Kinetic and inhibition studies on substrate channelling in the bifunctional enzyme catalysing C-terminal amidation

Allison B. MOORE^{*1} and Sheldon W. MAY^{*2}

School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332-0400, U.S.A.

A series of experiments has been conducted to investigate the possibility that substrate channelling might occur in the bifunctional forms of enzymes carrying out C-terminal amidation, a post-translational modification essential to the biological activity of many neuropeptides. C-terminal amidation entails sequential action by peptidylglycine mono-oxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5), with the mono-oxygenase catalysing conversion of a glycine-extended pro-peptide into the corresponding α -hydroxyglycine derivative, which is then converted by the lyase into amidated peptide plus glyoxylate. Since the mono-oxygenase and lyase reactions exhibit tandem reaction stereospecificities, channelling of the α -hydroxy intermediate might occur, as is the case for some other multifunctional enzymes. Selective inhibition of the mono-oxygenase domain by competitive ester inhibitors, as well as mechanism-based mono-oxygenase inactivation by the novel olefinic inhibitor 5-acetamido-4-oxo-6-phenylhex-2-enoate (N-

acetylphenylalanyl acrylate), has little to no effect on the kinetic parameters of the lyase domain of the AE from *Xenopus laevis*. Similarly, inhibition of the lyase domain by the potent dioxo inhibitor 2,4-dioxo-5-acetamido-6-phenylhexanoate has little effect on the activity of the monooxygenase domain in the bifunctional enzyme. A series of experiments on intermediate accumulation and conversion were also carried out, along with kinetic investigations of the reactivities of the monofunctional and bifunctional forms of PAM and PGL towards substrates and inhibitors. Taken together, the results demonstrate the kinetic independence of the mono-oxygenase and lyase domains, and provide no evidence for substrate channelling between these domains in the bifunctional amidating enzyme.

Key words: lyase, mono-oxygenase, peptidylamidoglycolate, peptidylglycine.

INTRODUCTION

Many peptide hormones require a C-terminal amide moiety for full biological activity [1,2]. The enzymic formation of C-terminally amidated peptides from their glycine-extended precursors is a two-step process catalysed by two enzymic activities, namely peptidylglycine mono-oxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5), as shown in Scheme 1 [3–7]. The mono-oxygenase, PAM, catalyses the production of the α -hydroxyglycine derivative of the glycine-extended precursor in a process dependent upon ascorbate, copper and molecular oxygen. The lyase, PGL, mediates the conversion of the α -hydroxyglycine derivative into C-terminally amidated peptide and glyoxylate.

Amidating enzymes, which have been isolated from various tissues and species [8–12], exist as monofunctional proteins

which contain only the mono-oxygenase or the lyase, as well as bifunctional proteins containing both enzymic activities. Previous work has shown that, by the expression of truncated peptide amidating enzyme cDNAs, which encode only one enzymic domain, monofunctional mono-oxygenase or lyase protein can be produced, and that the catalytic activities of these monofunctional proteins are independent of each other [3,4,13–18]. Furthermore, Husten et al. demonstrated that endoprotease treatment of a bifunctional amidating enzyme (AE) increased the mono-oxygenase activity 2–2.5-fold [18]. Cleavage between the mono-oxygenase and lyase domains was not required for this increase in mono-oxygenase activity, but removal of a non-catalytic C-terminal region was sufficient for the increase, consistent with catalytic independence of the two domains.

Sequential reactions in biochemical processes are often catalysed by either multienzyme complexes, in which the enzymic

Scheme 1 Enzymic formation of C-terminally amidated peptides from their glycine-extended precursors

Abbreviations used: PAM, peptidylglycine mono-oxygenase (EC 1.14.17.3); PGL, peptidylamidoglycolate lyase (EC 4.3.2.5); CAD, the multifunctional enzyme which catalyses the first three reactions of pyrimidine biosynthesis; AE, bifunctional amidating enzyme; dAE, AE with a truncated transmembrane domain; TNP, trinitrophenyl; p.f.u., plaque forming unit; Sf9, *Spodoptera frugiperda* cells; Sf9-BEV, Sf9-baculovirus expression vector system; DMPD, *N,N*-dimethylphenylene-1,4-diamine; SP-, sulphopropyl; N-Ac-Phe acrylate, *N*-acetylphenylalanyl acrylate; N-Ac-Phe pyruvate, 2,4-dioxo-5-acetamido-6-phenylhexanoate.

¹ Present address: Hercules Incorporated, Research Center, 500 Hercules Road, Wilmington, DE 19808-1599, U.S.A.

² To whom correspondence should be addressed (e-mail sheldon.may@chemistry.gatech.edu).

activities are found on separable proteins, or multifunctional enzymes, which contain all the requisite enzymic activities on a single polypeptide chain. In both types of multifunctional systems, reaction intermediates may be 'channelled' from one reaction site to the next without entering the bulk solution. Such substrate channelling has been reported to occur in the tryptophan synthase system [19–21] and the polyketide biosynthetic enzymes [22]. CAD, the multifunctional enzyme which catalyses the first three reactions of pyrimidine biosynthesis, exhibits efficient [23], but possibly incomplete [24], substrate channelling. Channelling is sometimes quite leaky, as in the lumazine synthase–riboflavin synthase complex [25] and the methylene-tetrahydrofolate dehydrogenase–cyclohydrolase system [26,27]; in both cases approximately half of the intermediate is channelled, with the remaining intermediate being released into the bulk solution. In the cases of bifunctional GlmU protein [28] and phosphoribosyl-anthranilate isomerase–indoleglycerol-phosphate synthase from *Escherichia coli* [29,30], substrate channelling either does not occur or is extremely inefficient.

We have previously demonstrated that PAM and PGL exhibit tandem reaction stereospecificities in carrying out the two requisite steps of C-terminal amidation, with PAM producing exclusively α -hydroxyglycine moieties of absolute configuration (*S*), and PGL being reactive only toward (*S*)- α -hydroxyglycines [5,31]. This stereochemical consistency raises the possibility that substrate channelling might occur between the mono-oxygenase and lyase domains of the bifunctional forms of enzymes catalysing C-terminal peptide amidation. We have therefore carried out a series of experiments designed to investigate this issue. Selective targeting or quenching of each individual enzymic activity, as well as kinetic experiments on intermediate accumulation and conversion, were utilized to investigate the possibility of substrate channelling between the mono-oxygenase and lyase domains. Our results clearly confirm the kinetic and functional independence of the mono-oxygenase and lyase domains, and we see no evidence for substrate channelling between the domains of AE. These results are inconsistent with the recent suggestions of a possible role for a bivalent metal atom in the transport of the α -hydroxy intermediate from the mono-oxygenase to the lyase active site [32] or the potential involvement of the lyase domain in release of the α -hydroxy intermediate into solution in AEs [33].

