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A Novel Enzyme from Bovine Neurointermediate Pituitary Catalyzes Dealkylation of α-Hydroxyglycine Derivatives, Thereby Functioning Sequentially with Peptidylglycine α-Amidating Monooxygenase in Peptide Amidation

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ABSTRACT: We report here the isolation of a novel enzyme from bovine neurointermediate pituitary which catalyzes the conversion of α-hydroxybenzoylglycine to benzamide. This enzyme, termed HGAD (α-hydroxyglycine amidating dealkylase), is a soluble protein with an apparent molecular mass of 45 kDa and no apparent cofactor requirement. Addition of HGAD to purified neurointermediate pituitary PAM (peptidylglycine α-amidating monooxygenase, EC 1.14.17.3) increases the rate of formation of amide products by an order of magnitude. Sequential additions of PAM and HGAD gave results consistent with PAM first catalyzing the formation of an intermediate that is subsequently, in a separate reaction, converted by HGAD to the final amide product. Experiments with olefinic inactivators demonstrate that HGAD is not required for turnover-dependent inactivation of PAM and, correspondingly, that HGAD activity is not affected by inactivators of PAM. As expected, HGAD has no effect on the rate of PAM-catalyzed sulfoxidation, where a reaction analogous to that occurring during amidation of glycine-extended substrates is not possible. On the basis of these results, we propose that peptide C-terminal amidation in neurointermediate pituitary is a two-step process, with PAM first catalyzing the conversion of a glycine-extended peptide to the α-hydroxyglycine derivative, which is in turn converted to the final amide product by HGAD.

Carboxy-terminal amidation is a feature necessary for bioactivity in approximately 50% of the known neuropeptide hormones (Bradbury & Smyth, 1987; Eipper & Mains, 1988). The enzyme peptidylglycine α-amidating monooxygenase (PAM,[1] EC 1.14.17.3) catalyzes the production of peptide amides from their glycine-extended precursors (Eipper & Mains, 1988). Because this enzyme is central to the biosynthesis of a wide variety of neurohormones and is present in almost all neural tissue, both its biochemical role and its mechanism of action are under active investigation. PAM has been isolated from various sources, and there exists both a soluble form, having a molecular mass of ~40 kDa depending on the source (Murthy et al., 1986; Mizuno et al., 1986; Kizer et al., 1986), and a membrane-bound form with a molecular mass of 113 kDa (Eipper et al., 1988). Complementary DNAs encoding PAM have been cloned from bovine pituitary, frog skin, and rat atrium (Eipper et al., 1987; Mizuno et al., 1987; Oshuye et al., 1988; Stoffers et al., 1989), and most code for an approximately 100-kDa protein. It has therefore been suggested that PAM is transcribed as a 100-kDa protein and the smaller, soluble, forms of the enzyme arise from post-translational proteolysis (Eipper et al., 1987).

PAM is a copper-dependent and ascorbate-requiring monooxygenase which catalyzes oxygenative cleavage at the C-terminal glycine to produce a C-amidated peptide and glyoxylic acid (Eipper et al., 1983; Bradbury et al., 1982). The enzyme stereospecifically abstracts the *pro-S* hydrogen of glycine (Ramer et al., 1988), and the glycine nitrogen is incorporated into the amide product (Bradbury et al., 1982). Recently, PAM from rat medullary thyroid carcinoma was shown to catalyze the conversion of dansyl-D-Tyr-Val-α-hydroxyglycine to dansyl-D-Tyr-Val-NH_2 in a reaction inde-

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[1] Abbreviations: PAM, peptidylglycine α-amidating monooxygenase; TNP-D-Tyr-Val-Gly,*N*-(trinitrophenyl)-D-Tyr-Val-Gly; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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pendent of copper and ascorbate (Young & Tamburini, 1989). This was interpreted as evidence that an α-hydroxyglycine derivative is an intermediate along the pathway of PAM catalysis at a point subsequent to the oxygen-transfer step. In addition to amidation, we have shown that PAM catalyzes several other monooxygenase reactions, including sulfoxidation, amine N-dealkylation, and O-dealkylation, and that PAM catalyzes olefin oxygenation leading to turnover-dependent enzyme inactivation (Katopodis & May, 1990).

