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A Radioimmunoassay for Measuring α-Amidating Enzyme Activity

Arco Y. Jeng, Mary Wong, Susan J. Lovato,* Mark D. Erion, and James P. Gilligan*

*Research Department, Pharmaceuticals Division, CIBA-GEIGY, Corporation, Summit, New Jersey 07901 and *Unigene Laboratories, Fairfield, New Jersey 07006*

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A sensitive α-amidating enzyme (αAE) assay using C-terminal glycine-extended substance P (SP-Gly) as a substrate was developed. The product, substance P (SP), was measured by a radioimmunoassay with specific polyclonal antibodies which recognize SP with an affinity 10,000-fold higher than that of SP-Gly. The sensitivity of the radioimmunoassay was 5 fmol. Enzyme activity could be readily detected with 25 ng αAE partially purified from the conditioned medium of rat medullary thyroid carcinoma CA-77 cells. The K_m and V_{max} values were 2.0 ± 0.2 μM and 1.7 ± 0.1 nmol/mg/min (mean ± SE, $n = 3$), respectively. The assay enabled the kinetic characterization of αAE from a single rat pituitary homogenate. Optimal Cu^{2+} required was 30 μM and greater than 3 mM of ascorbate was needed for maximal enzyme activity. The sensitivity of this assay will aid efforts to examine the regulation of *in vivo* αAE activity.

α-Amidating enzyme (αAE)[1] is a Cu^{2+}- and ascorbate-dependent monooxygenase that catalyzes the final step in the biosynthesis of amidated peptides. The enzyme converts the C-terminal glycine-extended prohormone to the C-terminal primary amide, which is a prerequisite for biological activity (1,2). This enzyme was first identified in tissues of neural origin including pituitary (3–6) and hypothalamus (7). Subsequent studies have documented a diverse tissue distribution of αAE activity (8,9). The pituitary represents one of the richest sources of αAE and has therefore served as the primary source for enzyme purification (10,11).

The αAE from a variety of sources has been characterized extensively *in vitro*. It is effectively inhibited by a number of Cu^{2+} chelators such as diethyldithiocarbamate (DDC), *o*-phenanthroline, and EDTA (7,12). This observation has been extended to animal studies by Mains *et al.* who demonstrated that acute treatment with DDC in rats decreased the ability of the intermediate pituitary to amidate α-melanotropin (13). Chronic treatment with these agents for 7 to 11 days yielded higher levels of the glycine-extended precursor of melanotropin (13). Similarly, Glembotski reported that depletion of ascorbate, the other essential cofactor of αAE, from tissue culture media caused intermediate pituitary cells to stop producing amidated melanotropin (14). The αAE is also inhibited by ascorbate oxidase (7) and a diet deficient in vitamin C has been reported to produce an accumulation of glycine-extended gastrin in guinea pig antrum (15).

The regulation of *in vivo* αAE as a post-translational processing enzyme, however, is not well understood. A major problem involves the inability to measure accurately αAE activity in specific tissues. The highest levels of αAE activity have been identified in rat medullary thyroid carcinomas (16) and the CA-77 cell line derived from these tumors (17). Tissues of interest, however, have very low αAE activity, thereby severely limiting an *in vivo* study of the enzyme. We have hence devoted an effort to develop a sensitive assay capable of measuring αAE levels in nontransformed tissues. Previous studies of the substrate structure–activity relationships for αAE have shown that SP-Gly has the highest affinity for αAE among a series of the prohormones examined (18). We utilized this observation in the development of a sensitive αAE assay capable of measuring αAE activity from a single rat pituitary.

MATERIALS AND METHODS

Materials. SP and SP antiserum (AC95) were from Cambridge Research Biochemicals (Valley Stream,

[1] Abbreviations used: αAE, α-amidating enzyme; DDC, diethyldithiocarbamate; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; phosphoramidon, [*N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan]; RIA, radioimmunoassay; SP, substance P; SP-Gly, C-terminal glycine-extended SP; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; BSA, bovine serum albumin.

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NY). Goat anti-rabbit γ-globulin antibodies and normal rabbit serum were purchased from Calbiochem (La Jolla, CA). ^{125}I-labeled SP was obtained from New England Nuclear (Boston, MA). Catalase, phosphoramidon, *n*-octyl β-D-glucopyranoside, pepstatin A, PMSF, soybean trypsin inhibitor, Tes, and polyethylene glycol (8000) were purchased from Sigma (St. Louis, MO).

