± 0.27                      |

![Figure 6. The effect of Mg²⁺ on Ni GTPase. Ni, 0.14 μg/assay, freed from detergent and ethylene glycol on a PD 10 column as described under “Experimental Procedures” was assayed for GTPase activity in the presence of increasing concentrations of Mg²⁺. Each assay also contained 1 μM [γ-³²P]GTP (80,000 cpm), 20 mM Tris-chloride, pH 7.5, and 1 mM EGTA. Data points are the mean ± S.E. of triplicate determinations.](image)

![Figure 5. The effects of Na⁺ and Mg²⁺ on the hydrolysis of GTP by Ni. Ni, 0.14 μg/assay, freed from detergent and ethylene glycol on a PD 10 column as described under “Experimental Procedures” was assayed for GTPase activity in the presence of increasing concentrations of NaCl and in the absence (——) or presence (○—○) of 5 mM MgCl₂. Each assay also contained 1 μM [γ-³²P]GTP (80,000 cpm) and 20 mM Tris-chloride, pH 7.5. Data points are the mean ± S.E. of triplicate determinations.](image)

![Figure 7. Time course of GTP hydrolysis by Ni. Ni, 0.14 μg/assay, prepared from stock on a PD 10 column as described under “Experimental Procedures” was assayed for GTPase activity at either pH 6.3 (——) or pH 7.5 (○—○). Assays also contained either 20 mM Tris-3,3-dimethylglutarate, pH 6.3, at 37 °C (——) or 20 mM Tris-Cl (○—○), pH 7.5, and 1 μM [γ-³²P]GTP (70,000 cpm). Data points are the mean ± S.E. of triplicate determinations.](image)

The protein purified from bovine brain membranes in this work is Ni because it is a substrate for ADP-ribosylation, strength alone because it is saturable (Fig. 5) and because K⁺ is much less effective (data not shown). Thus, the GTPase activity of purified Ni is specifically affected by Na⁺, but in a direction opposite to that occurring in membranes. Mg²⁺ inhibits the GTPase activity of Ni at very low concentrations, half-maximal effects being seen at <50 μM (Fig. 6). Surprisingly, therefore, EDTA, but not EGTA reduces the GTPase activity of Ni to negligible levels (Table I). Because the only ion known to be chelated well by EDTA and not EGTA is Mg²⁺, it would appear that Ni GTPase requires some Mg²⁺ for activity but that excess is inhibitory.

The pH optimum of Ni GTPase is near 6.3. The peak is broad and experiments at higher pH values show a slow decrease in activity as the pH is raised to above 9 (data not shown). In order to allow comparison with earlier results of experiments with intact membranes, most of the measurements made in the present studies were at the slightly suboptimal pH of 7.5. The time courses of Ni-catalyzed GTP hydrolysis at pH 6.3 and 7.5 (Fig. 7) confirm that the rates are similar at these two pH values. Interestingly, the data obtained at pH 6.3 suggest a burst of activity at early times which could reflect a rapid phosphorylation of Ni, as an early step in catalysis.

Several agents were tested for potential effects on GTPase activity. Hormones known to function via Ni-mediated inhibition of adenylate cyclase such as carbachol, norephinephrine, and the enkephalin analogue [α-Ala',Met'']enkephalinamide have no effect on the GTPase activity of Ni at concentrations of at least 10 μM. Similarly adenosine, NAD, and forskolin at 10 μM concentrations are without effect as is platelet-activating factor (1 μM). Fluoride (10 mM) has, at most, a small inhibitory effect on GTPase, both with purified Ni and with intact membranes (13). Ca²⁺ (1 μM) inhibited the GTPase activity by about 20% and calmodulin reversed the effect, presumably by binding calcium.

**DISCUSSION**

The abbreviations used are: EGTA, ethylene glycol bis(β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; GppNHzp, guanosine 5'-[β,γ-imido]triphosphate.