We report here the presence, in bovine neurointermediate pituitary, of a novel enzyme that functions in carboxy-terminal amidation. We report the isolation of this protein, which we term HGAD (α-hydroxyglycine amidating dealkylase), and demonstrate that it catalyzes the facile dealkylation of α-hydroxybenzoylglycine to benzamide, in a reaction which is neither copper nor ascorbate dependent. This dealkylating activity is not exhibited by purified soluble bovine neurointermediate PAM. The catalytic activity of HGAD has been characterized with a number of substrates and turnover-dependent PAM inactivators, and its relationship to PAM catalysis in peptide amidation has been explored. Our results provide evidence that amidation of glycine-extended substrates in neurointermediate pituitary is a two-step process, with the function of HGAD being to catalyze conversion of an α-hydroxyglycine intermediate to the final amide product.

Materials and Methods

Enzyme Assays. PAM amidation assays, assays with (4-nitrobenzyl)thioacetic acid, glyoxylate assays, and inactivation of PAM by *trans*-benzoylacrylic acid or 4-phenyl-3-butenoic acid were performed as described previously (Katopodis & May, 1990).

HGAD dealkylation assays were performed in 100 mM Na-MES, pH 6.6, containing 2 mM α-hydroxybenzoylglycine as a substrate and various amounts of HGAD in a total volume of 0.5 mL, at 37 °C for 15–30 min. Aliquots of 200 μL were withdrawn from the incubation mixture at the appropriate time, quenched with 20 μL of 2 M H_2ClO_4, centrifuged, and analyzed on a C18 reversed-phase column, in a solvent system of acetonitrile/30 mM ammonium acetate, pH 5.5 (15:85). Detection was at 225 nm, and product was quantified by comparing the peak heights to a standard curve constructed with authentic benzamide.

Enzyme Purification. Bovine pituitary PAM was isolated according to a modification of the method of Murthy et al. (1986), in which a hydrophobic chromatography step was introduced following ammonium sulfate fractionation (see below). The 50% ethylene glycol eluate from the hydrophobic column was applied to a chelating Sepharose column charged with copper, and the rest of the purification was performed as described previously (Katopodis & May, 1990). The final PAM isolated after gel chromatography was homogeneous, producing a single band at 39 kDa on SDS–PAGE.

HGAD was purified from frozen bovine pituitaries (Pel-Freeze), and all procedures were performed at 4 °C. Typically, 50 pituitaries were partially thawed; the neurointermediate lobe was dissected and homogenized in 500 mL of 25 mM Na-HEPES, pH 8.5. The solution was centrifuged (45000*g* for 30 min), and the supernatant was subjected to ammonium sulfate precipitation. The 25–60% ammonium sulfate pellet was suspended in approximately 40 mL of 50 mM Na-HEPES, pH 7.0 (buffer A), by use of a ground-glass homogenizer and frozen at −70 °C for storage. The frozen ammonium sulfate cut was thawed, diluted with 2 volumes of buffer A containing 5% ammonium sulfate, and centrifuged (100000*g* for 30 min). The solution was then loaded onto a phenyl-Sepharose column (1.6 × 10 cm) equilibrated with the same buffer. The flow rate was 50 mL/h, and the column was washed successively with loading buffer, buffer A, and buffer A/ethylene glycol (50:50). The absorbance at 280 nm of the cluent was allowed to return to base line between washings.

Breakthrough material from the phenyl-Sepharose column was combined with the loading buffer wash and loaded on a chelating Sepharose column (1.6 × 8 cm, flow rate 60 mL/h) which had been charged with Cu^{2+} and preequilibrated with buffer A containing 500 mM NaCl. The column was washed with loading buffer until A_{280} of the eluent returned to base line and then eluted with a linear gradient of 0–35 mM imidazole, pH 7.0, at a flow rate of 120 mL/h over a period of 3 h. Absorbance at 280 nm was monitored, and 6-mL fractions were collected. Fractions with HGAD activity (about 96 mL) were pooled and concentrated in a stirred cell, by use of an Amicon YM30 membrane, to approximately 10 mL. The solution was dialyzed against buffer A and applied to a Mono Q HR 10/10 column equilibrated with buffer A. The column was eluted at a flow rate of 3 mL/min with a stepwise gradient from 0 to 100 mM NaCl over 10 min and then from 100 to 225 mM NaCl over 60 min. Fractions of 6 mL each were collected and pooled according to HGAD activity. The pooled fractions were concentrated to approximately 1.5 mL, made 50% in ethylene glycol, and stored at −20 °C, where they retained full activity for at least 6 months. Aliquots of 0.2 mL were analyzed on Superose 12 HR 10/30 equilibrated with 50 mM KP_i and 125 mM NaCl, pH 7.0, at a flow rate of 0.2 mL/min. Fractions of 0.2 mL were collected and analyzed for activity.

