***NN*-Dimethyl-1,4-phenylenediamine as an alternative reductant for peptidylglycine α -amidating mono-oxygenase catalysis**

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C-terminal α -amidation is a structural feature essential to the biological activity of many peptide hormones. Peptidylglycine α -amidating mono-oxygenase (PAM; EC 1.14.17.3) catalyses conversion of glycine-extended peptide hormone precursors into their corresponding α -hydroxyglycine derivatives. This reaction is the first step in the C-terminal amidation process. We report here that in the presence of molecular O₂, copper and PAM substrate, *NN*-dimethyl-1,4-phenylenediamine (DMPD) serves as the requisite electron donor for the mono-oxygenase, being oxidized in the process to a stable and highly chromophoric cation radical. By monitoring the rate of increase in absorbance at 515 nm, PAM activity can be easily followed. This provides a spectrophotometric assay for PAM, which represents the first continuous assay reported for this enzyme. DMPD-supported PAM-catalysed mono-oxygenation exhibits normal Michaelis-Menten kinetic behaviour. Steady-state kinetic studies estab-

lished that both the ascorbate-supported and DMPD-supported PAM reactions exhibit apparent 'Ping Pong' kinetics. In addition, both electron donors give rise to similar pH profiles and identical inhibition patterns towards known competitive inhibitors of PAM. The stoichiometry between formation of the DMPD cation radical and the α -hydroxyglycine PAM product was determined to be 2:1, the value expected for a mono-oxygenase-catalysed reaction. The optimum pH for the DMPD-supported continuous PAM assay was found to be about 5.5. The major advantage of this assay over all previously reported methods is that it is continuous; thus accurate initial rates are easily obtained. Moreover, unlike previous assay methods, ¹²⁵I-labelled or chromophorically modified substrates are not required. Kinetic parameters for a broad range of PAM substrates and inhibitors have been successfully obtained using this assay.

INTRODUCTION

Neuropeptide hormones are synthesized as biologically inactive precursors which are converted into mature bioactive forms by a series of post-translational modifications [1]. One very prevalent prohormone peptide-processing event, essential to the bioactivity of many neuropeptides, is C-terminal amidation. Recently, we and others have demonstrated that formation of peptide amides from their glycine-extended precursors is not catalysed by a single enzyme, as previously thought, but is a two-step process resulting from the sequential action of two enzyme activities [2–6]. The first enzyme, peptidylglycine α -amidating mono-oxygenase (PAM; EC 1.14.17.3), catalyses formation of the α -hydroxyglycine derivative of the glycine-extended precursor in a process dependent on ascorbate, copper and molecular O₂. The second enzyme, peptidylamidoglycolate lyase (PGL, EC 4.3.2.5), catalyses breakdown of the α -hydroxyglycine derivative to produce glyoxylate and the C-terminal amidated peptide [2].

PAM has been identified in, and isolated from, a variety of tissues [7–10]. As expected, the mono-oxygenase activity of PAM is strictly dependent on a reducing agent; reduced ascorbate supports PAM activity, and the amount of ascorbate consumption is approximately equimolar to the amount of glycine-extended peptide conversion [7,11]. It has also been shown that dopamine and noradrenaline can replace ascorbic acid as electron donors for the PAM reaction [12].

The first assay method for PAM, reported by Bradbury et al. in 1982 [13], utilized ¹²⁵I-D-Tyr-Val-Gly as the peptide substrate. Subsequently, several other PAM assay methods have been reported [14–17]. All of these assay methods are based on

monitoring the PAM-catalysed conversion of a radiolabelled or chromophorically modified synthetic substrate to the corresponding product. PAM activity is then determined by first separating the enzyme product using h.p.l.c. or other chromatography methods, and then quantifying radioactivity or absorbance. Thus all extant PAM assays suffer the major disadvantage of being discontinuous methods, which precludes accurate and reproducible determinations of true initial rates. Moreover, none of these methods can be directly used for comparative kinetic evaluations of a series of substrates or turnover-dependent inactivators, unless labelled derivatives are first synthesized for each substrate or inactivator of interest.

Recently, Wimalasena and co-workers [18] demonstrated that the well-characterized reducing agent, *NN*-dimethyl-1,4-phenylenediamine (DMPD) [18–20], supports the activity of dopamine β -mono-oxygenase, leading to a spectrophotometric assay for that enzyme. We report here that DMPD is also capable of functioning as the requisite electron donor for PAM turnover, thus providing a spectrophotometric assay for PAM catalysis based on monitoring DMPD oxidation. This represents the first continuous assay reported for PAM, and it provides a convenient method for obtaining initial rates for a broad range of PAM substrates and inhibitors for this important enzyme.