The protein purified from bovine brain membranes in this work is Ni because it is a substrate for ADP-ribosylation,
catalyzed by pertussis toxin, and able to restore opiate-mediated inhibition of adenylate cyclase to membranes of NG 108-15 cells which had been pre-treated, in vivo, with pertussis toxin. The purification scheme used is a modified, and slightly simplified version of those used for the purification of Ni from rabbit liver (10) and human erythrocyte membranes (5). A major difference is the use of octyl-Sepharose which affords complete separation of Ni from Nc.

Bovine brain is a particularly rich source of Ni, as might be expected because of the extraordinarily high activity of adenylate cyclase in this tissue. The protein is homogenous by some criteria after only a 50-fold purification from a cholate extract of brain membranes. Thus, Ni accounts for approximately 2% of the protein of these extracts or 1% of the membranes. Pertussis toxin catalyzes the introduction of 1 mol of ADP-ribose/mol of protein. Thus, essentially all of the protein in the purified material is a pertussis toxin substrate. Very little further purification was achieved by a variety of techniques including sucrose gradient centrifugation, DEAE-Affigel blue, and repeated DEAE-Sephadex and hydroxyapatite columns. The protein migrates as a single boundary with hydroxyapatite columns. Possibly, suitable reconstitution with lipids and/or the absence of lipids and receptors is approximately one-fifth of the activity expected based on low K_m, GTPase to pertussis toxin inactivated NG 108-15 membranes. Thus, they represent different forms of Ni.

Close examination of the SDS gel patterns shows that the alpha band actually consists of a doublet corresponding to M, of 40,000 (alpha(1)) and 39,000 (alpha(2)). Although both bands are ADP-ribosylated with pertussis toxin, they can be distinguished by an antibody raised against bovine transducin. This antisemur, which recognizes Ni(1) from human erythrocytes binds to our alpha(1) but not the alpha(2) band. We do not know precisely how different the two polypeptides are but we have been able to effect a partial separation of the two with hydroxyapatite columns. The preparations relatively enriched either in alpha(1) or alpha(2) are indistinguishable in the amounts required to half-maximally restore opiate inhibition of adenylate cyclase to pertussis toxin inactivated NG 108-15 membranes. They, thus, represent different forms of Ni.

Bovine brain Ni is a GTPase, albeit a relatively weak one. However, the turnover number of 0.3 is within a factor of 2-3 of the activity expected based on low K_m, GTPase in brain membranes. Possibly, suitable reconstitution with lipids and/or inhibitory receptors would produce a stimulation of the GTPase activity. Experiments are in progress to test these possibilities. The stimulatory GTP-binding protein, Nc, isolated from rabbit liver displays a GTPase activity which, in the absence of lipids and receptors is approximately one-fifth of that of our preparations (34). Reconstitution with alpha adrenergic receptors resulted in a hormone-dependent GTPase activity with a turnover number of approximately 1 (34). The relationships among the GTPase activities associated with Ni, Nc, and that of the intact adenylate cyclase system need further study. The time course of Nc, GTPase near its pH 6.3 has a burst of GTP hydrolysis at early (<1 min) times which might suggest the initial accumulation of a polyribosylated intermediate which then breaks down more slowly.

Interestingly, the ionic dependences of Ni as a GTPase are not the same as those of intact membranes. With purified Ni, Na^+ stimulates GTPase activity whereas Mg^2+ inhibits; the two in conjunction cancel each other effects. The fact that EDTA, but not EGTA, inhibits Ni, GTPase suggests that low concentrations of Mg^2+ are necessary for activity. In membranes, Na^+ and Mg^2+ have little effect alone but the combination of the two ions inhibits GTPase. Furthermore, opiate inhibition of adenylate cyclase and stimulation of GTPase are completely dependent on Na^+ and Mg^2+ (33). Perhaps other proteins contribute significantly to the opiate-stimulated GTPase. Further experiments are necessary to clarify the relationships between Ni, GTPase and the low K_m, GTPase of intact membranes.