Results

Assays of HGAD. The HGAD-catalyzed production of benzamide from α-hydroxybenzoylglycine exhibits all the characteristics of an enzyme-catalyzed reaction. This dealkylating activity is independent of copper and ascorbate and is abolished when HGAD is boiled for 5 min or when 2 μg of HGAD (pool A) in 1 mL of assay buffer is treated with 1 μg of trypsin for 30 min at 25 °C. Purified PAM does not catalyze the dealkylation of α-hydroxybenzoylglycine, in either the presence or absence of ascorbate or copper. Benzamide formation is linear for at least 2 h under assay conditions and shows normal saturation kinetics. A K_m value of 0.44 mM was calculated for α-hydroxybenzoylglycine, from an inverse plot obtained with purified HGAD. The pH dependence of HGAD dealkylating activity was measured in the range of 5.1–7.0, and a pH maximum at 6.6 was observed. No data were obtained above pH 7 because the nonenzymatic background dealkylation rate increases rapidly. The nonenzymatic rate of α-hydroxybenzoylglycine dealkylation under assay conditions is more than an order of magnitude lower than the enzymatic rate. HGAD retains its full activity when 0.5 mM *o*-phenanthroline is added to the assay mixture.

In order to further characterize the HGAD reaction, we compared the ratio of glyoxylate to benzamide produced during HGAD turnover. A sample of 2 μg of purified HGAD was incubated in 2 mL of assay solution, and 0.5-mL aliquots were withdrawn at 10-min intervals and analyzed for benzamide and glyoxylate. A control experiment was performed with no HGAD added. Over a period of 30 min the ratio of glyoxylate to benzamide detected from HGAD turnover was 1.08 ± 0.05.

Purification of HGAD. A summary of the purification data from a single representative isolation is given in Table I. In a typical purification 17 g of bovine pituitary neurointermediate lobe tissue was homogenized in buffer. After centrifu-

Table I: Isolation of HGAD from Bovine Neurointermediate Pituitary[a]

fraction	vol (mL)	total protein (mg)	HGAD act. nmol/h	HGAD act. nmol mg^{-1} h^{-1}	yield (%)	purification (x-fold)
cell supernatant	425	1233	105×10^3	85	100	1
25–60% ammonium sulfate (after centrifugation)	39	468	72×10^3	154	69	1.8
phenyl-Sepharose eluent	97	233	62×10^3	266	59	3.1
chelating sepharose (after concentration)	50	27.5	37×10^3	1350	35	15.9
Mono Q pool A (after concentration)	1.9	1.3	11×10^3	8500	10.5	100
Mono Q pool B (after concentration)	1.4	1.1	5.4×10^3	4900	5.1	58

[a] Data from a single isolation using 50 pituitaries (17 g of dissected tissue). Activity was measured as nanomoles of benzamide produced; see Materials and Methods. Protein was quantitated by the method of Bradford (1976).

gation to remove both cell debris and membranes, the protein was precipitated with ammonium sulfate, and the 25–60% pellet was found to contain about 70% of the HGAD activity. After resuspension of the pellet and centrifugation, the protein mixture was made 5% in ammonium sulfate and passed through a phenyl-Sepharose column equilibrated with the same buffer. About 90% of the HGAD activity passed through the hydrophobic interaction column at this salt concentration, but more than 90% of the PAM activity was retained. Washing the column with 0% ammonium sulfate buffer released proteins with little PAM or HGAD activity. Subsequent washing with 50% ethylene glycol buffer released all the remaining PAM activity with negligible amounts of HGAD present.