EXPERIMENTAL

Materials

Ascorbic acid, L-mimosine (β -[*N*-(3-hydroxy-4-pyridone)]- α -aminopropionic acid) and all buffers were obtained from Sigma Chemical Co. DMPD, hippuric acid and 4-nitrohippuric acid were from Aldrich. Ac-Tyr-Val-Gly (Ac-YVG) was from Pen-

Abbreviations used: PAM, peptidylglycine α -amidating mono-oxygenase; DMPD, *NN*-dimethyl-1,4-phenylenediamine; PGL, peptidylamidoglycolate lyase; Ac-YVG, *N*-acetyl-L-Tyr-Val-Gly; TNP-YVG, *N*-trinitrophenyl-D-Tyr-Val-Gly; TNP-YV(OH)G, *N*-trinitrophenyl-D-Tyr-Val- α -hydroxyGly.

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insula Laboratories, Inc. All other peptides were from Bachem (Torrance, CA, U.S.A.). Bovine liver catalase was from Boehringer-Mannheim. *N*-Trinitrophenyl-D-Tyr-Val-Gly (TNP-YVG), 4-methoxyhippuric acid and [(4-methoxybenzoyl)-oxy]acetic acid were synthesized as previously described [21].

Methods

H.p.l.c. 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. All the spectrophotometric measurements were obtained with a Hewlett-Packard 8451A diode-array spectrophotometer equipped with a temperature-regulated cell compartment. Kinetic parameters (K_m , V_{max} and K_i) for various substrates and inhibitors were calculated with the computer program, EnzymeKinetics (Trinity Software), using a non-linear fit of the data to the Michaelis-Menten equation.

PAM isolation

PAM isolations were performed at 4 °C. In a typical isolation, 100 frozen bovine pituitaries (Pel-Freez Biologicals) were dissected and the neurointermediate fractions were homogenized with a hand-held homogenizer in 400 ml of 50 mM Tris/HCl, pH 8.5. The solution was centrifuged (45000 *g* for 25 min), and the supernatant was subjected to $(NH_4)_2SO_4$ precipitation. The 0–40 % satd. $(NH_4)_2SO_4$ pellet was suspended in 60 ml of 100 mM Tris/HCl buffer, pH 7, containing 200 mM NaCl, and loaded on a column packed with 10 ml of chelating Sepharose (Pharmacia) charged with copper by passing through it 45 ml of 10 mg/ml $CuSO_4$ in water and equilibrated with the same buffer. The column was flushed with starting buffer and eluted with a 0–40 mM imidazole hydrochloride gradient in starting buffer; the total volume of the gradient was 405 ml and the flow rate was 0.5 ml/min. Fractions with PAM activity were pooled and concentrated. The concentrated material was diluted with 100 mM Mes, pH 6.5, and then loaded on a substrate-affinity column. After being washed with 100 mM Mes/200 mM NaCl, pH 6.5, the column was eluted with 1 M imidazole, pH 8.5. The 40 ml imidazole wash was dialysed and concentrated with 50 mM Hepes/200 mM NaCl, pH 7, in an Amicon microdialysis apparatus. The concentrated material was loaded on a Superose 12 column (Pharmacia) and eluted with the same Hepes buffer. The PAM activity was found in a single protein peak. The fractions with PAM activity were collected, concentrated and stored in 50 % ethylene glycol at –20 °C.

Discontinuous assay of PAM

Assays were carried out as previously described [2,10] in 100 mM Mes, pH 6.5, containing various amounts of TNP-YVG, 4 μ M $CuSO_4$, various amounts of L-ascorbate or DMPD, 1 mg/ml catalase and various amounts of PAM in a total volume of 0.25 ml at 37 °C. A 200 μ l portion was withdrawn from the incubation mixture at the appropriate time, quenched with 20 μ l of 3 M $HClO_4$, centrifuged and analysed quantitatively for TNP-D-Tyr-Val- α -hydroxyGly [TNP-YV(OH)G] product by h.p.l.c. (C8 reversed-phase column; detection at 344 nm) using a standard curve based on the peak height. The elution buffer was 44 % (v/v) acetonitrile, 55.9 % water and 0.1 % trifluoroacetic acid. The production of each molecule of TNP-YV(OH)G was coupled with the oxidation of one ascorbate molecule or two DMPD molecules in the PAM-mediated reaction. Therefore the concentration of oxidized ascorbate or DMPD cation radical in

the discontinuous assay mixtures was calculated according to the concentration of TNP-YV(OH)G produced.