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GTP Hydrolysis by Purified $N_i$

Supplemental Material to

THE INITIATION GUYIATION NUCLEOTIDE BINDING PROTEIN (NI) PURIFIED FROM BOSTON BRAIN IS A HIGH AFFINITY GTPASE

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EXPERIMENTAL PROCEDURES

Materials - [α-32P]ATP and [32P-γGTP] were obtained from ICN. [32P]GTP was obtained from New England Nuclear. DEAE-sephacel, octyl-Sepharose and PH-10 columns, prepared with nephages D-05 were from Pharmacia. Urea R-6K was from Research Organics. GTP was a gift of Dr. S. Rose-O’Hanlon. All other materials were purchased from commercial sources and were the highest purity available. Fresh calf brains were obtained from a local abattoir, transported to the laboratory on ice and membranes immediately prepared as described below. The membranes were stored as a paste at -70°C until use.

Cell growth and membrane preparation - Neuroblastoma x glioma NG 108-15 hybrid cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics as prepared for the NG 108-15 cells.

Protein Determinations - Protein was measured titratically either by absorbance at 280 nm or by the Lowry method using bovine serum albumin as a standard. Amino acids were determined on a Beckman 121A analyzer using the methods of Bokoch et al.

Methods - Pertussis toxin treated NG 108-15 cells. [32P]NAD was assayed by the method of Salamon, et al. and was eluted with a linear gradient of NaCl 0.05-0.5 M

Step 1 - Purification of membranes. The cerebral cortex, soma and brain stem were removed from six fresh calf brains and dissected. Tonal weight was approximately 1500 g. The brains were removed rapidly and placed in 1000 ml of 20% cold 0.2 M sucrose. 300 ml aliquots + 900 ml, further 0.3M sucrose were homogenized in a Waring blinder with 5 x 30 min bursts interspersed with 5 min breaks to allow high speed dispersal. The homogenate (5000 ml total volume) was centrifuged (2500 rpm/15 min) in a Sorvall GS rotor. The supernatant fluid was decanted and reconstituted (600 rpm/1 min) in a Sorvall GS rotor. This produced 500 ml of membrane-rich samples which was stored at -70°C.

Step 2 - Solubilization of membranes. The 500 ml membrane paste was thawed in 1.1 final volume of 20 mM pH 5.5 NaPO4, 1M, 0.5M, bovine trypsin triton-X buffer 12 mg/ml and 0.1% phenylmethylsulfonyl fluoride (buffer A). After addition of .) buffer A + 25 (w/v) sodium cholate and stirring at 4°C for 1 h the mixture was centrifuged (1500 rpm/1 hr) in a Sorvall GS rotor. The supernatant fraction was taken as the chloroform extract.

Step 3 - DEAE-sephacel ion exchange chromatography. A 5 x 50 cm column was prepared with DEAE-sephacel which had been brought to pH 8.0 with trias base. The column was washed with 2.1 l of buffer A + 0.9% sodium cholate, 25 mM NaCl, at 200 ml/hr. The column extract (1600 ml) was applied, and the column was eluted with a linear gradient (0.5 l total volume) of NaCl 0.1-0.5M in buffer A + 0.9% sodium cholate at 200 ml/hr. Fractions of 20 ml were collected. eluted at a single symmetrical peak between 1200 and 1900 ml at a conductivity of 4 mmoles.

Step 4 - Hydroxyapatite concentration column. A 2.5 x 20 cm column of BioGel HTP hydroxyapatite was prepared and washed extensively with 20 mM pH 6.0, 0.1M sodium phosphate, 1ml buffer A. The column was eluted with 200 ml sodium phosphate, 0.1M, pH 6.8 (buffer C). 150 ml plus 100 ml NaCl and 0.9% (w/v) sodium cholate, then with buffer A + 0.9% sodium cholate. The pooled peak fractions from DEAE sephacel chromatography (Fig. 1) were applied as such to the column and the column was eluted with buffer A + 500 mM potassium phosphate, 0.9% sodium cholate. 5 ml fractions were collected. The majority of the protein eluted as a single broad peak starting near 50 ml. Only was eluted throughout the column. Fractions 14-16 and 19-20 were pooled separately and then further purified independently but identically.