The HGAD activity from the breakthrough of the phenyl-Sepharose column was next applied to a chelating Sepharose column charged with copper and eluted with an imidazole gradient. HGAD activity eluted in a broad peak, the beginning of which coincided with the major protein band. Fractions with specific activity higher than 1200 nmol mg^{-1} h^{-1} were pooled. Further purification of HGAD was achieved on a Mono Q anion exchange column at pH 7.0. HGAD activity eluted in two fractions, one at approximately 150–165 mM and the other at approximately 170–200 mM NaCl. The fractions were pooled separately and labeled pool A and pool B, respectively. No obvious difference in reactivity was observed between the two fractions.

Characterization of HGAD. Purified HGAD obtained from the ion exchange column was applied to an analytical gel filtration column. Activity eluted from the gel column as a single peak with an apparent molecular mass of 45 kDa. The activity peak, however, does not coincide with the major protein peak, indicating that the purified HGAD is not homogeneous. SDS–PAGE analysis of the HGAD obtained from a gel filtration column (stained with both silver and Coomassie blue stain) showed the presence of three major and several minor protein bands. Similarly, analysis of the same sample by isoelectric focusing showed the presence of six bands of variable intensity, with isoelectric points in the pH range of 4.5–5.5. The fact that HGAD has a low isoelectric point was confirmed by chromatofocusing experiments using a Mono P HR 10/20 column, where HGAD activity elutes in the pH range of 5–4.6.

Functional Studies of HGAD. The function of HGAD and its relationship to the overall amidation reaction were examined with the various substrates we have developed for PAM. Assays were performed to measure the conversion of TNP-D-Tyr-Val-Gly to TNP-D-Tyr-Val-NH_2, in the presence and absence of HGAD and/or PAM. The results in Figure 1A show that inclusion of HGAD in the PAM assay solution greatly enhances the rate of amide product formation and that HGAD alone cannot effect this conversion. Similar experiments were carried out with 4-nitrohippuric acid, a small-molecule amidation substrate that we have previously introduced (Katopodis & May, 1990). For this substrate the amidation reaction is monitored by following formation of 4-nitrobenzamide. As was observed for the tripeptide substrate, addition of HGAD causes a 7-fold increase in the rate of formation of 4-nitrobenzamide, under standard assay conditions. HGAD also greatly increases the PAM-catalyzed production of aPY-amide (a neuropeptide Y-like peptide from *Lophius americanus*) from aPY-Gly (Bryan D. Noe, personal

