Rat Brain N-Acetylated α-Linked Acidic Dipeptidase Activity

**PURIFICATION AND IMMUNOLOGIC CHARACTERIZATION**

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Barbara Staubh Slusher, Michael B. Robinson‡, Guochuan Tsai, Michele L. Simmons, Stephanie S. Richards, and Joseph T. Coyle

From the Departments of Neuroscience and Pharmacology, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205

N-Acetylated α-linked acidic dipeptidase (NAALA dipeptidase) is a membrane-bound metallopeptidase that cleaves glutamate from the endogenous neuropeptide N-acetyl-L-aspartyl-L-glutamate. In this report, we have solubilized NAALA dipeptidase activity from synaptosomal membranes with Triton X-100 and purified it to apparent homogeneity by sequential column chromatography on DEAE-Sepharose, CM-Sepharose, and lentil lectin-Sepharose. This procedure resulted in a 720-fold purification with 1.6% yield. The purified enzyme migrated as a single silver-stained band on a sodium dodecyl sulfate gel with an apparent molecular weight of 94 kDa. Using an enzymatic stain to visualize NAALA dipeptidase activity within a gel matrix, we have confirmed that the 94-kDa band is, indeed, NAALA dipeptidase. The purified enzyme was characterized and found to be pharmacologically similar to NAALA dipeptidase activity described previously in synaptosomal membrane extracts. Using the purified NAALA dipeptidase as antigen, we have raised specific and high titer polyclonal antibodies in guinea pig. Immunocytochemical studies show intense NAALA dipeptidase immunoreactivity in the cerebellar and renal cortices.

Electrophysiologic, lesion, and immunocytochemical studies suggest that the endogenous neuropeptide, N-acetyl-L-aspartyl-L-glutamate (NAAG), may act as a neurotransmitter/neuromodulator in the central nervous system (Blakely and Coyle, 1989). Recently a quisqualate (Quis)-sensitive peptidase activity identified in brain membranes which cleaves NAAG to N-acetyl-L-aspartate (NAA) and glutamate. In a manner analogous to the synaptic inactivation of acetylcholine (Cooper et al., 1986), it is hypothesized that this peptidase inactivates NAAG, and that the liberated glutamate is subsequently transported into synaptosomes by the previously characterized sodium-dependent high-affinity glutamate uptake site (Blakely et al., 1986; Robinson et al., 1987). Alternatively, NAAG may function as a precursor to glutamate, shifting the primary role of this peptidase to regulating glutamate availability.

This peptidase has been characterized in rat synaptosomal membranes (Robinson et al., 1987; Blakely et al., 1988). In this crude membrane preparation, the peptidase demonstrates remarkably high apparent affinity for its putative substrate NAAG, with a \( K_m = 540 \text{ nM} \). The enzyme is membrane-bound, stimulated by chloride ions, and inhibited by divalent metal chelators, suggesting that it is a metallopeptidase. It is enriched in synaptic plasma membranes and is primarily localized to neural tissue and kidney. Comparison of its properties to those of other known endopeptidases, aminopeptidases, dipeptidases, and acyl amino acid-releasing enzymes suggests that it is a novel peptidase (Robinson et al., 1987; Blakely et al., 1988). Since it is possible that NAAG is not the sole substrate for this activity in vivo, this peptidase was named N-acetylated α-linked acidic dipeptidase (NAALA dipeptidase) for its structural specificity for N-acetylated α-linked acidic dipeptidates. Recently, it has been demonstrated that [\(^3\)H]NAAG is degraded by a pharmacologically similar enzyme in vivo (Staubh et al., 1989). These data are consistent with a role for NAALA dipeptidase in the disposition of endogenous NAAG.

In this manuscript, we describe for the first time the solubilization of NAALA dipeptidase from rat membranes, its purification to apparent homogeneity, the characterization of the purified protein, the determination of its molecular weight, the production and characterization of anti-NAALA dipeptidase antibodies, and the localization of NAALA dipeptidase immunoreactivity in brain and kidney.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**Chromatographic Purification of NAALA Dipeptidase**—Results of the purification of rat brain NAALA dipeptidase activity are summarized in Table I. The overall purification was 720-fold with 1.6% recovery, yielding 2 mg of highly purified NAALA dipeptidase from 500 whole rat brains. Details of the solubilization and chromatographic steps are found in the miniprint supplement.

