The source of the oxygen atom in the α -hydroxyglycine intermediate of the peptidylglycine α -amidating reaction

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Peptidylglycine α -amidating activity catalyses the oxidation of a C-terminally glycine-extended peptide to a desglycine α -amidated peptide at the expense of ascorbate and O_2 in the presence of Cu^{2+} . The reaction involves oxidative N-dealkylation within the terminal glycine residue, with retention of the glycine N atom and release of the remainder as glyoxylate. Recent studies by us and others have revealed that the reaction consists of two steps via a carbinolamide as an intermediate (peptidyl α -hydroxyglycine), and also that two separate enzymes derived from a common precursor protein catalyse these steps, formation of the carbinolamide and its conversion into α -amide and glyoxylate. As for the mechanism of carbinolamide formation, two distinct pathways can be considered: direct mono-oxygenation at the glycine α -C atom and dehydrogenation leading to an imine followed by hydration. To draw a distinction between them, we carried out the reaction with D-Tyr-Val-Gly as the substrate either in the $H_2^{18}O$ -enriched medium or under an atmosphere of $^{18}O_2$, and isolated the α -hydroxyglycine intermediate. The fast-atom-bombardment mass-spectral analysis demonstrated that the hydroxy O atom comes from O_2 , but not from H_2O , indicating that the α -hydroxylation should be a mono-oxygenase reaction.

INTRODUCTION

Peptidylglycine α -amidating activity (EC 1.14.17.3), which catalyses the oxidation of a C-terminally glycine-extended peptide to a desglycine α -amidated peptide, was originally detected in the pig pituitary in 1982 by Bradbury *et al.*, who used a synthetic tripeptide, D-Tyr-Val-Gly, as a model substrate [1]. The reaction occurs at the expense of ascorbate and molecular O_2 in the presence of Cu^{2+} [2–4] and involves oxidative N-dealkylation within the terminal glycine residue, with retention of the glycine N atom and release of the remainder as glyoxylate [1].

During the course of clarifying the relation between the neutral and alkaline pH optima of the α -amidating reaction, which have been observed in various rat tissues [5–7], we first identified in the rat brain a protein of 41 kDa (41K protein) that could stimulate the α -amidation reaction catalysed by an α -amidating enzyme of 36 kDa (36K enzyme) at neutral pH [8]. It has already been proposed that the α -amidating reaction proceeds in a two-step mechanism via, possibly, an α -hydroxy derivative in the terminal glycine residue [9,10]. In fact, we were able to detect an α -hydroxyglycine derivative in a reaction with horse serum α -amidating enzyme [11]. We then showed that α -amidation proceeded via an intermediate that was initially formed by the 36K enzyme and then converted into an amide by the 41K protein; the intermediate was stable at neutral to acidic pH values, but was non-enzymically cleaved into the product at alkaline pH [12]. Katopodis *et al.* also isolated an enzyme of 45 kDa, similar to the 41K protein, from the bovine pituitary, and showed that it catalysed dealkylation of α -hydroxybenzoylglycine to benzamide [13]. Subsequently several investigators have identified a number of enzymes, from various sources, that were able to dismutate peptidylglycine α -hydroxyglycine derivatives into an amidated peptide and glyoxylate [14–16]. Thus it was established that peptide amide is formed by the

sequential action of two separate enzymes, and the apparent alkaline pH optimum observed could be explained by a form of compromise between a nearly neutral pH optimum of the α -amidating enzyme (hydroxylating enzyme) and non-enzymic breakdown of the hydroxylated intermediate at alkaline pH [17]. Recently, the two enzymes have been shown to originate from a common precursor protein encoded by a single mRNA molecule [18–20]. A variety of names have been proposed for the first enzyme [9,14] and the second enzyme [13,14,16,20]. In the present paper we call the first enzyme peptidylglycine α -hydroxylase (PH) [9] and the second one peptidylamidoglycolate lyase (PGL, IUB Nomenclature Committee draft recommended name) [20].