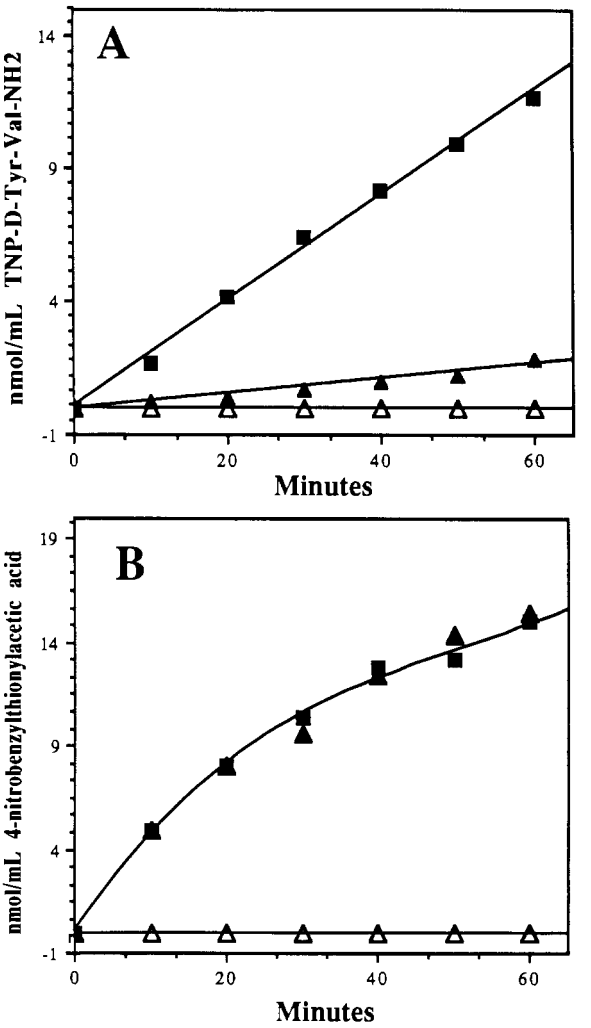

FIGURE 1: Time dependence of amide and sulfoxide production. (Panel A) 4 μg of purified PAM and/or 2 μg of HGAD (pool A) was incubated at 37 °C in 2 mL of 100 mM Na-MES, pH 6.6, containing 2 mM ascorbate, 4 μM Cu^{2+}, 120 μM TNP-D-Tyr-Val-Gly, and 20000 units of catalase. Aliquots were quenched and analyzed for the presence of TNP-D-Tyr-Val-NH_2 at the indicated times. (Panel B) Incubations were performed as above but with twice the amount of PAM and HGAD. The substrate was 1.0 mM (4-nitrobenzyl)thioacetic acid, and the product detected was (4-nitrobenzyl)thionylacetic acid. Aliquots of 0.2 mL were quenched and analyzed on a C8 reversed-phase HPLC column in a solvent system of acetonitrile/50 mM ammonium acetate, pH 5.5 (15:85). In both panels, (▲) represents the data obtained when only PAM was present, (△) when only HGAD was present, and (■) when both PAM and HGAD were present in the assay mixture.

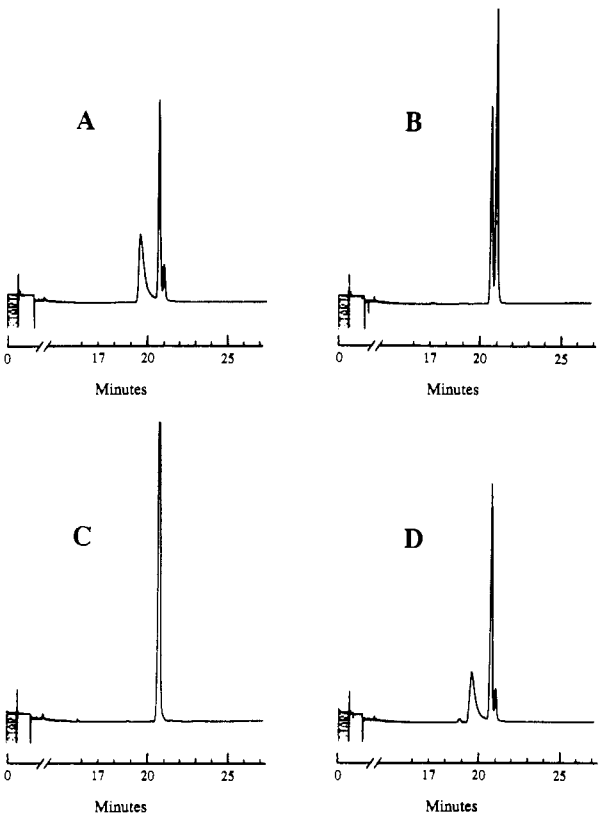

FIGURE 2: Sequential addition of PAM and HGAD. As described under Results, purified PAM was incubated in the complete PAM assay mixture containing TNP-D-Tyr-Val-Gly as the substrate. Panel A is the HPLC trace of the mixture after incubation for 20 min and ultrafiltration to remove all proteins. HPLC analysis was performed on a C8 reversed-phase column at a flow rate of 1.5 mL/min with a 25-min gradient from water (0.1% TFA) to 65% acetonitrile/35% water (0.1% TFA); detection was at 344 nm. The peak at 19.8 min is the product from PAM turnover, the peak at 20.7 min is TNP-D-Tyr-Val-Gly, and the peak 21.0 min is TNP-D-Tyr-Val-NH_2. Panel B is the HPLC trace of the ultrafiltrate after addition of HGAD and incubation for 5 min. The peak at 19.8 min has disappeared, while the TNP-D-Tyr-Val-NH_2 peak is greatly increased. The same experiment was repeated except that HGAD was added first to the complete assay mixture, followed by addition of PAM after removal of HGAD by ultrafiltration. Panel C is the trace of the mixture after incubation with HGAD and ultrafiltration, and panel D is the trace of the ultrafiltrate after addition of PAM and incubation for 20 min.

communication). Thus, HGAD causes a marked increase in the rate of amide production from a wide variety of glycine-extended substrates.