Measurement of Ni during purification - [32P]NI activity in membranes and peripheral toxins treated NG 108-15 cells - Membranes of cells treated with peripheral toxins in vivo were suspended in 1ml of 50 mM Tris pH 7.5, 5 mM MgCl2, 5mM KCl and 2% glycerol were added to a 50 ml final volume containing 20 μM NaPO4, 2 mM MgCl2 pH 7.5, 100 mM NaCl, 0.1% protease, 400 μg/l neuporphinase, and 25 μg/ml phosphatase. All of the supernatant was used 24 μM of the lipid substrate, [32P]-GTP (about 0.1 μM) was placed in a 100 μl final volume routinely containing 20 μM Tris pH 7.5, 1 ml of 5% [32P]-GTP (100,000 cpm), 10% dTT, soybean trypsin phosphatase, 1 ml of BioGel HTP (15,000 c.p.m), 0.2 ml 10-3 M ATP (by c.p.m) and 50 μg cryst. membranes. Assays were incubated for 1 hr at 37°C and 20% (v/v) acetic acid was added to 100 μl of the reaction mixture and 1 ml of ice cold 0.32 H2O containing 1 ml of 10% TCA (each wash for 3 min) was added. The samples were vortexed and allowed to stand at 0°C for 5 min before filtration through Whatman 3MM paper with a vacuum. The radioactivity in the filter paper was determined by liquid scintillation counting. Membrane blanks were typically only 0.1% of the added radioactivity. Pertussis toxin labelling of NI was generally linear with protein concentration over the range of dilutions used, but dead time of injections caused high protein concentration Cohnate extract and hydroxypatite concentration at high linearity was only achieved at the higher dilutions. Cohnate concentra-

Fig. 1. Hydroxyapatite chromatography of DEAE Sephacel peak. In this experiment fractions 1 - 10 and 11 - 20 were pooled separately and then further purified independently. Pertains to analysis of DEAE-Sepharose (A) and protein as measured by D0 280 are shown.

Step 5 - Ultrapure [3H]-niacin adenosine chromotography. The 2 pools of NI from the hydroxyapatite concentration (55 x 50 ml) which had been equilibrated with buffer A + 100 mM NaCl, 0.9% sodium cholate and eluted in the same buffer at a flow rate of 10 ml/hour. Fractions of 10 ml were collected. NI from both preparations eluted in fractions centered near 750 ml.

Step 6 - [3H]-Niacin Chromatography. In contrast to other purification procedures for NI (5, 11) NI was used as the hydrophobic matrix rather than heparin sulfate sepharose, as this resin afforded sufficiently complete resolution of NI from Nα (Fig. 2).

An NI sepharose column (1.2 x 34 cm) was equilibrated with 100 mM buffer A + 100 mM NaCl, 0.13 sodium cholate. The peak NI fraction from [3H]-niacin der-ived from the hydroxyapatite column fractions 1 - 20 (155 ml) were diluted to 650 ml with buffer A + 100 mM NaCl and applied to the column, which was then washed with 150 ml buffer A + 100 mM NaCl, 0.38 sodium cholate and then with 150 ml buffer A + 500 mM NaCl, 0.38 sodium cholate. The column subsequently was eluted with a rewash gradient of NaCl and sodium cholate (1500 total volume) starting with buffer A + 250 ml NaCl, 0.35 sodium cholate and ending with buffer A + 50 mM NaCl, 1.2% sodium cholate at a flow rate of 7 ml/hr. Fractions of 10 ml were collected. Fractions 1-5, 9-16 and 18-20 were pooled. Results derived from hydroxyapatite fraction 1-10 except that the peak fraction volume was reduced to 200 ml, which was eluted to 150 ml with buffer A + 0.75 sodium cholate in both cases. NI eluted toward the end of the gradient. The peak pooled fractions, 4-5 (in Fig. 2, from octyl sepharose chromatography) were pooled, dialyzed, and lyophilized in acetone at -70°C after elution of impurities. There was essentially no NI activity as measured by cpm resolution in the final pooled fractions. NI was more tightly bound to octyl sepharose than in NI.
GTP Hydrolysis by Purified Ni

**Fig. 2.** GTPase chromatography of 48-55 peak fractions

Fractions 11 - 20 from Fig. 1 were purified on an A4-34 column as described in the text and the peak fractions applied to the octyl-sepharose column. Pur- tussis toxin catalyzed GDP-ribosylation as a measure of Ni (■), activity of Na+ stimulated adenylate cyclase as a measure of Na (○), and OD 280 as a measure of protein (□) are shown.