On the basis of the similarities between the peptidylglycine α -amidating and dopamine β -hydroxylation reactions with respect to the cofactors required and the hydroxylated product, it has been suggested that the α -amidation is a mono-oxygenase reaction [4]. However, as has been pointed out by some investigators [21,22], two different mechanisms can be considered for the peptidylglycine α -hydroxylation, as illustrated in Scheme 1: direct mono-oxygenation at the glycine α -C atom to yield a carbinolamide (peptidylglycine α -hydroxyglycine, II) (pathway A), which subsequently dissociates to the products (IV and V), or dehydrogenation leading to an imine (III), followed by hydration, resulting in the same carbinolamide (pathway B). Pathway A corresponds to a typical mono-oxygenase reaction, whereas pathway B corresponds to an oxidase reaction involving tandem dehydrogenation of one substrate and one auxiliary reductant [21]. Although pathway A seems to be more plausible, pathway B cannot be ruled out. In fact, it has been shown that in the N-demethylation reaction of *NN*-dimethylaniline catalysed by chloroperoxidase or horseradish peroxidase, a reaction mechanism is partly operating in which a carbinolamine is formed via an imine cation followed by hydration [23,24]. Owing to rapid exchange of the carbonyl O atom of glyoxylate with water O

Abbreviations used: PH, peptidylglycine α -hydroxylase; PGL, peptidylamidoglycolate lyase; f.a.b.-m.s., fast-atom-bombardment mass spectrometry.

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Scheme 1. Possible mechanisms for the α -amidating reaction of C-terminally glycine-extended peptides

atoms, determination of the origin of the O atom incorporated into glycoylate is hampered. However, now that the α -hydroxyglycine intermediate has turned out to be isolable, experimental distinction between the two possible mechanisms has become possible.

In the present paper we describe characterization of PH- and PGL-catalysed reactions in some detail, isolation of the α -hydroxyglycine derivative of D-Tyr-Val-Gly used as a substrate and the determination of its chemical structure as D-Tyr-Val- α -hydroxy-Gly. Finally we demonstrate that the O atom incorporated into the hydroxy intermediate is derived from O_2 .

EXPERIMENTAL

Materials

The 36K enzyme exhibiting pH activity and the 41K protein exhibiting PGL activity (referred to below as the PH and PGL enzymes respectively) were purified from rat brain as previously described [11]. H_2^{18}O (99.0 atom %) was purchased from Isotec (Miami, OH, U.S.A.). $^{18}\text{O}_2$ (97.7 atom %; 0.5 litre at 0.2 MPa in a carbon-steel cylinder) is a product of Commissariat à l'Energie Atomique (Saclay, France). The other chemicals used were obtained from sources previously described [7,8,11].

Enzyme reactions

The 80 μl assay systems of PH and PGL activities employing ^{125}I -labelled D-Tyr-Val-Gly as a tracer have been previously detailed [8,11]. This time a scaled-up reaction system was designed for the purpose of characterization of the PH- and PGL-catalysed reactions. The system consisted of 200 μM -D-Tyr-Val-Gly, 3 μM - CuSO_4 , 0.2 mM-ascorbate, 50 μg of catalase/ml, 100 mM-Hepes/KOH buffer, pH 7.0, and indicated amounts of the purified PH and/or PGL enzyme in a final volume of 240 μl . After incubation at 37 $^\circ\text{C}$ for 3 h, the reaction was terminated by the addition of 1.5 ml of cold 2 mM-sodium phosphate buffer, pH 5.0. The solution was applied to a 3 ml column of SP-Sephadex C-50 equilibrated with 2 mM-sodium phosphate buffer, pH 5.0 [7,8,11]. The column was washed with three 3 ml portions of the same buffer and the washings were combined (the substrate fraction of SP-Sephadex). As is shown in the Results section, the remaining D-Tyr-Val-Gly and its α -hydroxyglycine derivative were recovered in this fraction. The amidated product (D-Tyr-Val-NH $_2$) was eluted with three 1 ml portions of 50 mM-sodium phosphate buffer, pH 5.0, containing 0.5 M-NaCl (the product fraction of SP-Sephadex). Both fractions were freeze-dried and subjected to h.p.l.c. analysis. Reverse-phase h.p.l.c. was performed on an Asahipak ODP-50 column (0.6 cm $\times$ 15 cm; Asahi Chemical,

Tokyo, Japan) with a 50 min linear gradient from 0 to 24 % (v/v) acetonitrile in 0.1 % (v/v) trifluoroacetic acid at a flow rate of 1 ml/min. Elution was monitored by measuring the absorbance at 280 nm.