In order to gain additional insight into the action of HGAD, the following experiments were performed. A mixture of 8.5 μg of purified PAM, catalase (20 000 units), ascorbate (2 mM), copper (4 μM), and TNP-D-Tyr-Val-Gly (120 μM), in 3 mL of 100 mM Na-MES, pH 6.6, was incubated for 20 min at 37 °C. The mixture was then subjected to ultrafiltration through an amicon YM10 membrane, to remove PAM, and the filtrate analyzed by HPLC (Figure 2A). After HPLC analysis, 4 μg of HGAD was added, and the solution was incubated at 37 °C for 5 min and reanalyzed by HPLC. The resulting trace (Figure 2B) shows disappearance of the intermediate and appearance of a greatly increased amount of amide product. The entire experiment was then repeated, except that the order of addition of the two enzymes (PAM and HGAD) was reversed. As is evident from panels C and D of Figure 2, HPLC analysis showed neither the appearance of the intermediate nor the increased amount of amide product that was evident in the first experiment. On the basis of these results, we conclude that HGAD and PAM act sequentially, with PAM first catalyzing the formation of an intermediate species that is then converted by HGAD to the final amide product. Furthermore, the catalytic action of HGAD is independent of PAM, and the simultaneous presence of both proteins in the reaction mixture is not required for catalytic activity of either enzyme.

The effect of HGAD on PAM substrates that do not form amide products was also examined. One such substrate is (4-nitrobenzyl)thioacetic acid, which we have previously shown to be an active substrate for PAM, being enzymatically oxygenated to the corresponding sulfoxide, (4-nitrobenzyl)-thionylacetic acid (Katopodis & May, 1990). Sulfoxidation requires the presence of active PAM, ascorbate, and copper. As shown in Figure 1B, HGAD does not catalyze sulfoxidation, consistent with the view that this enzyme is not a monooxygenase. Furthermore, no stimulation of PAM-catalyzed sulfoxide production is observed when HGAD is added to the reaction mixture. Thus, it is clear that in the case of the sulfoxide PAM product, where a ketonization process analogous to that occurring during amidation of glycine-extended substrates is not possible, HGAD has no effect on the reaction.

We have previously reported that the olefinic substrate analogues, *trans*-benzoylacrylic acid and 4-phenyl-3-butenoic acid, are potent turnover-dependent inactivators of PAM, with inactivation exhibiting the characteristics expected for mechanism-based inactivation by a reactive species along the olefin oxygenation pathway (Katopodis & May, 1990). HGAD has no effect on the rate of PAM inactivation by *trans*-benzoylacrylic acid; K_I was calculated to be 0.11 mM in the absence and 0.16 mM in the presence of HGAD; the respective k_{inact} values were 3.3 and 3.6 min^{-1}. We also examined the effect of *trans*-benzoylacrylic acid on the dealkylating activity of HGAD. No inactivation of HGAD was evident after 30-min incubation with 1 mM *trans*-benzoylacrylic acid, in either the absence or presence of ascorbate. Similarly, inactivation of PAM but not of HGAD was also observed when samples were incubated with 4-phenyl-3-butenoic acid. These results are in accord with our expectation that olefinic substrate analogues would not be turnover-dependent inactivators of HGAD.

As a further demonstration of the differential effects of an olefin inactivator on the monooxygenase activity of PAM vs the dealkylating activity of HGAD, we incubated HGAD and the full PAM system with 1 mM *trans*-benzoylacrylic acid for 30 min. At this point PAM activity toward TNP-D-Tyr-Val-Gly was totally abolished, whereas HGAD dealkylating activity was unaffected. In order to demonstrate that there was also no change in the ability of HGAD to function together with PAM in amidating glycine-extended peptide substrates, an aliquot of the inhibited mixture was added to an assay solution containing fresh PAM and TNP-D-Tyr-Val-Gly. HPLC analysis confirmed that the HGAD sample withdrawn from the inactivation mixture produced the same degree of enhancement in production of TNP-D-Tyr-Val-NH_2 as an equivalent amount of fresh HGAD. Taken together, the experiments with olefinic inactivators demonstrate that HGAD is not required for turnover-dependent inactivation of PAM and that neither HGAD dealkylating activity toward α-hydroxybenzoylglycine nor its activity with PAM toward glycine-extended substrates is affected by inactivators of PAM. An additional implication is that any reactive intermediates produced by the action of PAM on olefin inhibitors either are not released in free solution or are not capable of inactivating HGAD.