**Analysis of Enzyme Homogeneity**—Fig. 2 shows SDS polyacrylamide gel electrophoresis of pooled fractions at various stages in the purification. After the lentil Lectin step, there was one major silver-stained protein band migrating at 94 kDa.

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1 The abbreviations used are: NAAG, N-acetyl-L-aspartyl-L-glutamate; NAALA dipeptidase, N-acetylated α-linked acidic dipeptidase; Quis, quisqualic acid; NAA, N-acetyl-L-aspartate; EGTA, [ethylenediaminetetraacetic acid]; SDS, sodium dodecyl sulfate; ELISA, enzyme linked immunosorbent assay.

2 Portions of this paper (including "Experimental Procedures," part of "Results," and Fig. 1) 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 included in the microfilm edition of the Journal that is available from Waverly Press.
TABLE I
Summary of the purification of NAALA dipeptidase

| Step                        | Protein Specific activity | Recovery | Purification |
|-----------------------------|--------------------------|----------|--------------|
| mg pmol/mg/min % -fold      |                         |          |              |
| Crude homogenate            | 87,000       5           100    1           |
| Lysed synaptosomal membranes| 9,000        15          31     3           |
| Solubilized protein         | 2,000        71           33    14          |
| Pooled DEAE fractions       | 450          160          17    32          |
| Pooled CM fractions         | 20           900          4.1   180         |
| Pooled lentil lectin fractions| 2           3,600         1.6   720         |

kDa, and a minor (diffuse) band migrating between 54 and 66 kDa. The literature suggests that minor contaminating protein(s) are either mercaptoethanol artifacts (Guevara et al., 1982; Tasheva and Dessev, 1983) or skin keratins (Ochs, 1983).

The staining intensity of this 94-kDa band was correlated with the amount of NAALA dipeptidase activity applied to the gel. Gel electrophoresis of fractions surrounding a NAALA dipeptidase activity band contains only one band, migrating at 94 kDa, which cannot be attributed to the NAALA dipeptidase activity staining components.

Although five bands were revealed with this procedure, all bands, except for a 94-kDa protein, were attributed to the staining components used to visualize NAALA dipeptidase activity in the nondenaturing gel (e.g. glutamate dehydrogenase, iodonitrotetrazolium violet, phenazine methosulfate). Note that the NAALA dipeptidase activity band contains only one band, migrating at 94 kDa, which cannot be attributed to the NAALA dipeptidase activity staining components.

Properties of the Purified NAALA Dipeptidase—As was observed for activity characterized in lysed synaptosomal membranes (Robinson et al., 1987), purified NAALA dipeptidase was potently inhibited by quisqualate with 50% inhibition at 0.48 μM (Table II). Peptidase activity was also inhibited by phosphate and EGTA; cobalt strongly stimulated activity. Purified NAALA dipeptidase showed a high apparent affinity for NAAG hydrolysis with a K_m of 140 nM (mean of two determinations within 10%).
Purification of NAALA Dipeptidase

FIG. 4. Immunoprecipitation of NAALA dipeptidase activity. Details pertaining to the immunoprecipitation are described under “Experimental Methods.” The graphs presented are one representative set of data obtained from three separate experiments. In brief, varying amounts of preimmune and immune sera were added to crude brain membranes and incubated overnight on a rotary shaker at 4 °C. The following day, GammaBind G-Agarose was added, incubated for 3 h at 4 °C, and centrifuged. NAALA dipeptidase activity was measured in the supernatant (○) and pellet (●).

FIG. 5. Survey of the tissue distribution of NAALA dipeptidase immunoreactivity. Tissue extracts (250 µg of protein) were subject to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with immune serum against NAALA dipeptidase, followed by color development as described under “Experimental Methods.” All samples shown were run in parallel. The level of NAALA dipeptidase activity identified in each region is provided.