EXPERIMENTAL

Materials

All buffers, ascorbate oxidase (1390 units/mg) and TNM-FH insect medium were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N,N*-Dimethylphenylene-1,4-diamine (DMPD) and L-ascorbic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Bovine liver catalase (65 000 units/mg) was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and Coomassie Plus protein assay mix was from Pierce (Rockford, IL, U.S.A.). EX-CELL 401 insect medium was obtained from JRH Biosciences (Lenexa, KS, U.S.A.), and heat-inactivated fetal-bovine serum was from Atlanta Biologicals (Norcross, GA, U.S.A.). Antibiotic–antimycotic (100 $\times$) and Pluronic F-68 (10%) solutions were from Life Technologies (Gaithersburg, MD, U.S.A.). Acetyl-L-Tyr-L-Val-Gly was from Peninsula Laboratories (Belmont, CA, U.S.A.), and D-Tyr-L-Val-Gly was from Bachem (Torrance, CA, U.S.A.). Ac-L-Phe-Gly [31], Ac-L-Phe-L-Phe-Gly [31], Ac-L-Phe-OCH₂CO₂H [31], [(4-methoxybenzoyl)oxy]acetic acid [39] and the pyruvate-extended *N*-acetyl amino acids [16] were synthesized

as described previously. *N*-Acetylphenylalanyl acrylate (*N*-Ac-Phe acrylate; 5-acetamido-4-oxo-6-phenylhex-2-enoate) was generously given by Dr. Corinne E. Mounier-Lee of our laboratory. All other chemicals were of the highest quality available.

Methods

HPLC analyses were performed on a Spherisorb C₈ reversed-phase column (Alltech), using an LDC Constametric III system outfitted with an LDC Spectromonitor 3100 variable-wavelength detector. Product quantification was by standard curves based on peak height. The elution buffer for trinitrophenyl (TNP)-labelled compounds was acetonitrile/water/trifluoroacetic acid (44:56:0.1, by vol.) with detection at 344 nm [4]. The elution buffer for (4-nitrobenzyl)thionylacetic acid was 50 mM ammonium acetate buffer (pH 5.5)/acetonitrile (17:3,v/v) with detection at 280 nm. PAM and PGL assays were carried out as described previously [3,4,39,40] with the following modifications. In the continuous PAM assay, a 295-nm-UV-cut-off filter was used, and the increase in absorbance at 514 nm due to the oxidation of DMPD was recorded every 2 or 5 s for a total period of 120 or 200 s. Values for $V_{\max}$ and K_m were obtained from the inverse plots by using the least-squares-fit program EnzymeKinetics (Trinity Software, Campton, NH, U.S.A.). Inhibition parameters were calculated using the curve-fitting program SigmaPlot (Jandel Scientific, San Rafael, CA), to perform an iterative fit of the data to the appropriate form of the Michaelis–Menten equation. Dixon plots ($1/v$ versus $[I]$) were used to determine inhibition type.

Insect cell culture

Spodoptera frugiperda (Sf9) cells (A.T.C.C. CRL1711) were maintained at 27 °C in monolayer and suspension cultures in TNM-FH medium supplemented with 10% fetal-bovine serum and 10 ml/l antibiotic–antimycotic solution (100 $\times$). The cells were subcultured twice a week. After infection with a recombinant baculovirus, the medium was replaced by the serum-free medium, EX-CELL 401, supplemented with the above antibiotics and 0.1% Pluronic F-68.

The recombinant baculovirus vectors for the monofunctional and bifunctional *Xenopus laevis* skin amidating enzymes were obtained from Dr. Y. Nishikawa [36–38]. Amplification was accomplished by infecting Sf9 cultures at a multiplicity of infection of 0.1 plaque-forming unit (p.f.u.)/cell and expression by infecting at a multiplicity of infection of 10 p.f.u./cell. Media was collected 4 days post infection by centrifugation.

Enzyme isolations

Four forms of the *X. laevis* amidating enzymes were expressed. As can be seen in Table 1, enzyme activity levels in the culture media were lower for the full AE (*X. laevis* AE) than for the monofunctional enzymes (*X. laevis* PAM and PGL) and the AE with a truncated transmembrane domain (*X. laevis* dAE), consistent with the results of Iwasaki et al. [37]. While the protein levels are similar across the enzyme forms, the specific activity is consistently much lower for the full AE.

X. laevis PAM, PGL and dAE were isolated at 4 °C as follows. The medium supernatant was diluted (2:1) with deionized water and applied to a sulphopropyl (SP)-Sephacrose Fast Flow column (1.6 cm $\times$ 90 cm, Pharmacia) equilibrated with 50 mM sodium Mes buffer, pH 6.2. Elution was achieved with a linear NaCl gradient (0–0.5 M). The concentrated material was applied to a

Table 1 Typical expression levels in the medium supernatant*

Enzyme form	Protein level (mg/l)	Activity (munits/ml)†	Specific activity (munits/mg)
<i>X. laevis</i> PAM	130	8.0	62
<i>X. laevis</i> PGL	72	13	180
<i>X. laevis</i> AE	59		
PAM domain		0.74	13
PGL domain		1.4	23
<i>X. laevis</i> dAE‡	58		
PAM domain		1.2	21
PGL domain		4.4	76

* The medium supernatant is the resulting supernatant after the collected media has been centrifuged to remove cellular debris.

† One munit of PAM activity is defined as the amount of enzyme required to produce 1 nmol of TNP-D-Tyr-Val-(S)- α -hydroxy-Gly/min at 37 °C; one munit of PGL activity is defined as the amount of enzyme required to produce 1 nmol of TNP-D-Tyr-Val-NH₂/min at 37 °C.

‡ Bifunctional amidating enzyme with a truncated transmembrane domain [37].