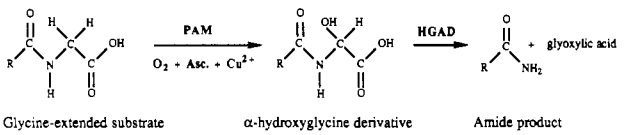

FIGURE 3: Minimal scheme depicting the formation of amide products by neurointermediate pituitary PAM and HGAD. The monooxygenase site of PAM catalyzes the conversion of the glycine-extended substrate to an α-hydroxyglycine derivative, which is then converted to amide by HGAD.

Discussion

In early work with the tripeptide substrate TNP-D-Tyr-Val-Gly, we discovered that while whole-cell extracts of neurointermediate pituitaries exhibit high amidating activity with this substrate, separation on phenyl-Sepharose produces two fractions. Each fraction exhibits very low amidation activity by itself, but when the fractions were recombined, the original activity is regained. From the more hydrophobic of these fractions PAM was purified to homogeneity. PAM purified in this manner exhibits all the characteristics of the monooxygenase-catalyzed amidation reaction reported by many different laboratories (Murthy et al., 1986; Mizuno et al., 1986; Kizer et al., 1986; Mehta et al., 1988). The more hydrophilic fraction contains a protein—which we later termed HGAD—which enhances the appearance of amide products during PAM turnover.

While examining several small-molecule substrates of PAM, we observed that crude pituitary fractions, high in PAM activity, also catalyze the conversion of α-hydroxybenzoylglycine to benzamide, with this dealkylation process occurring even in the absence of ascorbate and copper. However, using the same crude fractions, we determined that α-hydroxybenzoylglycine is not a competitive inhibitor for the amidation of either TNP-D-Tyr-Val-Gly or 4-nitrohippuric acid. This result led us to hypothesize the existence of a separate active site for α-hydroxybenzoylglycine dealkylation. This hypothesis was supported by subsequent purification work in which the two activities were completely separated from each other. Using dealkylation activity as a specific assay for HGAD, we developed a purification scheme which provides a 100-fold purification, although the final protein preparation is clearly not yet homogeneous.

The experimental data presented here are consistent with the catalytic sequence in the scheme shown in Figure 3. According to this scheme, soluble bovine neurointermediate PAM, in the presence of copper and ascorbate, catalyzes the oxygenative conversion of the glycine-extended substrate to an α-hydroxyglycine derivative, which is subsequently converted by HGAD to the final amide product.[2] Indeed, experiments in our laboratory have shown that purified neurointermediate PAM incubated with *N*-benzoylglycine produces an HPLC peak that coelutes with authentic α-hydroxybenzoylglycine. The kinetics of this reaction, as well as the large-scale isolation and structure determination of this intermediate PAM product, are currently in progress.

While we have no information yet regarding the intracellular location of HGAD in the neurointermediate pituitary, we have found soluble HGAD activity in highly purified bovine chromaffin granules where it is colocalized with soluble PAM. In bovine atrial granules, which contain high amounts of predominantly membrane-bound PAM (Eipper et al., 1988), we find that HGAD activity is also predominantly membrane bound (A. G. Katopodis, unpublished observations). As mentioned in the introduction, while the PAM gene encodes for an ca. 100-kDa protein, soluble forms of PAM have a molecular mass in the 40-kDa range. The physiological function of the protein encoded by the remainder of the PAM gene is currently unknown, although Eipper and co-workers have pointed out that the high homology predicted from the DNA sequence of the gene in several species suggests a physiological role for this fragment (Stoffers et al., 1989). The apparent molecular mass of 45 kDa reported here for HGAD is consistent with the intriguing possibility that, in neurointermediate pituitary, soluble HGAD may represent the protein encoded by the remainder of the PAM gene (Eipper et al., 1987). If this is indeed the case, then PAM and HGAD activities in neurointermediate pituitary may initially be present in a single protein translation product, with the monooxygenase and dealkylating reactions combining to catalyze neuropeptide C-terminal amidation.[3] Tissue-specific endoproteolytic cleavage may then produce two soluble and still functional proteins. Clearly, additional information will be required in order to determine whether a physiological function for a HGAD-type activity exists in tissues other than the neurointermediate pituitary. Furthermore, primary structure information will be needed to clarify the relationship between HGAD, PAM, and the translation product of the PAM gene.