In order to isolate the α -hydroxyglycine intermediate, a reaction mixture containing PH alone as enzyme (0.05–0.1 μg) and Mes/KOH buffer, pH 6.5, instead of Hepes/KOH buffer, pH 7.0, was incubated for 5 h under conditions otherwise the same as those stated above. Although the yield of intermediate varied with the activities of the PH enzyme employed, 5–15 μg of purified intermediate was usually obtained from ten incubations.

^{18}O experiments

We adopted a reaction system similar to that for isolating the intermediate in the following ^{18}O experiments.

For H_2^{18}O experiments, a concentrated solution of each chemical employed, i.e. Mes/KOH buffer, pH 6.5, ascorbate and CuSO_4 , was prepared with H_2^{18}O , freeze-dried once and then redissolved in H_2^{18}O . Enzymes (PH and catalase) were subjected to two cycles of freeze-drying and redissolving in H_2^{18}O . This treatment hardly affected the activities of PH and catalase.

An anaerobic train was constructed according to the description by Beinert *et al.* [25] in order to conduct anaerobic and $^{18}\text{O}_2$ experiments. The Ar gas used was ultrapure (99.9995 %, Nihon Sanso, Tokyo, Japan). The PH reaction under the $^{18}\text{O}_2$ atmosphere was performed with the aid of a specially designed vessel, as illustrated in Fig. 1. D-Tyr-Val-Gly was placed in the side arm (A) and other components were mixed in the main container (C). The reaction volume was scaled up to 2.4 ml (equivalent to ten incubations of the 240 μl reaction system) without changing the relative proportions of each component. After both parts had been assembled, the vessel was connected to the anaerobic train and to the $^{18}\text{O}_2$ -containing cylinder with vacuum hose as indicated. With the main cock of the cylinder closed, the gas phase within the vessel was replaced with Ar by four cycles of Ar-flushing and evacuation. After the final evacuation, stopcock S_2 was closed and $^{18}\text{O}_2$ was introduced into the vessel by opening the main cock of the cylinder. The reaction was started by tilting the vessel.

Mass spectrometry

Fast-atom-bombardment mass spectra (f.a.b.-m.s.) were run on a Jeol JMS-HX110 mass spectrometer at 10 kV accelerating voltage, scanned from m/z 0 to m/z 1500 in about 6 s, and recorded with a JMA-DA5500 data system. The atom-emission gun was operated at 3 kV for generation of the Xe fast atom beam. Freeze-dried samples were dissolved in $^3\text{H}_2\text{O}$, mixed with glycerol and then deposited on a stainless-steel stage.

Fig. 1. Diagrammatic sketch of the anaerobic vessel used for the $^{18}\text{O}_2$ experiment on the PH reaction

Features of the vessel include: A, side arm for D-Tyr-Val-Gly solution; C, main container (approx. 3.5 ml in volume), which was tightly attached to the upper side arm part by springs; S_1 and S_2 , $\frac{1}{2}$ stopcocks.

^1H -n.m.r. measurements

^1H -n.m.r. spectra were recorded at 30 °C on a Bruker AM 600 spectrometer equipped with an ASPECT 3000 computer. Freeze-dried samples were dissolved in $^2\text{H}_2\text{O}$ containing 0.1% trifluoro[^2H]acetic acid and 0.002% dioxan. Chemical shifts were reported in p.p.m. relative to dioxan resonance (3.70 p.p.m.).

RESULTS

Characterization of PH- and PGL-catalysed reactions

Fig. 2 shows a series of h.p.l.c. analyses of compounds produced from D-Tyr-Val-Gly during incubation with a combination of the PH and PGL enzymes (Fig. 2b) or with the PH enzyme alone (Fig. 2c). When the reaction mixture incubated with both the PH and PGL enzymes was directly analysed, two peaks with retention times of 28 and 31 min were observed (Fig. 2b1). By comparison with the elution positions of the authentic D-Tyr-Val-Gly and D-Tyr-Val-NH $_2$ (Fig. 2a), the 31 min peak was assigned to the remaining D-Tyr-Val-Gly and the 28 min peak was considered to indicate the product, D-Tyr-Val-NH $_2$. However, h.p.l.c. analyses of the substrate and product fractions eluted from the SP-Sephadex column revealed that the 28 min peak seen in Fig. 2(b1) contained two components, one being D-Tyr-Val-NH $_2$ (Fig. 2b3) and the other possibly an intermediate derived from D-Tyr-Val-Gly (Fig. 2b2). This was supported by the finding that the 28 min peak detected in the direct analysis of the reaction mixture incubated with the PH enzyme alone (Fig. 2c1) was mostly retained in the corresponding peak of the substrate fraction of SP-Sephadex (Fig. 2c2).