Mono Q HR 5/5 column (Pharmacia) which was equilibrated in 50 mM Tris/HCl buffer, and eluted with a stepwise NaCl gradient. The concentrated Mono Q pool was loaded on to a Superose 6 or 12 HR 10/30 column (Pharmacia) and eluted with 50 mM Hepes buffer, pH 7.0, containing 200 mM NaCl. Active fractions were pooled, concentrated and stored in 50% (v/v) ethylene glycol at -20 °C and used as purified enzyme. The full *X. laevis* AE was isolated by a modification of this procedure. The medium supernatant dilution and SP-Sepharose chromatography were carried out in 50 mM sodium acetate buffer, pH 5.5. The concentrated SP-Sepharose pool was loaded on to two Superose 12 HR 10/30 columns connected in series and eluted with 50 mM Hepes buffer, pH 7.0, containing 200 mM NaCl. Bovine pituitary PAM and PGL were isolated from the neuro-intermediate tissue of mature bovine pituitaries as described previously [3,4].

Bifunctional time-course experiments

Assays were performed in 100 mM sodium Mes buffer, pH 6.5, containing 100 μ M TNP-D-Tyr-Val-Gly, 4 μ M CuSO₄, 1 mg/ml catalase, 4 mM L-ascorbic acid and enzyme in a total volume of 3 ml at 37 °C. A 100 μ l aliquot was withdrawn from the incubation mixture at the appropriate time, quenched with 10 μ l of 3 M HClO₄, centrifuged, and analysed quantitatively by HPLC for TNP-D-Tyr-Val- α -hydroxy-Gly and TNP-D-Tyr-Val-NH₂. In experiments where the *in situ* generation of TNP-D-Tyr-Val- α -hydroxy-Gly was desired, ascorbate oxidase was used to quench the mono-oxygenase reaction. The conversion of the TNP-D-Tyr-Val- α -hydroxy-Gly into amide was monitored by HPLC.

Selective time-dependent inactivation of the mono-oxygenase domain of the AE by *N*-Ac-Phe acrylate

Selective inactivation of mono-oxygenase domain of the AE by *N*-Ac-Phe-acrylate was determined by the dilution method [34]. Inactivation reactions in 100 mM sodium Mes buffer, pH 6.5, containing 4 μ M CuSO₄, 4 mM ascorbate, 1 mg/ml catalase, 0.2% Triton X-100, 60 mM KI, purified AE, and various concentrations of *N*-Ac-Phe acrylate in a total volume of 540 μ l were incubated at 37 °C. The inactivation reactions were initiated by the addition of *N*-Ac-Phe acrylate. Aliquots of 10 μ l were withdrawn from the inactivation mixtures at 100 s intervals and

diluted into 0.5 ml of the appropriate assay solution. Assay solutions for residual mono-oxygenase activity contained 4 μ M CuSO₄, 4 mM ascorbate, 1 mg/ml catalase, 60 mM KI and an excess amount of TNP-D-Tyr-Val-Gly (26 μ M) in 100 mM sodium Mes buffer, pH 6.5. Assay solutions for residual lyase activity contained an excess amount of TNP-D-Tyr-Val- α -hydroxy-Gly (100 μ M) in 100 mM sodium Mes buffer, pH 6.5. After 30 min of incubation at 37 °C, the residual assay reactions were quenched by the addition of 50 μ l of 3 M HClO₄, the mixtures being centrifuged then analysed quantitatively by HPLC. Kitz and Wilson plots were used to determine the values of K_i and k_{inact} .

The effect of *N*-Ac-Phe acrylate on the full AE reaction time course was investigated by pre-incubating the full AE in the absence or presence of 100 μ M *N*-Ac-Phe acrylate as described above for the dilution method. An aliquot (100 μ l) was withdrawn from the appropriate inactivation mixture after 30 min and added to the appropriate assay solution. Mono-oxygenase activity assays contained 4 μ M CuSO₄, 4 mM ascorbate, 1 mg/ml catalase, 60 mM KI and 26 μ M TNP-D-Tyr-Val-Gly in 100 mM sodium Mes buffer, pH 6.5, in a total volume of 2.5 ml. Lyase activity assays contained 100 μ M TNP-D-Tyr-Val- α -hydroxy-Gly in 100 mM sodium Mes buffer, pH 6.5, in a total volume of 2.5 ml. The assay mixtures were incubated at 37 °C. Aliquots (100 μ l) were withdrawn over time, quenched with 10 μ l of 3 M HClO₄, centrifuged, and analysed quantitatively by HPLC for TNP-D-Tyr-Val- α -hydroxy-Gly and TNP-D-Tyr-Val-NH₂.

RESULTS

Recombinant amidating enzymes

Four forms of the *X. laevis* amidating enzymes were expressed in the Sf9/BEV system: the full AE (*X. laevis* AE), the AE with a truncated transmembrane domain (*X. laevis* dAE) and the monofunctional mono-oxygenase and lyase (*X. laevis* PAM and PGL). Kinetic parameters for these enzymes as well as the corresponding monofunctional bovine enzymes are shown in Table 2. It is evident from the Table that the kinetic parameters for the mono-oxygenase and lyase activities of the various enzyme forms towards peptide substrates are quite similar. The full AE, *X. laevis* AE, generally has lower K_m values for the entire glycine-extended substrate series. This is consistent with the results of Husten et al. in which the rat AE had a slightly lower K_m value for Ac-Tyr-Val-Gly (14 μ M) than that of the monofunctional mono-oxygenase (18 μ M) [18]. Kinetic experiments were also carried out to compare the K_m values of the *in vivo* electron donor, L-ascorbate [9], and that of the alternative electron donor, DMPD, towards the various enzyme forms. The K_m values obtained using peptide substrates for both electron donors were approx. 1–2 mM for all forms of the mono-oxygenase enzyme.

The first potent inhibitors for bovine pituitary PGL were recently introduced by this laboratory [16]; these compounds are 2,4-dioxo-5-acetamidoalkanoic acid derivatives (or, more simply, pyruvate-extended *N*-acetylamino acid derivatives). As shown in Table 2, these dioxo compounds are potent competitive inhibitors for the lyase domain of both the monofunctional and bifunctional *X. laevis* skin enzymes. 2,4-Dioxo-5-acetamido-6-phenylhexanoate (*N*-Ac-Phe pyruvate) is the most potent of these dioxo lyase inhibitors, with K_i values in the 50 nM range. In addition, these pyruvate-extended *N*-acetylamino acid derivatives also bind to the ascorbate site in ascorbate-dependent mono-oxygenases, such as PAM and dopamine β -mono-oxygenase, giving rise to inhibition that is competitive with respect to ascorbate and uncompetitive with respect to the organic substrates [16].