Measurement of SP by RIA. Vials of SP antiserum, goat anti-rabbit γ-globulin antibodies, and normal rabbit serum obtained commercially were reconstituted with 250 μl, 12.5 ml, and 47.6 ml of RIA buffer, respectively. The RIA buffer contained 0.2% BSA, 0.1% Triton X-100, and 0.02% NaN_3 in PBS. The SP RIA was carried out at room temperature as described (19) with minor modifications. Briefly, samples to be assayed were diluted in 300 μl of RIA buffer and mixed with 100 μl each of 1:200 diluted SP antiserum and ^{125}I-labeled SP (10,000 cpm/tube). After 2 h of incubation, 100 μl each of the goat anti-rabbit γ-globulin antibodies and the normal rabbit serum was added and incubated further for 45 min. The antigen–antibody complex was then precipitated with 1 ml of 17.5% polyethylene glycol and centrifuged in a tabletop centrifuge. The supernatant was decanted and the radioactivity in the pellet was counted in a gamma counter. The total binding and nonspecific binding were measured in the absence of nonradioactive SP and SP antiserum, respectively. In general, the radioactivities measured in the total and nonspecific binding were 6000 and 300 cpm, respectively.

Chemical synthesis of SP-Gly. SP-Gly was synthesized by the solid-phase method of Merrifield (20) and purified by reverse-phase HPLC. The composition of peptide was verified by amino acid analysis using phenylisothiocyanate precolumn derivatization (21).

Partial purification of αAE from the conditioned medium of CA-77 cells. αAE was partially purified from the conditioned medium of CA-77 cells using a combination of DEAE cartridge and Sephacryl 300 SF column chromatography as described (18). This enzyme preparation could amidate at least 5 nmol of *N*-dansyl-Tyr–Val–Gly/mg protein/min at 37°C. The partially purified enzyme preparation was determined to be free of other protease activity since it did not cleave various prohormones or amidated peptides up to 45 amino acids in length when incubated at room temperature for up to 4 h.

Extraction of αAE from pituitary. Male Sprague–Dawley [Tac:N(SD)fBR] rats (Taconic Farms, Germantown, NY) were decapitated and the pituitaries removed and individually homogenized in 130 μl of buffer containing 0.2 mg/ml pepstatin A, 0.2 mg/ml soybean trypsin inhibitor, 0.25% *n*-octyl β-D-glucopyranoside, 0.1 mM PMSF in 100 mM Tes, pH 7.0 at 4°C. The homogenates were centrifuged at 12,000*g* for 6 min and the resulting supernatants were used in assays. Protein content was determined by the method of Bradford (22) using bovine γ-globulin as the standard.

αAE assay. The αAE assay using the partially purified enzyme from the conditioned medium of CA-77 cells was carried out in a reaction mixture containing SP-Gly at the desired concentration, 150 ng αAE of the partially purified enzyme preparation, and 3 mM of ascorbate in 150 mM Tes, pH 7.0, in a total volume of 60 μl. After incubation at room temperature for various time periods, 10-μl aliquots of the mixture were removed and mixed with 2 μl of 100 mM EDTA to stop the reaction. This reaction mixture was then diluted to 1 ml with the RIA buffer and appropriate aliquots were used to measure the formation of SP by RIA. The αAE assay using the rat pituitary extract was performed by the same method except that the reaction mixture contained 10 μM SP-Gly, 60 μg rat pituitary extract, 0.5 mg/ml catalase, and varying concentrations of Cu^{2+} and ascorbate in 150 mM Tes, pH 7.0, in a total volume of 60 μl. In reactions where a low αAE activity was present or a prolonged incubation time was required the addition of catalase was mandatory in order to prevent the enzyme from being inactivated by hydrogen peroxide generated in the presence of exogenous Cu^{2+} and ascorbate (7,17).