The possible intermediate was prepared in a large quantity as described in the Experimental section and was subjected to

Fig. 2. H.p.l.c. analyses of the products formed in the reactions catalysed by the combined PH and PGL system (b) and by PH alone (c)

Conditions for h.p.l.c. are described in the Experimental section. (a) Elution profile of D-Tyr-Val-NH $_2$ and D-Tyr-Val-Gly; the retention times were about 28 and 31 min respectively. (b) α -Amidating reactions were carried out in duplicate at pH 7 (100 mM-Hepes/KOH buffer) with 0.066 μg of PH and 0.426 μg of PGL as described in the Experimental section. One incubation was directly analysed by h.p.l.c. (1), the other was analysed after fractionation into the substrate (2) and product (3) fractions by SP-Sephadex. (c) PH reactions were carried out with 0.066 μg of PH under conditions otherwise the same as those in (b). (1) Direct analysis of the reaction mixture; (2) and (3) analysis of the substrate and product fraction of SP-Sephadex respectively. The early and late peaks, shown in (b1)–(c3) are referred to as the 28 min and 31 min peak respectively. Variations in the elution positions (± 1 min) and the shapes (sharpness) of peaks were considered to be due to variations in the injection volumes (varied from 0.5 to 2 ml) and also possibly to the column condition during each run. However, the deviations in the interval between the retention times of the 28 min and 31 min peaks were less than 0.2 min.

incubation with or without the PGL enzyme at pH 7. As shown in Fig. 3, D-Tyr-Val-NH $_2$ was produced from the isolated material in the presence of the PGL enzyme [compare (1) and (2) in Fig. 3a], whereas it remained almost unchanged in the absence of PGL (compare (1) and (2) in Fig. 3b). Thus the compound isolated was proved to be the intermediate that we had been searching for. The requirements for the amide formation from the intermediate catalysed by PGL are summarized in Table 1. This reaction did not require Cu^{2+} , O_2 or ascorbate as cofactors.

Chemical structure of the intermediate

The intermediate isolated was subjected to 600 MHz ^1H -n.m.r. and f.a.b.-m.s. analysis to determine its structure.

The chemical shifts of signals (in p.p.m.) and their assignments of D-Tyr-Val-Gly and the intermediate were as follows. D-Tyr-

Fig. 3. Conversion of the intermediate into D-Tyr-Val-NH₂ by PGL

(a) The intermediate isolated (about 5 µg) was incubated with 0.426 µg of PGL in 240 µl of 100 mM-Hepes/KOH buffer, pH 7.0, containing 0.2 mM-ascorbate and 3 µM-Cu²⁺ at 37 °C for 3 h. The substrate (1) and product (2) fractions eluted from the SP-Sephadex were analysed by h.p.l.c. (b) The intermediate was incubated without PGL under conditions otherwise the same as those in (a). (1) and (2) indicate the substrate and product fractions respectively.

Table 1. Requirements for amide formation from the intermediate

The complete system is the same as that in Fig. 3(a). The amounts of PGL enzyme used in Expt. 1 and 2 were 0.426 and 0.400 µg respectively. Anaerobic experiments were done under Ar with a gas-tight Warburg flask. Replacement of the gas phase in the flask was achieved by four cycles of evacuation and Ar-flushing with the aid of the anaerobic train. The percentage conversion of the intermediate into D-Tyr-Val-NH₂ was calculated from the areas under the corresponding peaks as shown in Fig. 3. Care was taken to make the injection volume at each h.p.l.c. run nearly equal (approx. 1 ml). The mean values in duplicate assays are presented. Abbreviation: DDC, diethyldithiocarbamate.