Table 2 PAM and PGL kinetic parameters

(a) Mono-oxygenase activity											
Enzyme form ...		<i>X. laevis</i> AE			<i>X. laevis</i> dAE		<i>X. laevis</i> PAM			Bovine PAM	
Substrate	Inhibitor	K_m^* (μ M)	$V_{max}^\dagger$ (nmol/min per μ g)	$K_i^\ddagger$ (μ M)	K_m (μ M)	V_{max} (nmol/min per μ g)	K_m (μ M)	V_{max} (nmol/min per μ g)	$K_i^\ddagger$ (μ M)	K_m (μ M)	V_{max} (nmol/min per μ g)
Ac-Tyr-Val-Gly§		11	0.61		31	220	17	34		24	790
TNP-D-Tyr-Val-Gly§		1.8	0.278		4.4	10.1	3.2	39.0		2.9	4.4
Ac-Phe-Gly§		2.7	0.70		8.7	11.2	13	46		12	51
Ac-Phe-Phe-Gly§		3.7	0.82		6.9	11.5	8	38		1.2**	3.3**
Ascorbate†††		1.0	0.035				1.7	8.62		0.79‡‡‡	57.4‡‡‡
DMPD§§		1.4	1.22				2.4	6.0		1.4‡‡‡	52‡‡‡
TNP-D-Tyr-Val-Gly***	Ac-Phe pyruvate			137 (U)					59 (U)		
	Ac-Tyr pyruvate			340 (U)					133 (U)		
	Ac-Met pyruvate			78 (U)					52 (U)		
Ascorbate†††	Ac-Phe pyruvate			48 (C)					32 (C)		
	Ac-Tyr pyruvate			210 (C)					150 (C)		
	Ac-Met pyruvate			59 (C)					43 (C)		
(4-Nitrobenzyl)thioacetic acid		680	0.0071		410	0.0272	900	0.49		150	0.186
(b) Lyase activity											
Enzyme form ...		<i>X. laevis</i> AE			<i>X. laevis</i> dAE		<i>X. laevis</i> PGL			Bovine PGL	
Substrate	Inhibitor	K_m^* (μ M)	$V_{max}^\dagger$ (nmol/min per μ g)	$K_i^\ddagger$ (μ M)	K_m (μ M)	V_{max} (nmol/min per μ g)	K_m (μ M)	V_{max} (nmol/min per μ g)	$K_i^\ddagger$ (μ M)	K_m (μ M)	V_{max} (nmol/min per μ g)
TNP-D-Tyr-Val-OH-Gly		18	0.35		14	4.3	19	2.8		33	11.9
Ac-Phe-pyruvate				0.071 (C)					0.049 (C)		
	Ac-Tyr-pyruvate			0.92 (C)					0.55 (C)		
	Ac-Met-pyruvate			4.8 (C)					3.2 (C)		

* All values had calculated S.E.M. values of 5–13%, except for bovine PAM towards TNP-D-Tyr-Val-Gly (17%).
† All values had calculated S.E.M. values of 2–5%; steady-state kinetic measurements were carried out at atmospheric oxygen concentration; monooxygenase V_{max} (apparent) differs from the 'true' V_{max} by the factor $1 + K_{O_2}/[O_2]$.
‡ All values had calculated S.E.M. values of 3–11% and represent the average of at least duplicate experiments; inhibition type, competitive (C) or uncompetitive (U), was determined by a Dixon plot of $1/v$ versus $[I]$.
§ Determined by the continuous spectrophotometric assay method with DMPD as the electron donor.
** From [31].
†† Determined by the discontinuous HPLC assay method with TNP-D-Tyr-Val-Gly as the peptide substrate.
‡‡ From [35].
§§ Determined by the continuous spectrophotometric assay method with Ac-Tyr-Val-Gly as the peptide substrate.
*** Determined by the discontinuous HPLC assay method with TNP-D-Tyr-Val-Gly as the variable substrate and L-ascorbate as the electron donor.
††† Determined by the discontinuous HPLC assay method with L-ascorbate as the variable substrate and TNP-D-Tyr-Val-Gly as the peptide substrate.

Table 3 Effect of competitive PAM inhibitors on bifunctional PGL*

Enzyme	K_m (μ M)	V_{max} (nmol/min per μ g)
<i>X. laevis</i> dAE	14 ± 1	4.3 ± 0.1
<i>X. laevis</i> dAE with 1.26 mM <i>N</i> -Ac-L-Phe-OCH ₂ CO ₂ H	13.6 ± 0.9	3.69 ± 0.08
<i>X. laevis</i> dAE with 1.10 mM 4-CH ₃ O-C ₆ H ₄ -CO ₂ CH ₂ CO ₂ H	15 ± 3	3.9 ± 0.3
<i>X. laevis</i> AE	18 ± 2	0.35 ± 0.01
<i>X. laevis</i> AE with 1.26 mM <i>N</i> -Ac-L-Phe-OCH ₂ CO ₂ H	16.8 ± 0.7	0.262 ± 0.004
<i>X. laevis</i> AE with 1.10 mM 4-CH ₃ O-C ₆ H ₄ -CO ₂ CH ₂ CO ₂ H	19 ± 2	0.32 ± 0.01

* K_m and V_{max} of the bifunctional PGL domains were measured using TNP-D-Tyr-Val- α -hydroxy-Gly as the substrate.

However, pyruvate-extended *N*-acetylamino acid derivatives possessing hydrophobic side chains are much more potent inhibitors of the lyase than of the mono-oxygenase; thus the data in Table 2 demonstrate that *N*-Ac-Phe pyruvate is a 1000-fold more potent an inhibitor for *X. laevis* PGL than for *X. laevis* PAM.