RESULTS

Measurement of SP by RIA. In the presence of appropriate cofactors, αAE catalyzes the conversion of SP-Gly to SP. In order to develop a quantitative RIA for SP, a specific antiserum which recognize SP but not SP-Gly is required. Three commercially available SP antisera were tested and the antiserum from Cambridge Research Biochemicals was found to be satisfactory. SP displaced ^{125}I-labeled SP binding with an IC_{50} of 140 ± 16 fmol (mean ± SE, n = 14). As low as 5 fmol of SP could be detected. Figure 1A shows that SP-Gly is about 10,000 times weaker in displacing ^{125}I-labeled SP binding than SP. The validity of the difference between the IC_{50} values of SP and SP-Gly in the displacement of ^{125}I-labeled SP was further substantiated when samples containing SP-Gly were treated with αAE. Upon this treatment, samples originally containing SP-Gly then showed the same potency as SP in the RIA (Fig. 1B).

Kinetic parameters of αAE from the conditioned medium of CA-77 cells. With the specific RIA for SP described above, a sensitive assay for αAE was developed using SP-Gly as the substrate. At least 80% of the substrate was converted to SP by αAE in a linear fashion with respect to time (Fig. 2A). Only 25 ng of αAE from the partially purified conditioned medium of CA-77 cells was required for the assay. The K_m and V_{max} values for SP-Gly obtained from the double reciprocal plots of the kinetics data were 2.0 ± 0.2 μM and 1.7 ± 0.1 nmol/mg/min (mean ± SE, n = 3), respectively (Fig. 2B), similar to those obtained with the same enzyme source using dansyl-Tyr–Val–Gly as the substrate (18).