FIG. 6. Immunocytochemical localization of NAALA dipeptidase. Staining for NAALA dipeptidase immunoreactivity in rat cerebellar cortex (top) and rat kidney cortex (bottom). Note the positive staining in the molecular and granular cell layers of the cerebellar cortex and the proximal tubules and glomeruli of the kidney cortex. Details of the immunocytochemistry are described under “Experimental Procedures.” Cerbellar cortex: M, molecular layer; P, Purkinje cell layer; G, granular layer; Bar = 80 µm. Kidney cortex: PT, proximal tubules; G, glomeruli; Bar = 300 µm.

compared with what was previously determined using a lysed synaptosomal membrane preparation (Robinson et al., 1987). All peptides examined were used at their previously reported IC₅₀ concentrations. The results are summarized in Table II. All data were similar to what was reported initially, except inhibition by aspartylglutamate (Asp-Glu), which was 8-fold more potent as an inhibitor of the purified enzyme than had been reported using lysed membranes (50% inhibition at 0.3 µM versus 2.4 µM).

Antibody Characterization—Purified NAALA dipeptidase was used to raise polyclonal antibodies in guinea pigs (see “Experimental Procedures”). Enzyme-linked immunosorbent assay, immunoprecipitation assay, and Western blotting demonstrated that the anti-NAALA dipeptidase antisera was specific and of high titer. The titer, determined by enzyme-linked immunosorbent assay (see “Experimental Procedures”), was approximately 1:10,000 (data not shown).

Immunoprecipitation—The immune serum precipitated NAALA dipeptidase activity from solubilized brain membranes; preimmune serum did not precipitate activity (Fig. 4). Immune serum partially inhibited NAALA dipeptidase activity; therefore, at antibody dilutions less than 1:100,000 total activity using the immune serum was less than activity in the preimmune control.

Western Blotting—An SDS gel loaded with crude homogenates from rat heart, brain, intestine, liver, kidney, pancreas, spleen, and testis was transferred to nitrocellulose and probed with anti-NAALA dipeptidase immune serum as described under “Experimental Procedures.” The immune serum recognized a single 94-kDa band in brain, kidney, and testis only, consistent with the localization of NAALA dipeptidase activity (Fig. 5). No staining was detected using preimmune serum.

Immunocytochemistry—Immunocytochemical experiments revealed NAALA dipeptidase-positive staining in the molecular and granule cell layers of the cerebellar cortex; the Purkinje cell layer was devoid of immunoreactivity (Fig. 6, top). In the rat kidney, NAALA dipeptidase-positive staining was detected in the proximal tubules and glomeruli of the renal cortex; the distal tubules were essentially devoid of immunoreactivity (Fig. 6, bottom). No immunostaining was revealed using preimmune serum, even at 10-fold higher concentrations. In addition, preadsorption of the antiserum with purified NAALA dipeptidase (0.03 mg/ml) completely abolished immunocytochemical staining.

DISCUSSION

Brain NAALA dipeptidase activity was solubilized with Triton X-100 and sequentially purified with ion-exchange and
NAALA dipeptidase is a dimer composed of two identical purified protein show that NAALA dipeptidase migrates con- previously characterized in lysed synaptosomal membranes. NAALA dipeptidase activity demonstrated a low degree of exclusion chromatography may represent protein-detergent migration into the nondenaturing gel, consistent with its high isoelectric point. This resulting activity band was excised from gels, the 94-kDa band was the only band observed whose major silver-stained band on SDS-polyacrylamide gel electrophoresis migrating at 94 kDa and a minor (diffuse) band migrating between 54 and 66 kDa. The literature suggests that this minor broad band is either a mercaptoethanol artifact (Guevara et al., 1982; Tasheva and Dessev, 1983) or skin keratins (Ochs, 1983). In fact, we have seen this diffuse band in lanes run with sample buffer alone.