The *X. laevis* PAMs are also capable of catalysing the alternate mono-oxygenase reaction, sulphoxidation (Table 2), as previously reported for the bovine enzyme by Katopodis and May [34]. This sulphoxidation reaction is much less facile than the corresponding α -hydroxylation reaction that occurs with glycine-extended peptide substrates. As is evident from Table 2, the $V_{max,app}$ values are 24-fold (bovine PAM) to 370-fold (*X. laevis* dAE) greater for TNP-D-Tyr-Val-Gly than for the sulphoxidation of (4-nitrobenzyl)thioacetic acid, and the $V_{max,app}/K_m$ ratios of TNP-D-Tyr-Val-Gly/(4-nitrobenzyl)thioacetic acid range from 1.2×10^3 (bovine PAM) to 35×10^3 (*X. laevis* dAE). Since the chemical reactivity of sulphur toward mono-oxygenase-catalysed oxygenation would be expected to be much greater than that of carbon [39,40], this probably reflects altered substrate positioning in the active site. In this regard, we note that PAM exhibits high S_2 -subsite stereospecificity, with both substrate reactivity and inhibitor binding being markedly decreased by the presence of either a D-amino acid residue or a (*R*)-mandelyl residue at the P_2 position [31] {the nomenclature for the substrate residues, or analogues (P_1 , P_2 , etc.) and the corresponding enzyme subsites (S_1 , S_2 , etc.) is the nomenclature originally proposed by Schechter and Berger [41]}. In the case of the sulphide substrate, the positioning of the aromatic substituent is obviously altered from that of the side chain of an L-amino acid side chain such as L-Phe, resulting in diminished reactivity.

Reactivity of the lyase domain in the presence of mono-oxygenase inhibitors

Efficient substrate channelling has been shown to occur in the tryptophan synthase system [19–21] and, in this system, binding of a substrate analogue at one site can have a dramatic effect on the kinetic parameters for catalysis at the other site [42]. We have previously shown that esters are potent competitive inhibitors of PAM; typical K_i values are 45 μ M for the peptide inhibitor *N*-Ac-L-Phe-OCH₂CO₂H [31] and 160 μ M for the aryl ester inhibitor [(4-methoxybenzoyl)oxy]acetic acid [34]. These potent ester inhibitors were therefore used to investigate the effect of inhibitor binding at the mono-oxygenase domain on the sub-

Figure 1 Time course of production of the α -hydroxy intermediate and final amide product by the full AE

AE was incubated in 100 mM sodium Mes buffer, pH 6.5, containing 100 μ M TNP-D-Tyr-Val-Gly, 4 μ M CuSO₄, 1 mg/ml catalase, 4 mM L-ascorbic acid and enzyme in a total volume of 3 ml at 37 °C (A). The inset to (A) shows the first 15 min of the reaction. The reaction with the addition of 37.5 units of ascorbate oxidase at 20 min (B). Aliquots were withdrawn over time and analysed quantitatively by HPLC for TNP-D-Tyr-Val- α -hydroxy-Gly (●) and TNP-D-Tyr-Val-NH₂ (▲).

strate reactivity of the lyase domain of AEs. As shown in Table 3, inhibition of the mono-oxygenase domain by these ester inhibitors has no effect on K_m and very little effect on V_{max} for both forms of the AE.

Intermediate accumulation and product formation

The tryptophan synthase system, which exhibits efficient substrate channelling [19–21], generates a reaction intermediate that is undetectable in the bulk solution, and no lag is observed in the time course of formation of the final product. Moreover, it has been demonstrated that intermediate added to the bulk solution is converted into the final product at a rate that is much lower than that of the channelled intermediate [19]. Therefore a series of experiments was carried out in order to explore the possibility of substrate channelling in the bifunctional *X. laevis* amidating enzyme, AE.

Figure 1(A) shows the normal time course of the reaction of the substrate TNP-D-Tyr-Val-Gly with *X. laevis* AE. The α -

Figure 2 Comparison of the rate of *in-situ*-generated and added TNP-D-Tyr-Val- α -hydroxy-Gly conversion by the full AE

AE was incubated in 100 mM sodium Mes buffer, pH 6.5, containing either 130 μ M TNP-D-Tyr-Val-Gly (continuous line) or 5 μ M TNP-D-Tyr-Val- α -hydroxy-Gly (broken line), 4 μ M CuSO₄, 1 mg/ml catalase and 4 mM L-ascorbic acid in a total volume of 3 ml at 37 °C. Aliquots were withdrawn over time and analysed quantitatively by HPLC. For the *in situ* generation of TNP-D-Tyr-Val- α -hydroxy-Gly, AE was incubated for 20 min with 130 μ M TNP-D-Tyr-Val-Gly and then ascorbate oxidase was added to quench the mono-oxygenase reaction. The concentration of TNP-D-Tyr-Val- α -hydroxy-Gly was 5 μ M at the point at which the ascorbate oxidase was added. The concentrations of the *in-situ*-generated TNP-D-Tyr-Val- α -hydroxy-Gly (▲) and the externally added TNP-D-Tyr-Val- α -hydroxy-Gly (●) were measured over time.

hydroxy intermediate is easily detected and accumulates in the bulk solution, and there is an initial lag period before amide production (Figure 1A, inset). Clearly, this kinetic behaviour is sharply distinct from that observed in systems with substrate channelling.

As shown in Figure 1(B), initial experiments demonstrated that addition of ascorbate oxidase to the normally functioning bifunctional amidating system immediately quenches mono-oxygenase turnover via oxidation of the requisite electron donor, L-ascorbate, without affecting catalytic turnover at the lyase domain. It is evident from the Figure that, subsequent to ascorbate oxidase addition, no further production of TNP-D-Tyr-Val- α -hydroxy-Gly occurs, whereas formation of the final amide product continues unabated until all of the α -hydroxy intermediate is consumed. Therefore this ascorbate oxidase addition protocol was utilized to allow a quantitative comparison of the rates of conversion of the '*in-situ*-generated' and 'added' intermediate to the final amide product by the bifunctional enzyme. It is evident from the results of this experiment, shown in Figure 2, that the rates of conversion of the '*in-situ*-generated' and 'added' intermediate to the final amide product by the bifunctional enzyme are identical. Moreover, in contrast with the normal reaction time course, conversion of the externally added α -hydroxy intermediate to amide commences immediately upon the addition of AE, with no lag whatsoever evident in the reaction time course. Taken together, these results establish kinetic behaviour for the AE which contrasts sharply with that reported for the tryptophan synthase system [19].