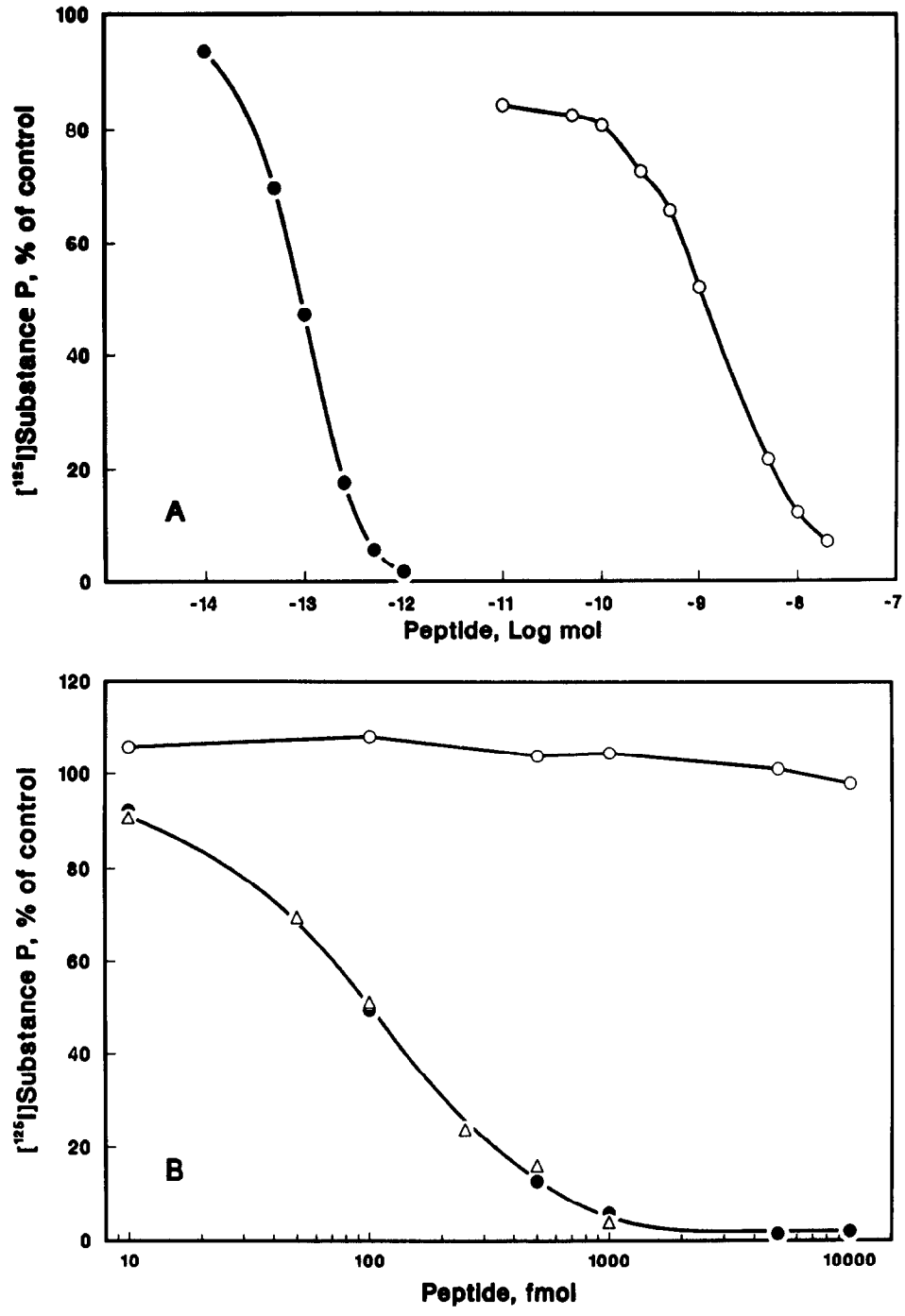

FIG. 1. Specificity of RIA for the measurement of SP. (A) SP RIA was preformed as described under Materials and Methods in the presence of varying amounts of nonradioactive SP (●) or SP-Gly (○). (B) Samples containing various amounts of SP-Gly were pretreated in the presence (●) or absence (○) of 25 ng of αAE from partially purified conditioned medium of CA-77 cells for 30 min at 37°C. The RIA was then carried out. In parallel experiments, the RIA was also performed with samples containing authentic SP (△).

Measurement of αAE activity using rat pituitary extract. The αAE activity in rat pituitary was measured using the method described above. Since bovine pituitary was reported to contain neutral endopeptidase (EC 3.4.24.11), an enzyme capable of hydrolyzing SP (23,24), the effect of phosphoramidon, a specific inhibitor of neutral endopeptidase (25), was tested in the αAE assay using rat pituitary extract as the enzyme source. The results from pretreatment of rat pituitary extract with 1 μM phosphoramidon showed no significant difference in αAE activity as compared with the control experiments (not shown), suggesting that SP in the samples was not hydrolyzed. Therefore, subsequent experiments were carried out in the absence of the neutral endopeptidase inhibitor.