Reaction system	Conversion (%)	Conversion (% of that in complete system)
Expt. 1		
Complete	83	100
- PGL	9	11
- Ascorbate	81	97
Expt. 2		
Complete	77	100
- Cu ²⁺ and + DDC (100 µM)	74	96
- O ₂	76	99
- Ascorbate, - O ₂ - Cu ²⁺ and + DDC (100 µM)	70	91

Val-Gly: δ7.11 (2H, d, *J* = 8.5 Hz, Tyr C2, 6H), 6.83 (2H, d, *J* = 8.5 Hz, Tyr C3, 5H), 4.19 (1H, dd, *J* = 6.4, 9.2 Hz, Tyr C_(α)H), 3.96 (1H, d, *J* = 17.9 Hz, Gly C_(α)H), 3.94 (1H, d, *J* = 7.0 Hz, Val C_(α)H), 3.93 (1H, d, *J* = 17.9 Hz, Gly C_(α)H), 3.13 (1H, dd, *J* = 6.4, 13.8 Hz, Tyr C_(β)H), 3.01 (1H, dd, *J* = 9.2, 13.8 Hz, Tyr C_(β)H), 1.87 (1H, oct, *J* ≈ 6.8 Hz, Val C_(β)H), 0.69 (3H, d, *J* = 6.8 Hz, Val C_(γ)H) and 0.63 (3H, d, *J* = 6.6 Hz, Val C_(γ)H). The intermediate: δ7.11 (2H, d, *J* = 8.5 Hz, Tyr C2, 6H), 6.83 (2H, d, *J* = 8.5 Hz, Tyr C3, 5H), 5.46 (1H, s, Gly C_(α)H), 4.18 (1H, dd, *J* = 6.3, 9.3 Hz, Tyr

Fig. 4. Positive-ion f.a.b.-m.s. spectrum of the intermediate obtained from the reaction either in the air (a) or in the ¹⁸O₂ atmosphere (b)

The intermediate was isolated and analysed as described in the Experimental section. G stands for the glycerol peak.

C_(α)H), 3.89 (1H, d, *J* = 7.1 Hz, Val C_(α)H), 3.13 (1H, dd, *J* = 6.3, 13.8 Hz, Tyr C_(β)H), 3.01 (1H, dd, *J* = 9.3, 13.8 Hz, Tyr C_(β)H), 1.85 (1H, oct, *J* ≈ 6.9 Hz, Val C_(β)H), 0.69 (3H, d, *J* = 6.8 Hz, Val C_(γ)H) and 0.62 (3H, d, *J* = 6.7 Hz, Val C_(γ)H). The signals at 3.96 and 3.93 p.p.m. observed in the spectrum of the substrate, which were assigned to the two C_(α)-protons of glycine, were missing from the spectrum of the intermediate. Instead, a one-proton signal in the downfield region (5.46 p.p.m.) was observed, suggesting that one of the two C_(α)-protons of glycine was replaced by an electron-withdrawing functionality such as the hydroxy group. This was confirmed by f.a.b.-m.s. analysis of the intermediate. Fig. 4(a) shows a f.a.b.-m.s. spectrum of the intermediate isolated from the incubation in the air. The (M+H)⁺ ion peak at *m/z* 354 corresponds to a hydroxylated derivative of D-Tyr-Val-Gly. Thus we concluded that the structure of the intermediate is D-Tyr-Val-NH-CH(OH)-CO₂H.

¹⁸O experiments

To probe the origin of the hydroxy O atom incorporated into the intermediate, we carried out a series of experiments using H₂¹⁸O and ¹⁸O₂. First, an experiment was done to determine whether the α-hydroxylated intermediate exchanges O atoms with aqueous medium. The intermediate was incubated in 100 mM-sodium phosphate buffer, pH 5, prepared with 99% H₂¹⁸O for 30 min at 37 °C. Its mass spectrum showed an (M+H)⁺ ion peak at *m/z* 354, indicating that the hydroxy group introduced did not exchange with the solvent water (results not shown).

Reactions in the 99% H₂¹⁸O medium were carried out in air. The intermediate isolated showed an (M+H)⁺ ion peak at *m/z* 354 (results not shown). Then we carried out reactions in the ¹⁸O₂ atmosphere as described in the Experimental section and isolated the intermediate. Its mass spectrum (Fig. 4b) exhibited an (M+H)⁺ ion peak at *m/z* 356, greater by 2 mass units than that of the intermediate produced in air. An ion peak at *m/z* 337 was considered to be a fragment ion produced by cleavage of the hydroxy group introduced, because the differences in the mass

Scheme 2. Possible reaction pathways of the formation of carbinolamide (III) and its conversion into D-Tyr-Val NH_2 (VI) and glyoxylate (VII) from D-Tyr-Val-Gly (I), sequentially catalysed by PH and PGL enzymes

R stands for D-Tyr-Val moiety. The fact that the *pro-S*-H atom of the glycine residue is removed [34] is included. Modified after the Scheme shown in ref. [21].

units between the m/z 337 peak and the $(M+H)^+$ ion peaks of the $[^{16}O]$ - and $[^{18}O]$ -hydroxyglycine intermediates were 17 and 19 respectively (Fig. 4). Therefore it was concluded that the α -hydroxy O atom came from O_2 , and not from the water medium.