Mono-oxygenase-catalysed sulfoxidation in the presence of a lyase inhibitor

Since the lyase domain does not process the sulfoxide product of mono-oxygenase-catalysed sulfoxidation, catalytic turnover of sulphide substrates reflects only the activity of the mono-

Figure 3 Time course of final product formation by the full AE

AE was incubated in 100 mM sodium Mes buffer, pH 6.5, containing 2.28 mM (4-nitrobenzyl)thioacetic acid, 4 μ M CuSO₄, 1 mg/ml catalase, and 4 mM L-ascorbic acid in a total volume of 3 ml at 37 °C. Aliquots were withdrawn over time and analysed quantitatively by HPLC. The final sulfoxide product formation in the absence (●) or presence (○) of 1.4 μ M *N*-Ac-Phe pyruvate is shown; at this concentration lyase activity is completely inhibited. Also shown is the time course of production of the amidated product, TNP-D-Tyr-Val-NH₂, from TNP-D-Tyr-Val-Gly by the full AE in the absence (▲) or presence (△) of 1.1 μ M *N*-Ac-Phe pyruvate (data taken from Figure 4 in [16]). A 10-fold higher enzyme concentration was present in sulfoxidation experiments, since turnover is less facile than for amidation.

oxygenase domain. Accordingly, we investigated the effect of inhibition of the lyase domain on sulfoxidation; if the two domains are truly independent, the presence of a competitive lyase inhibitor should have no effect on enzymic sulfoxidation. As can be seen in Figure 3, the presence of *N*-Ac-Phe-pyruvate under conditions where the activity of the lyase domain is completely inhibited has only a small effect on the rate of enzymic sulfoxidation. In contrast, as shown in the Figure, the presence of *N*-Ac-Phe pyruvate at this concentration virtually abolishes formation of the amidated product, TNP-D-Tyr-Val-NH₂, from TNP-D-Tyr-Val-Gly (results taken from Figure 4 in [16]).

N-Ac-Phe acrylate as a selective mono-oxygenase inactivator

It has been previously reported that the olefinic substrate analogues, 4-phenylbut-3-enoic acid, *trans*-benzoylacrylic acid and monoethyl fumarate, have been shown to be mechanism-based inactivators of PAM [34,43]. On the basis of these results, the novel olefinic compound *N*-Ac-Phe acrylate was synthesized in our laboratory as a potential PAM inactivator capable of enhanced binding at the active site owing to secondary binding interactions with the *N*-Ac-Phe moiety [44]. *N*-Ac-Phe acrylate is a time-dependent inactivator of the mono-oxygenase domain of the AE with weighted linear regression of the double-reciprocal plot of $1/k_{\text{obs}}$ versus $1/[N\text{-Ac-Phe acrylate}]$ yielding a K_i of 360 ± 20 μ M and a k_{inact} of 1.47 ± 0.07 min⁻¹, whereas it does not inactivate the lyase domain.

We then proceeded to utilize *N*-Ac-Phe acrylate to investigate the effect of mechanism-based inactivation of the mono-oxygenase domain on the catalytic activity of the lyase domain. In these experiments, the full AE reaction time course was monitored after 30 min preincubation in the absence or presence of 100 μ M *N*-Ac-Phe acrylate. The residual activity of each domain was followed by either reaction with TNP-D-Tyr-Val-Gly (mono-oxygenase activity) or reaction with TNP-D-Tyr-Val-(*S*)- α -hydroxy-Gly (lyase activity). Preincubation with *N*-Ac-Phe acrylate

has virtually no effect on the rate of lyase-catalysed production of TNP-D-Tyr-Val-NH₂ from TNP-D-Tyr-Val- α -hydroxy-Gly (initial rate of 1.1 μ M/min in both cases). Parallel experiments demonstrated that mono-oxygenase activity is completely abolished by preincubation with *N*-Ac-Phe acrylate, with production of TNP-D-Tyr-Val- α -hydroxy-Gly from TNP-D-Tyr-Val-Gly being virtually undetectable. These results represent further evidence for the kinetic independence of the two domains of the AE.

DISCUSSION

Four forms of the recombinant *X. laevis* amidating enzymes were expressed in the baculovirus system and investigated in the present study; two of these forms are AEs, possessing both mono-oxygenase and lyase domains, while the remaining two forms were the soluble mono-oxygenase and lyase enzymes respectively. The kinetic parameters of all of these enzyme forms towards a series of substrates and inhibitors were determined (Table 2). While some differences in K_m , V_{max} and K_i were observed, these kinetic experiments revealed no evidence for kinetic interdependence or co-operativity between the mono-oxygenase and lyase domains of the AEs.

Our results further demonstrate that selective inhibition of either the mono-oxygenase or lyase domain has only a minimal effect on the kinetic parameters of the other domain of the AE. Thus the initial rate of production of TNP-D-Tyr-Val- α -hydroxy-Gly by the mono-oxygenase domain in the AE is unaffected when the lyase domain is completely inhibited by *N*-Ac-Phe pyruvate [16]. Similarly, inhibition of the mono-oxygenase domain by competitive ester inhibitors has no effect on K_m and very little effect on V_{max} for both forms of the AE. Furthermore, inactivation of the mono-oxygenase domain by the potent mechanism-based inactivator *N*-Ac-Phe acrylate, has no effect on the rate of the lyase-catalysed production of TNP-D-Tyr-Val-NH₂ from TNP-D-Tyr-Val- α -hydroxy-Gly.

If the two domains are truly independent, the presence of a competitive lyase inhibitor should not affect the rate of sulphoxidation by the *X. laevis* bifunctional enzyme. This alternative mono-oxygenase reaction was chosen because the sulfoxide product is not acted upon by the lyase. Thus this system serves as an excellent substrate probe for mono-oxygenase turnover. Saturation of the lyase with *N*-Ac-Phe pyruvate, under conditions where the lyase domain is completely inhibited, has only a very small effect on enzymic sulphoxidation. Taken together, these results demonstrate that the mono-oxygenase and lyase domains of bifunctional *X. laevis* AE are kinetically independent.

When substrate channelling which entails direct transfer of an intermediate occurs in a multifunctional enzyme or multi-enzyme complex, there is no detectable lag between the sequential reactions [45] as in the tryptophan synthase system [19]. In contrast, in systems where substrate channelling does not occur, the product of the first reaction must find the second active site by free diffusion, resulting in a distinct lag [46]. This is the case for phosphoribosyl-anthranilate isomerase/indoleglycerol-phosphate synthase, where the intermediate, *o*-carboxyphenylamino-deoxyribulose phosphate, accumulates in the bulk solution, and there is a lag before the second reaction begins [29]. In the case of the amidating enzymes described here, the initial lag period before amide production, and the presence of the α -hydroxy intermediate in the bulk solution (Figure 1A), suggest that either there is no direct substrate channelling or that substrate channelling is extremely inefficient. An initial lag period before amide production and accumulation of the α -hydroxy intermediate are also evident in the data presented by Consalvo et al. for the

reaction time course of dansyl-Tyr-Val-Gly with recombinant rat AE [47].