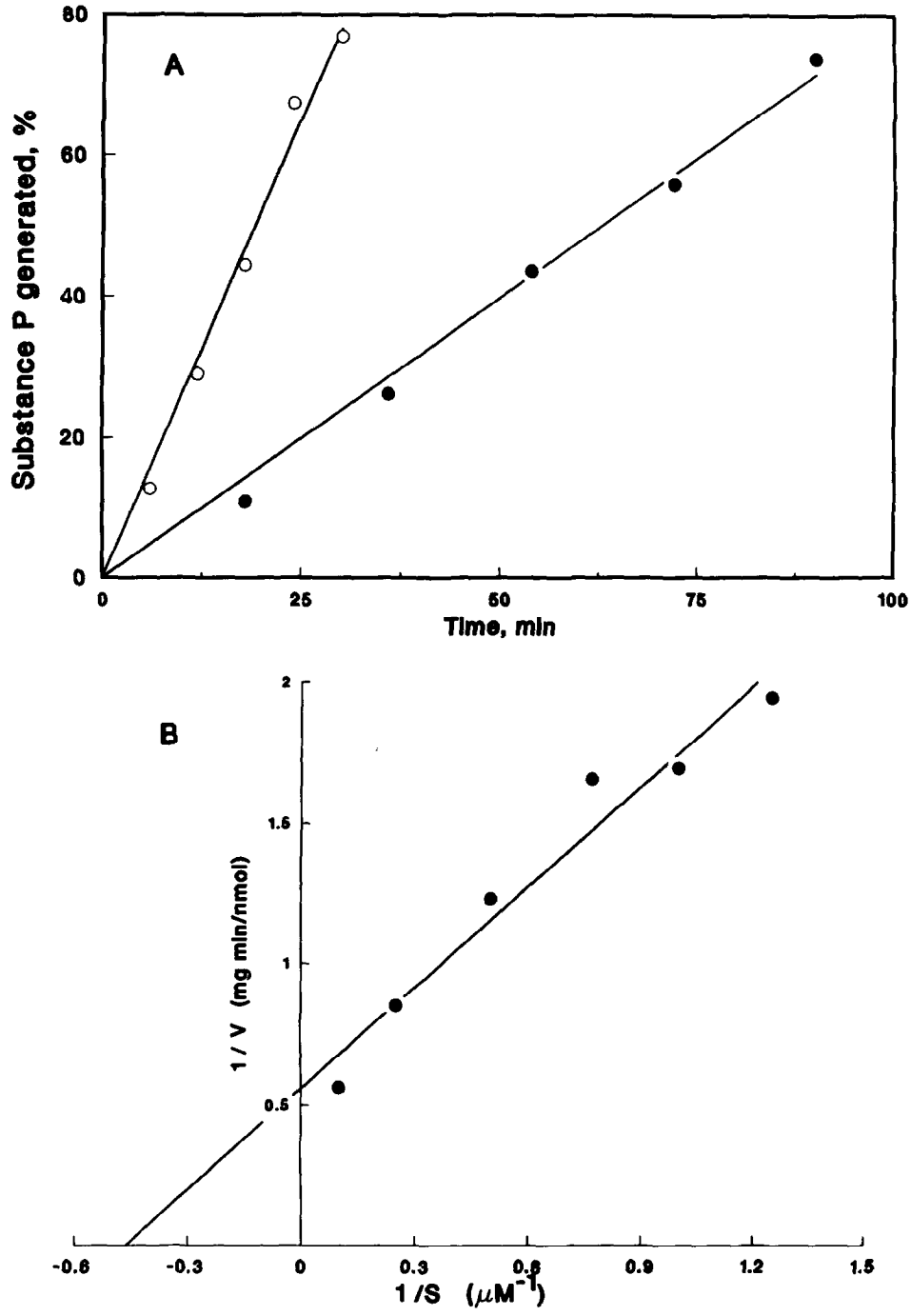

FIG. 2. Determination of kinetic parameters of αAE from the conditioned medium of CA-77 cells. (A) SP-Gly ((○) 1.3 μM; (●) 10 μM)) was amidated by 25 ng αAE from partially purified conditioned medium of CA-77 cells at room temperature as described under Materials and Methods. The formation of SP at different time points was measured by RIA. (B) The rate of SP formation from SP-Gly by αAE was measured by similar experiments described in A. The results were analyzed by double reciprocal plots. Representative plots from one experiment are shown. The K_m and V_{max} values obtained were 2.0 ± 0.2 μM and 1.7 ± 0.1 nmol/mg/min (mean ± SE, n = 3), respectively.

Typically, 30 assays could be performed with the extract from a single rat pituitary. Figure 3A shows experiments performed using one-half of a rat pituitary homogenate. SP generated by the rat pituitary αAE was linear with respect to time and was dependent on the concentration of exogenously added Cu^{2+}. The optimal Cu^{2+} concentration required for this enzyme was 30 μM (Fig. 3B). The effects of ascorbate were also examined. A broad optimal ascorbate concentration was found and at least 3 mM ascorbate was required for optimal enzyme activity (Fig. 4).

DISCUSSION

The αAE is the key enzyme involved in the post-translational α-amidation of many biologically active neuro-