DISCUSSION

The identification of the source of the O atom incorporated into the α -hydroxyglycine intermediate is pivotal for defining the reaction mechanism of the α -amidating enzyme-catalysed *N*-dealkylation of peptidylglycine. The present study clearly demonstrated for the first time that the hydroxy O atom incorporated is derived from O_2 , indicating that the α -amidating reaction should be regarded as a mono-oxygenase reaction.

In Scheme 2, several possible pathways leading to carbinolamide (III) formation from D-Tyr-Val-Gly (I) are depicted. In the light of the results of the present study and by an analogy to the cytochrome *P*-450-catalysed reaction [26,27], the most probable reaction pathway catalysed by PH would be: initial reduction of O_2 by two electrons from ascorbate, with one O atom being reduced to water and the other being retained on the active-site Cu(II) as a Cu(II)-oxo [or Cu(II)-hydroxy] species, concurrent or subsequent H atom abstraction (or electron transfer and proton transfer) from the substrate, resulting in an α -C-centred radical (II), and finally recombination with the copper-bound O atom to form the carbinolamide (III). Then it dismutates into α -amide peptide (VI) and glyoxylate (VII) in the reaction catalysed by the second enzyme, PGL.

On the other hand, there is a good possibility that the radical intermediate (II) can transfer an electron to Cu(II) leading to an imine formation (IV). As an alternative pathway to form the imine, a mechanism via a chelate, in which active-site copper coordinates with the amide N atom (V), has been proposed based on model studies with Cu(II)-peptide-like complexes [21]. In any case, if the imine is to be formed, it must react with a water molecule to transform into the carbinolamide. The $H_2^{18}O$ ex-

periment showed that the O atom from aqueous medium was never incorporated into the carbinolamide. Thus the intermediacy of the imine structure seems to be unlikely, although the possibility cannot be completely excluded that an O_2 -derived water molecule bound to the active-site copper is specifically delivered to the imine intermediate [21].

A radical intermediate (II) is postulated to intervene between the substrate (I) and the carbinolamide (III). For dopamine β -hydroxylase, generation of a substrate-derived radical intermediate has been well established [28,29], but there has been controversy regarding the O-insertion chemistry and the nature of the hydroxylating agent [30]. May and co-workers have favoured a mechanism analogous to the cytochrome *P*-450-dependent reaction [31,32]. On the other hand, Millar & Klinman have proposed a mechanism in which O-O homolysis [from a putative Cu(II)-OOH species] and C-H homolysis (from substrate) occur in a concerted fashion to give a Cu(II)-alkoxide complex [29]. To date very little is known about this intermediary step of the PH reaction. Kizer *et al.* [33] have reported that the primary isotope effect on the velocity of the α -amidating reaction is fairly large (> 5) when the α -C H atoms of the glycine residue are replaced by deuterium. This observation implies that the abstraction of the H atom at the glycine α -C atom also takes place in the PH reaction, resulting in the formation of a radical intermediate. A detailed kinetic study is required to obtain a more complete picture of this step.

Note added in proof (received 17 February 1992)

Recently, Zabriskie *et al.* [35] have reported results that also support the direct α -carbon hydroxylation mechanism.