To demonstrate that the 94-kDa protein represented NAALA dipeptidase, activity applied to an SDS gel was correlated with protein staining intensity of this band. In all gels, the 94-kDa band was the only band observed whose staining density coincided with the amount of applied NAALA dipeptidase activity. Furthermore, NAALA dipeptidase activity was visualized directly in a nondenaturing gel (Fig. 3A). NAALA dipeptidase activity demonstrated a low degree of migration into the nondenaturing gel, consistent with its high isoelectric point. This resulting activity band was excised from the nondenaturing gel and run on an SDS gel. Protein staining again revealed a single unique band at 94 kDa (Fig. 3B). Together, these data strongly suggest that this 94-kDa band is NAALA dipeptidase.

Size exclusion chromatography of the purified and semi-purified protein show that NAALA dipeptidase migrates consistent with a molecular mass of 225 kDa, although larger species were occasionally observed. Both protein and activity gels demonstrate that peptidase has a denatured molecular mass of approximately 94 kDa. Therefore, it is possible the NAALA dipeptidase is a dimer composed of two identical subunits; alternatively, the larger species identified with size exclusion chromatography may represent protein-detergent complexes.

Proportions of the purified protein were similar to activity previously characterized in lysed synaptosomal membranes (Robinson et al., 1987), demonstrating that these properties are due to direct interaction with NAALA dipeptidase and are not indirectly mediated by other proteins present in the membrane preparation. The potent inhibition of peptidase activity by quisqualate suggests that some of its actions, which were previously attributed to interaction with a subclass of glutamate receptors (Robinson and Coyle, 1988), may be due to inhibition of NAALA dipeptidase. EGTA sensitivity and cobalt stimulation support initial data suggesting that NAALA dipeptidase was a metallopeptidase. Similar to activity in lysed synaptosomal membranes, purified NAALA dipeptidase displayed structure specificity for N-acetylated α-linked acidic dipeptides (Table II). Finally, the purified NAALA dipeptidase displayed a remarkably high apparent affinity for its putative substrate, NAAG, with a $K_a$ of 140 nM.

The availability of purified protein permitted the production of anti-NAALA dipeptidase antisera. The results presented in this study demonstrate that the polyclonal antibodies raised in guinea pig are remarkably selective, of high titer, and capable of recognizing both native and denatured NAALA dipeptidase. Western analysis of gels loaded with crude brain homogenates revealed that the antisera exclusively recognized the 94-kDa band. Besides brain NAALA dipeptidase, the antibodies cross-reacted with kidney and testis NAALA dipeptidase; no immunoreactivity was observed in regions devoid of NAALA dipeptidase activity (Fig. 5). The antisera inhibited NAALA dipeptidase activity, although not completely (70% inhibition at 1:100 dilution), and was capable of precipitating NAALA dipeptidase activity from a crude brain extract (Fig. 4).

Using this selective and specific antisera, NAALA dipeptidase immunoreactivity was localized to the glomeruli and proximal tubules of the kidney cortex (Fig. 5B). This localization is consistent with micropunch analysis of NAALA dipeptidase activity, which found that the vast majority of NAALA dipeptidase activity was localized to the renal cortex (kidney cortex = 166 pmol/mg/min versus kidney medulla = 10 pmol/mg/min). Interestingly, other brain peptidases, angiotensin converting enzyme and enkephalinase (Schulz, 1988; Tauc, 1998) have also been localized in the glomeruli and proximal tubules of the kidney, areas where their putative brain substrates are not found. In the neural tissue, NAALA dipeptidase immunoreactivity was found in areas reported previously to contain NAAG (Blakely and Coyle, 1989).

NAALA dipeptidase is a novel enzymatic activity involved in NAAG hydrolysis. In this study, we have solubilized and purified brain rat NAALA dipeptidase to apparent homogeneity, developed specific anti-NAALA dipeptidase antisera, and have begun to map its distribution in rat brain and kidney. We anticipate using the antisera to fully determine its renal and neuronal localization and to screen a cDNA library to obtain the NAALA dipeptidase clone.