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[2] Recently, Noguchi et al. (1989) have identified a 41-kDa peptide in rat brain which lowers the pH optimum for appearance of amide product by rat brain PAM. Since we have found that *nonenzymatic* breakdown of α-hydroxyglycine derivatives in solution occurs readily only at alkaline pH, the function of HGAD demonstrated here may provide a chemical explanation for these findings. Thus, as the pH is raised the α-hydroxyglycine species produced by PAM could be nonenzymatically converted to amide product even in the absence of HGAD, and the pH optimum for overall amidation would then reflect the pH sensitivities of both PAM turnover and stability of the α-hydroxyglycine. It is noteworthy that the internal pH of secretory granules has been reported to be maintained at 5.5–6.0 (Gainer et al., 1985), consistent with a physiological role for HGAD catalysis in amidation.

[3] It is important to consider the relationship between the results reported in this paper and those of Young and Tamburini (1989). Our results clearly establish that α-hydroxyglycine dealkylation is catalyzed by a separate enzyme, distinct from soluble PAM, and that purified soluble PAM does not exhibit dealkylase activity. Thus, the α-hydroxyglycine species is not a PAM reaction intermediate but rather represents an enzymatic product. We note that the rat thyroid medullary carcinoma PAM utilized by Young and Tamburini has a molecular mass of 75 kDa (Mehta et al., 1988), which is sufficiently large to represent a translation product containing both the monooxygenase and dealkylating catalytic sites.

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Articles

Proton Electron–Nuclear Double-Resonance Spectra of Molybdenum(V) in Different Reduced Forms of Xanthine Oxidase†

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ABSTRACT: Electron–nuclear double-resonance (ENDOR) spectra of protons coupled to molybdenum(V) in reduced xanthine oxidase samples have been recorded. Under appropriate conditions these protons may be studied without interference from protons coupled to reduced iron–sulfur centers. Spectra have been obtained for the molybdenum(V) species known as Rapid, Slow, Inhibited, and Desulfo Inhibited. Resonances corresponding to at least nine protons or sets of protons are observed for all four species, with coupling constants in the range 0.08–4 MHz. Most of these protons do not exchange when ^2H_2O is used as solvent. Additional protons giving couplings up to 40 MHz are also detected. These correspond to EPR-detectable protons studied in earlier work. The strongly coupled protons may be replaced by ^2H, through appropriate use of ^2H_2O or of ^2H-substituted substrates, with consequent disappearance of the ^1H resonances. In most cases the corresponding ^2H ENDOR features have also been observed. The nature of the various coupled protons is briefly discussed. Results permit specific conclusions to be drawn about the structures of the Inhibited and Desulfo Inhibited species. In particular, the data indicate that the aldehyde residue of the Inhibited species has been oxidized and that the four protons derived from the ethylene glycol molecule in the Desulfo Inhibited species are not all equivalent. Recent assignments [Edmondson, D. E., & D'Ardenne, S. C. (1989) *Biochemistry 28*, 5924–5930] of the weakly coupled protons in the latter species appear not to be soundly based. The possibility of obtaining more detailed structural information from the spectra is briefly considered. In agreement with the above workers, ^{14}N ENDOR was not detected, indicating the absence of a nitrogen ligand of molybdenum in the enzyme.

A variety of spectroscopic methods is available for the determination of local structural information on the environment of paramagnetic centers in metalloenzymes. Thus, in the case of molybdenum enzymes knowledge of the environment of the metal comes almost exclusively from EPR and EXAFS spectroscopy. In the particular case of the molybdenum iron–sulfur flavoprotein xanthine oxidase, extensive studies by these methods [see Bray (1988) for review] have provided quite detailed information on the structure of the molybdenum center and the nature of the catalytic reaction. A prime feature of these EPR results is the finding that the molybdenum center of the enzyme, at the molybdenum(V) level, can exist in a number of clearly defined states of ligation of the metal. Each

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