Fractions 46 - 53 were pooled and stored in aliquots at -70°C after addition of 15% ethylene glycol. Most experiments in this paper refer to this preparation.

The purification scheme of Ni is summarized in Table 1.

| Purification of Ni from chlorate extracts of bovine brain membranes | Purification Step | Volume (μl) | Protein (mg) | Amount of Ni (nmol) | Specific Activity (nmol/mg) | Recovery (%) |
|---|---|---|---|---|---|---|
| Cholate extract | 1680 | 3230 | 664 | 0.21 | 100 |
| DEXA-Sepharose | 154 | 253 | 565 | 2.03 | 85 |
| Hydroxylapatite | 53 | 44.8 | 235 | 1.61 | 35 |
| (11-20) | | | | | |
| A4-34 | 150 | 26.7 | 193 | 7.50 | 29 |
| Octyl sepharose | 77 | 13.0 | 140 | 10.8 | 21 |

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) - Electrophoretic analysis of proteins was carried out in 5 - 20% polyacrylamide gradient gels as described in the text. The gels were used as standard proteins in the gradient range of the gels. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by Coomassie brilliant blue staining.

10 μg of purified Ni in Laemmli sample buffer (20) containing bromphenol blue as the tracking dye and boiled for 1 min prior to application. Electrophoresis was at room temperature overnight at a constant voltage of 50 volts. The next morning the gel was stained with 125 volts and electrophoresis continued until the tracking dye reached the edge of the gel. Approximate calibration of electrophoretic mobility as a function of molecular weight was obtained by electrophoresing standard proteins in the borders lanes of the gel. The standard proteins and their assumed molecular weights were phosphorylase A (Boehringer Mannheim) 97,000, bovine serum albumin (Ciba-Geigy) 68,000, catalase (Boehringer Mannheim) 58,000, foxtailmes (Boehringer Mannheim) 48,000, ovalbumin (Miles) 43,000, lactate dehydrogenase (Boehringer Mannheim) 35,000, soybean trypsin inhibitor (Burroughs) 21,500 and cytochrome C (equine heart, Ciba-Geigy) 12,300. These samples were mixed with Laemmli sample buffer to a final concentration of 0.2 mg/ml. At the termination of electrophoresis, gels were stained with 0.1% Coomassie Brilliant Blue containing 20% (v/v) isopropanol and 7% (v/v) acetic acid for 1 hr at 37°C and then destained with 10% (v/v) isopropanol and 10% (v/v) acetic acid at room temperature (Fig. 3). In some cases the gels were then stained with silver according to Merril et al. (21).

**Fig. 3.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of Ni after octyl sepharose chromatography.

10 μg of purified Ni in Laemmli sample buffer (19) was applied to a 5 - 20% SDS-polyacrylamide gel and run as described above. Proteins were stained with Coomassie Blue. The faintly visible band at Rf 22,000 in soybean trypsin inhibitor which was included at 2 μg/ml throughout the purification procedure.
Fig. 5. Equilibrium sedimentation of D1 in the analytical ultracentrifuge.

(a) After 50 minutes at 50,000 rpm the speed of the ultracentrifuge was reduced to 16,000 rpm in a continuation of the experiment described in Fig. 5. Scans taken at 18 and 20 hra were identical and indicated attainment of equilibrium. Data from the two scans were pooled and are shown here.

(b) Equilibrium pattern of the sample studied in part A after addition of 1.5 mM GTP and 20 mM Mg2⁺ and storage at 37°C for 20 min.