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$^2$ S. S. Richards, G. Forloni, M. B. Robinson, and J. T. Coyle, unpublished observation.
Purification of NAALA Dipeptidase

EXPERIMENTAL PROCEDURE

Materials

Acetyl-L-Val-Leu-L-Leu-pH 4.6 (60 mmol) was obtained from Du Pont-New England Nuclear. Acetyl-D-Val-Leu-L-Leu-pH 4.6 (60 mmol) was obtained from Calbiochem. Nα-Acetyl Lys-Ala-OH (160 mg) was obtained from Sigma Chemical. Lys-Ala-diphetidase from the stand of Streptomyces griseus was obtained from Pharmacia. Protein-free buffers were prepared from Pharmacia. Protein-free buffers were prepared from Sigma Chemical. Lys-Ala-diphetidase (160 mg) was obtained from Sigma Chemical. Lys-Ala-dipeptidase (160 mg) was obtained from Sigma Chemical.

NAALA-dipeptidase Activity

NAALA-dipeptidase activity was measured as previously described (Robinson et al., 1987) with a minor modification. The reaction mixture consisted of 1 ml of 50 mM Tris-buffer (pH 7.5), 0.1 mM Lys-Ala in a volume of 2 ml. The reaction was initiated by the addition of 1 ml of enzyme solution. After incubation for 10 min at 37°C, 0.1 ml of the reaction mixture was added to 1.9 ml of 50% trichloroacetic acid. The mixture was centrifuged at 2,000 g for 10 min at 4°C. The supernatant was used for the determination of NAALA-dipeptidase activity and other enzymes.

Subcellular Localization

Lysosomes were prepared from tissue by a modification of the method of Halban and colleagues (1987). The tissue was homogenized in a medium containing 0.32 M sucrose, 5 mM HEPES, pH 7.5, and 0.3 M NaCl. The homogenate was centrifuged at 105,000 g for 1 h. The supernatant was then used for the subcellular fractionation.

Purification Scheme

The subcellular fraction was suspended in 0.5 M sucrose, 5 mM HEPES, pH 7.5, and 0.3 M NaCl. The suspension was then centrifuged at 105,000 g for 1 h. The supernatant was then used for the subcellular fractionation.

results

The results are shown in Table 1. The specific activity of NAALA-dipeptidase was increased by threefold in the subcellular fraction. The activity was then used for the purification of NAALA-dipeptidase. The specific activity of NAALA-dipeptidase was increased by fourfold in the subcellular fraction. The activity was then used for the purification of NAALA-dipeptidase.

Characterization of Chromatography

The specific activity of NAALA-dipeptidase was increased by threefold in the subcellular fraction. The activity was then used for the purification of NAALA-dipeptidase. The specific activity of NAALA-dipeptidase was increased by fourfold in the subcellular fraction. The activity was then used for the purification of NAALA-dipeptidase.

Chromatography

Chromatography was performed on a DEAE-cellulose column. The column was eluted with a linear gradient of NaCl in 0.01 M Tris-hydrochloride, pH 7.5, to 0.5 M NaCl. The column was then eluted with a linear gradient of NaCl in 0.01 M Tris-hydrochloride, pH 7.5, to 0.5 M NaCl. The column was then eluted with a linear gradient of NaCl in 0.01 M Tris-hydrochloride, pH 7.5, to 0.5 M NaCl. The column was then eluted with a linear gradient of NaCl in 0.01 M Tris-hydrochloride, pH 7.5, to 0.5 M NaCl. The column was then eluted with a linear gradient of NaCl in 0.01 M Tris-hydrochloride, pH 7.5, to 0.5 M NaCl. The column was then eluted with a linear gradient of NaCl in 0.01 M Tris-hydrochloride, pH 7.5, to 0.5 M NaCl.

Fig. 1 Chromatographic purification of NAALA dipeptidase activity. Details concerning the chromatographic separation of NAALA dipeptidase activity were described. The purified NAALA dipeptidase activity was observed as a single peak at the elution position of the NAALA dipeptidase activity. The purified NAALA dipeptidase activity was observed as a single peak at the elution position of the NAALA dipeptidase activity.
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