An additional criterion which can be used to determine the extent of substrate channelling in a multifunctional system is an examination of the rate of conversion of added intermediate. Thus, in the bifunctional GlmU protein, externally added radiolabelled intermediate equilibrates in the bulk solution with the endogenous intermediate [28], and in the phosphoribosyl-anthranilate isomerase/indoleglycerol-phosphate synthase system, addition of intermediate to the reaction mixture eliminates the initial lag in product formation [48]. These results have been used to confirm that substrate channelling does not occur in these two enzyme systems. In contrast with these results, in the tryptophan synthase system, which exhibits efficient substrate channelling, the added radiolabelled indole intermediate is turned over at least 25-fold more slowly than indole produced at the α active site [19]. The results reported here demonstrate that externally added intermediate is turned over by the lyase domain at a rate which is identical with that for the *in-situ*-generated intermediate. This result is consistent with the absence of a direct channelling mechanism between the mono-oxygenase and lyase domains of AEs.

Substrate channelling between the domains of multifunctional enzymes may serve as a protection mechanism for labile intermediates along a reaction pathway. In pyrimidine biosynthesis, carbamoyl phosphate is efficiently channelled between sequential active sites in CAD [23], and this labile intermediate has a half-life of approx. 40 min under physiological conditions [24,49]. In the formiminotransferase-cyclodeaminase system, substrate channelling is beneficial, because the concentration of *N*-5-formiminotetrahydrofolate, the labile intermediate with no other known metabolic function, is decreased [50]. Adenosine 5'-phosphosulphate, the labile intermediate of the bifunctional ATP sulphurylase-adenosine-5'-phosphosulphate kinase system, is channelled with almost 100% channelling efficiency, and Lyle et al. suggested that channelling is a protection mechanism for the labile intermediate which is subject to both biochemical (sulphohydrolases) and spontaneous chemical degradation [51]. We have previously demonstrated the stability of acyl- α -hydroxyglycines under physiological conditions [16], and thus substrate channelling of the α -hydroxy intermediate between the mono-oxygenase and lyase active sites of AEs would not be required for intermediate protection.

Bell et al. have recently investigated the possible structural and functional role of zinc in peptide amidation and determined by X-ray-absorption spectroscopy and 4-(2-pyridylazo)resorcinol analysis that recombinant rat AE contains a tightly bound Zn(II) [32]. They have further suggested that the 'extra' bivalent-metal-binding site observed by Prigge et al. in the crystal structure of the PAM catalytic core [52] could be occupied by zinc in AE, and that this zinc atom may play a role in the transport of the α -hydroxy intermediate from the mono-oxygenase to the lyase active site [32]. Francisco et al. have also suggested the possibility of substrate channelling between the mono-oxygenase and lyase domains of the AE [33]. Isotope-effect studies on monofunctional and bifunctional mono-oxygenases indicate that release of the α -hydroxy intermediate occurs more slowly from the bifunctional mono-oxygenase, indicating potential involvement of the lyase domain in release of the α -hydroxy intermediate into solution [33]. However, our results do not indicate any mechanism of transport between the two active sites other than free diffusion through the bulk solution, and support the possibility that Zn plays either a structural role or is involved in PGL catalysis.

In different organisms, the enzyme systems catalysing sequential reactions may exist as individual monofunctional enzymes,

multienzyme complexes or multifunctional enzymes. This has led to the idea that multifunctional enzymes may have evolved from small monofunctional proteins through multienzyme complexes to the covalent linkage of the C-terminus of one protein to the N-terminus of another, thus forming the multifunctional enzyme [46]. This evolutionary hypothesis would be supported if it could be demonstrated that individual enzymes which become covalently linked bestow a selective advantage to the organism, and biochemical and physicochemical investigations may reveal the molecular basis for such an advantage [46]. However, investigations to date have not yielded an explanation for the *in vivo* existence of both mono- and bi-functional AEs. We note that the absence of substrate channelling opens up the possibility that, under physiological conditions with various peptides, steady-state or transient accumulation of α -hydroxy-glycinated peptides may occur. Whether or not such α -hydroxy-glycinated peptides exhibit distinct biological activities, as is apparently the case for the glycine-extended precursor of gastrin₁₇ [53,54], remains to be determined.

We thank Dr. Y. Nishikawa, Ciba-Geigy, Takarazuka, Japan, for generously providing the baculovirus vectors, Dr. C. Li for initial studies of the truncated bifunctional enzyme, Dr. C. E. Mounier-Lee for generously supplying *N*-Ac-Phe acrylate, Dr. C. D. Oldham for many technical discussions and recommendations, and the National Institutes of Health (grant no. GM 40540) for financial support.