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REFERENCES

1. Bradbury, A. F., Finnie, M. D. A. & Smyth, D. G. (1982) *Nature* (London) **298**, 686–688
2. Bradbury, A. F. & Smyth, D. G. (1983) in *Peptides 1982* (Bláha, K. & Maloň, P., eds.), pp. 381–386, Walter de Gruyter, Berlin
3. Bradbury, A. F. & Smyth, D. G. (1983) *Biochem. Biophys. Res. Commun.* **112**, 372–377
4. Eipper, B. A., Mains, R. E. & Glembotski, C. C. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5144–5148
5. Emeson, R. B. (1984) *J. Neurosci.* **4**, 2604–2613
6. Eipper, B. A., Myers, A. C. & Mains, R. E. (1985) *Endocrinology* (Baltimore) **116**, 2497–2504
7. Noguchi, M., Takahashi, K. & Okamoto, H. (1988) *Tohoku J. Exp. Med.* **156**, 191–207
8. Noguchi, M., Takahashi, K. & Okamoto, H. (1989) *Arch. Biochem. Biophys.* **275**, 505–513
9. Bradbury, A. F. & Smyth, D. G. (1987) *Eur. J. Biochem.* **169**, 579–584
10. Young, S. D. & Tamburini, P. P. (1989) *J. Am. Chem. Soc.* **111**, 1933–1934
11. Tajima, M., Iida, T., Yoshida, S., Komatsu, K., Namba, R., Yanagi, M., Noguchi, M. & Okamoto, H. (1990) *J. Biol. Chem.* **265**, 9602–9605
12. Takahashi, K., Okamoto, H., Seino, H. & Noguchi, M. (1990) *Biochem. Biophys. Res. Commun.* **169**, 524–530
13. Katopodis, A. G., Ping, D. & May, S. W. (1990) *Biochemistry* **29**, 6115–6120
14. Perkins, S. N., Husten, E. J. & Eipper, B. A. (1990) *Biochem. Biophys. Res. Commun.* **171**, 926–932
15. Perkins, S. N., Husten, E. J., Mains, R. E. & Eipper, B. A. (1990) *Endocrinology* (Baltimore) **127**, 2771–2778
16. Suzuki, K., Shimoi, H., Iwasaki, Y., Kawahara, T., Matsuura, Y. & Nishikawa, Y. (1990) *EMBO J.* **9**, 4259–4265
17. Bradbury, A. F. & Smyth, D. G. (1991) *Trends Biochem. Sci.* **16**, 112–115
18. Kato, I., Yonekura, H., Tajima, M., Yanagi, M., Yamamoto, H. & Okamoto, H. (1990) *Biochem. Biophys. Res. Commun.* **172**, 197–203
19. Eipper, B. A., Perkins, S. N., Husten, E. J., Johnson, R. C., Keutmann, H. T. & Mains, R. E. (1991) *J. Biol. Chem.* **266**, 7827–7833
20. Katopodis, A. G., Ping, D., Smith, C. E. & May, S. W. (1991) *Biochemistry* **30**, 6189–6194
21. Reddy, K. V., Jin, S.-J., Arora, P. K., Sfeir, D. S., Maloney, S. C. F., Urbach, F. L. & Sayre, L. M. (1990) *J. Am. Chem. Soc.* **112**, 2332–2340
22. Ranganathan, D. & Saini, S. (1991) *J. Am. Chem. Soc.* **113**, 1042–1044
23. Kedderis, G. L. & Hollenberg, P. F. (1984) *J. Biol. Chem.* **259**, 3663–3668
24. Kedderis, G. L., Rickert, D. E., Pandey, R. N. & Hollenberg, P. F. (1986) *J. Biol. Chem.* **261**, 15910–15914
25. Beinert, H., Orme-Johnson, W. H. & Palmer, G. (1978) *Methods Enzymol.* **54**, 111–132
26. White, R. E. & Coon, M. J. (1980) *Annu. Rev. Biochem.* **49**, 315–356
27. Coon, M. J. & White, R. E. (1980) in *Metal Ion Activation of Dioxigen* (Spiro, T. G., ed.), pp. 73–123, John Wiley and Sons, New York
28. Fitzpatrick, P. F., Flory, D. R., Jr. & Villafranca, J. J. (1985) *Biochemistry* **24**, 2108–2114
29. Miller, S. M. & Klinman, J. P. (1985) *Biochemistry* **24**, 2114–2127
30. Stewart, L. C. & Klinman, J. P. (1988) *Annu. Rev. Biochem.* **57**, 551–592
31. Wimalasena, K. & May, S. W. (1987) *J. Am. Chem. Soc.* **109**, 4036–4046
32. Wimalasena, K. & May, S. W. (1989) *J. Am. Chem. Soc.* **111**, 2729–2731
33. Kizer, J. S., Bateman, R. C., Jr., Miller, C. R., Humm, J., Busby, W. H., Jr. & Youngblood, W. W. (1986) *Endocrinology* (Baltimore) **118**, 2262–2267
34. Ramer, S. E., Cheng, H., Palcic, M. M. & Vederas, J. C. (1988) *J. Am. Chem. Soc.* **110**, 8526–8532
35. Zabriskie, T. M., Cheng, H. & Vederas, J. C. (1991) *J. Chem. Soc. Chem. Commun.*, 571–572

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