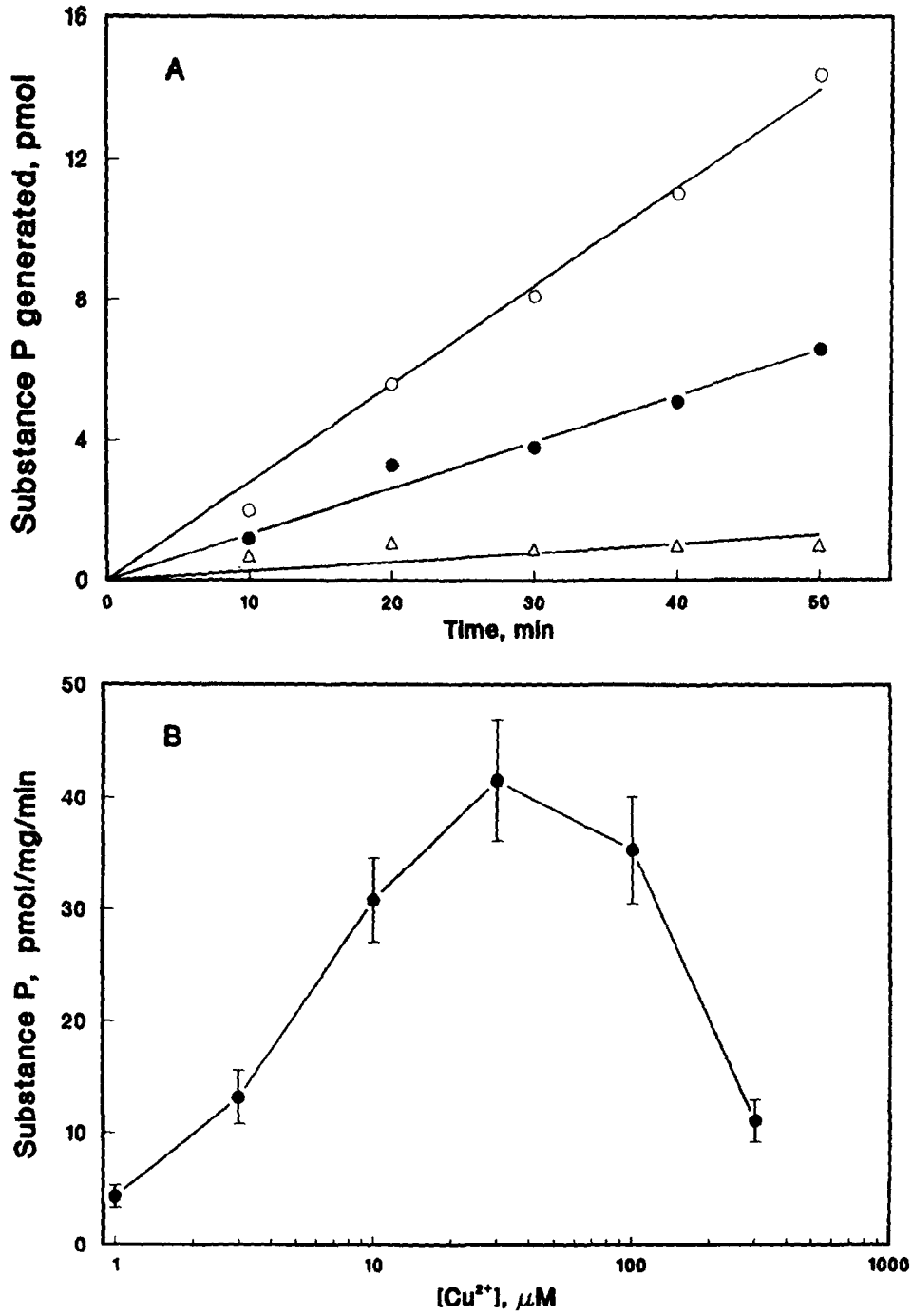

FIG. 3. Determination of optimal Cu^{2+} concentrations for αAE from rat pituitary. (A) SP-Gly (10 μM) was amidated by αAE from rat pituitary extract in the presence of 3 mM ascorbate and various concentrations of Cu^{2+} ((△) 3 μM; (●) 10 μM; (○) 30 μM)). The formation of SP at different time points was determined by RIA. The figure shows a representative experiment using one-half of a single rat pituitary extract. Similar results were obtained from three other experiments using different pituitaries. (B) The rates of SP formation by αAE from rat pituitary extract at various Cu^{2+} concentrations were measured as described in A. The concentration of ascorbate was fixed at 3 mM. The data show mean values with SE from four to eight experiments, except for results obtained using 300 μM Cu^{2+} where the error range from two experiments is shown.

peptides. A thorough study of the regulation of this enzyme *in vivo* should increase the understanding of the mechanisms controlling amidated neuropeptide production. To date, such studies have been limited due to the low levels of αAE activity in tissues and the lack of a sensitive and convenient assay for the detection of αAE.