REFERENCES

- Bradbury, A. F. and Smyth, D. G. (1987) *Biosci. Rep.* **7**, 907–916
- Eipper, B. A. and Mains, R. E. (1988) *Annu. Rev. Physiol.* **50**, 333–344
- Katopodis, A. G., Ping, D. and May, S. W. (1990) *Biochemistry* **29**, 6115–6120
- Katopodis, A. G., Ping, D., Smith, C. E. and May, S. W. (1991) *Biochemistry* **30**, 6189–6194
- Ping, D., Katopodis, A. G. and May, S. W. (1992) *J. Am. Chem. Soc.* **114**, 3998–4000
- Tajima, M., Iida, T., Yoshida, S., Komatsu, K., Namba, R., Yanagi, M., Noguchi, M. and Okamoto, H. (1990) *J. Biol. Chem.* **265**, 9602–9605
- Perkins, S. N., Husten, E. J. and Eipper, B. A. (1990) *Biochem. Biophys. Res. Commun.* **171**, 926–932
- Bradbury, A. F., Finnie, M. D. A. and Smyth, D. G. (1982) *Nature (London)* **298**, 686–688
- Eipper, B. A., Stoffers, D. A. and Mains, R. E. (1992) *Annu. Rev. Neurosci.* **15**, 57–85
- Mizuno, K., Sakata, J., Kojima, M., Kangawa, K. and Matsuo, H. (1986) *Biochem. Biophys. Res. Commun.* **137**, 984–991
- Murthy, A. S. N., Mains, R. E. and Eipper, B. A. (1986) *J. Biol. Chem.* **261**, 1815–1822
- Mehta, N. M., Gilligan, J. P., Jones, B. N., Bertelsen, A. H., Roos, B. A. and Birnbaum, R. S. (1988) *Arch. Biochem. Biophys.* **261**, 44–54
- Iwasaki, Y., Kawahara, T., Shimoi, H., Suzuki, K., Ghisalbal, O., Kangawa, K., Matsuo, H. and Nishikawa, Y. (1991) *Eur. J. Biochem.* **201**, 551–559
- Shimoi, H., Kawahara, T., Suzuki, K., Iwasaki, Y., Jeng, A. Y. and Nishikawa, Y. (1992) *Eur. J. Biochem.* **209**, 189–194
- Eipper, B. A., Perkins, S. N., Husten, E. J., Johnson, R. C., Keutmann, H. T. and Mains, R. E. (1991) *J. Biol. Chem.* **266**, 7827–7833
- Mounier, C. E., Shi, J., Sirimanne, S. R., Chen, B.-H., Moore, A. B., Gill-Woznichak, M. M., Ping, D. and May, S. W. (1997) *J. Biol. Chem.* **272**, 5016–5023
- Husten, E. J. and Eipper, B. A. (1991) *J. Biol. Chem.* **266**, 17004–17010
- Husten, E. J., Tausk, F. A., Keutmann, H. T. and Eipper, B. A. (1993) *J. Biol. Chem.* **268**, 9709–9717
- Anderson, K. S., Miles, E. W. and Johnson, K. A. (1991) *J. Biol. Chem.* **266**, 8020–8033
- Creighton, T. E. (1970) *Eur. J. Biochem.* **13**, 1–10
- Dunn, M. F., Aguilar, V., Brzovic, P., Drewe, Jr., W. F., Houben, K. F., Leja, C. A. and Roy, M. (1990) *Biochemistry* **29**, 8598–8607
- McDaniel, R., Ebert-Khosla, S., Hopwood, D. A. and Khosla, C. (1993) *Science* **262**, 1546–1550
- Irvine, H. S., Shaw, S. M., Paton, A. and Carrey, E. A. (1997) *Eur. J. Biochem.* **247**, 1063–1073
- Christopherson, R. I. and Jones, M. E. (1980) *J. Biol. Chem.* **255**, 11381–11395
- Kis, K. and Bacher, A. (1995) *J. Biol. Chem.* **270**, 16788–16795
- Pelletier, J. N. and MacKenzie, R. E. (1994) *Biochemistry* **33**, 1900–1906
- Smith, D. D. S. and MacKenzie, R. E. (1983) *Can. J. Biochem. Cell Biol.* **61**, 1166–1171
- Gehring, A. M., Lees, W. J., Mindiola, D. J., Walsh, C. J. and Brown, E. D. (1996) *Biochemistry* **35**, 579–585
- Bisswanger, H., Kirschner, K., Cohn, W., Hager, V. and Hansson, E. (1979) *Biochemistry* **18**, 5946–5953
- Eberhard, M., Tsai-Pflugfelder, M., Bolewska, K., Hommel, U. and Kirschner, K. (1995) *Biochemistry* **34**, 5419–5428
- Ping, D., Mounier, C. E. and May, S. W. (1995) *J. Biol. Chem.* **270**, 29250–29255
- Bell, J., Ash, D. E., Snyder, L. M., Kulathila, R., Blackburn, N. J. and Merkle, D. J. (1997) *Biochemistry* **36**, 16239–16246
- Francisco, W. A., Merkle, D. J., Blackburn, N. J. and Klinman, J. P. (1998) *Biochemistry* **37**, 8244–8252
- Katopodis, A. G. and May, S. W. (1990) *Biochemistry* **29**, 4541–4548
- Li, C., Oldham, C. D. and May, S. W. (1994) *Biochem. J.* **300**, 31–36
- Iwasaki, Y. and Nishikawa, Y. (1993) *U.S. Pat.* 5,196,316
- Iwasaki, Y., Shimoi, H., Saiki, H. and Nishikawa, Y. (1993) *Eur. J. Biochem.* **214**, 811–818
- Mizuno, K., Ohsuye, K., Wada, Y., Fuchimura, K., Tanaka, S. and Matsuo, H. (1987) *Biochem. Biophys. Res. Commun.* **148**, 546–552
- Wimalasena, K. and May, S. W. (1987) *J. Am. Chem. Soc.* **109**, 4036–4046
- Guengerich, F. P. and MacDonald, T. L. (1984) *Acc. Chem. Res.* **17**, 9–16
- Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
- Brzovic, P. S., Ngo, K. and Dunn, M. F. (1992) *Biochemistry* **31**, 3831–3839
- Bradbury, A. F., Mistry, J., Roos, B. A. and Smyth, D. G. (1990) *Eur. J. Biochem.* **189**, 363–368
- Shi, J. (1997) M.Sc. Thesis, Georgia Institute of Technology, Atlanta
- Schmincke-Ott, E. and Bisswanger, H. (1980) in *Multifunctional Proteins*, (Bisswanger, H. and Schmincke-Ott, E., eds.), pp. 1–29, John Wiley and Sons, New York
- Kirschner, K. and Bisswanger, H. (1976) *Annu. Rev. Biochem.* **45**, 143–166
- Consalvo, A. P., Young, S. D. and Merkle, D. J. (1992) *J. Chromatogr.* **607**, 25–29
- Creighton, T. E. (1970) *Biochem. J.* **120**, 699–707
- Allen, Jr., C. M. and Jones, M. E. (1964) *Biochemistry* **3**, 1238–1247
- Paquin, J., Baugh, C. M. and MacKenzie, R. E. (1985) *J. Biol. Chem.* **260**, 14925–14931
- Lyle, S., Ozeran, J. D., Stanczak, J., Westley, J. and Schwartz, N. B. (1994) *Biochemistry* **33**, 6822–6827
- Prigge, S. T., Kolhekar, A. S., Eipper, B. A., Mains, R. E. and Amzel, L. M. (1997) *Science* **278**, 1300–1305
- Seva, C., Dickinson, C. J. and Yamada, T. (1994) *Science* **265**, 410–412
- Todisco, A., Takeuchi, Y., Seva, C., Dickinson, C. J. and Yamada, T. (1995) *J. Biol. Chem.* **270**, 28337–28341

Received 30 September 1998/9 March 1999; accepted 8 April 1999