The first assay used for the detection of αAE activity was developed by Bradbury *et al.* (3) who utilized ^{125}I-labeled D-Tyr-Val-Gly as a substrate. The substrate and product were separated by a lengthy cation-exchange column chromatography. This assay has been used by several other laboratories (8,26). Other methods re-

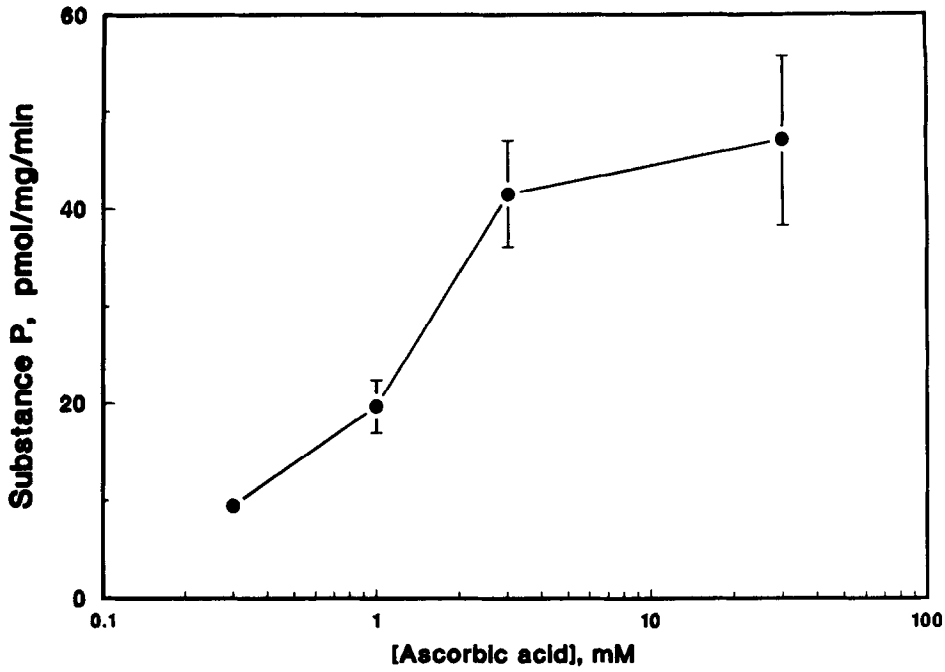

FIG. 4. Determination of optimal ascorbate concentrations for αAE from rat pituitary. The rates of SP formation by αAE from rat pituitary extract at various ascorbate concentrations were measured as described in Fig. 3A. The concentration of Cu^{2+} was fixed at 30 μM. The data show mean values with SE from three to six experiments.

ported include a RIA detection of D-Tyr–Val–NH_2 (27), an HPLC method to separate ⟨Glu–His–Pro–Gly from ⟨Gly–His–Pro–NH_2 (28), and an ethyl acetate extraction of ^{125}I-labeled Ac-Tyr–Phe–NH_2 (9). In these assays, the substrate turnover rate was slow; they ranged from 0.03 to 0.2 nmol/mg/min. As a result, long incubation times ranging from a few hours (3,9,27) to 1 day (28) at 37°C were required. The length of the assay may in itself lead to variability due to instability of αAE and/or substrate. The substrate used in this paper is SP-Gly, which has high affinity (K_m = 2 μM compared with 185 μM for Tyr–Val–Gly used in Ref. (27)) and high turnover rate (Fig. 2B) for αAE. These advantages coupled with a specific RIA for SP render the method reported here, to our knowledge, the most sensitive assay for measuring αAE activity. The procedure requires only 30 min of incubation at room temperature to produce accurate measurable levels of SP.

The αAE is dependent on Cu^{2+} for activity. Dialysis of the enzyme to remove Cu^{2+} resulted in a total loss of the αAE activity, while addition of Cu^{2+} restored the enzyme activity (data not shown). The αAE derived from CA-77 cell culture medium could be used in enzyme assays without the addition of exogenous Cu^{2+} (17) whereas those from rat brain and rat stomach required 5 and 50 μM Cu^{2+}, respectively, for optimal activity (9,27). The optimal Cu^{2+} for αAE from the rat pituitary extract was 30 μM (Fig. 3B). Therefore, the Cu^{2+} requirement for different tissues should be tested individually. In contrast, the optimal ascorbate concentrations for αAE activity were not significantly different among tissues.

The biosynthesis and regulation of C-terminal-amidated hormones are of considerable interest. The low abundance of αAE in animal tissues which produce amidated peptides has hindered research in this area. The sensitive assay described in this paper will enable the measurement of αAE activity in a single rat pituitary and can be extended to study αAE activity in other tissues (8,9).

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