Continuous spectrophotometric assay of PAM

The continuous PAM assay, with DMPD as an electron donor, was performed in 150 mM Mes, pH 5.5, containing 1 mg/ml catalase, 4 μ M $CuSO_4$ and various amounts of PAM substrate in a 1 ml spectrophotometric cuvette at 37 °C. Stock solutions of DMPD were stored on ice during the experiment. In each typical assay, the increase in absorbance at 515 nm due to DMPD auto-oxidation was initiated with 10 μ l of 250 mM DMPD and recorded every 5 s for a total period of 200 s. Then 3 μ l of PAM stock solution (52.9 μ g/ml protein) was immediately added to the reaction mixture to initiate the enzyme-mediated reaction, and the increase in absorbance at 515 nm was recorded for the same time interval and period. For initial enzyme reaction rate calculations, the rate of increase in absorbance due to enzyme-mediated oxidation of DMPD was the difference between the rate of increase in absorbance after and before addition of the enzyme. Absorption coefficients of 5200 $M^{-1}\cdot cm^{-1}$ for the protonated form and 7300 $M^{-1}\cdot cm^{-1}$ for the unprotonated form of the DMPD cation radical [19] were used in rate calculations, and relative concentrations of the two forms in enzyme reaction mixtures were calculated on the basis of a pK_a of 6.1 for the DMPD cation radical [19]. The kinetic parameters, K_m and V_{max} , for various PAM substrates were determined at 5.0 mM DMPD. Enzyme concentration and pH-dependence studies were carried out with various amounts of PAM or at various pH values of 150 mM Mes buffer containing 0.18 mM Ac-YVG and 5.0 mM DMPD. The [(4-methoxybenzyl)oxy]acetic acid (4- $CH_3O-C_6H_4-CO-O-CH_2-COOH$) inhibitor study was performed with 5.0 mM DMPD and various amounts of Ac-YVG and inhibitor. The L-mimosine inhibition study was carried out with a constant 0.36 mM Ac-YVG and various amounts of DMPD and mimosine.

Stoichiometry of the DMPD-supported PAM reaction

The DMPD-supported PAM reaction was carried out under the continuous assay conditions using TNP-YVG (60.2 μ M in the final assay mixture) as substrate and DMPD (5.0 mM in the final assay mixture) as electron donor in a total volume of 0.50 ml of Mes buffer, pH 5.0, 5.5 or 6.0 at 37 °C. The enzyme reaction was quenched after 200 s with 50 μ l of 3 M $HClO_4$, and the TNP-YV(OH)G was quantified by h.p.l.c. as described above. The amount of oxidized DMPD cation radical was measured spectrophotometrically at 515 nm using the molar absorption coefficients of the protonated and unprotonated oxidized DMPD [19], and the relative concentrations of these two forms were calculated from the pK_a [19].

Absorption spectra

DMPD stock solution (250 mM; 10 μ l) was mixed with 490 μ l of 150 mM Mes, pH 5.5, containing 1 mg/ml catalase, 4 μ M $CuSO_4$ and 120 μ M TNP-YVG in a 1 ml spectrophotometric cuvette at 37 °C. Then 16 μ l of purified PAM (0.594 μ g of protein) was added to the reaction mixture and incubated for 3 min. The absorption spectra of the reaction mixture were recorded before and 3 min after the addition of PAM.

RESULTS AND DISCUSSION

Initial experiments were aimed at demonstrating that DMPD is capable of serving as the electron donor for PAM-catalysed mono-oxygenation of our standard PAM substrate, TNP-YVG,

Figure 1 Identification of TNP-YV(OH)G as a product of DMPD-supported PAM reaction by h.p.l.c.-u.v. analysis of the enzyme reaction mixtures

The enzyme reactions were carried out using the standard PAM discontinuous assay as described in the Experimental section. The initial concentration of TNP-YVG in the standard assay mixture was $114 \mu\text{M}$. The peak at 4.50 min is TNP-YVG, the peak at 3.70 min is the product from PAM turnover, and the peak at 4.76 min is TNP-D-Tyr-Val-NH₂. (a) Chromatogram of 100 mM Mes buffer containing TNP-YVG only. (b) Chromatogram of the standard assay mixture containing no electron donor incubated with $0.167 \mu\text{g/ml}$ PAM for 25 min. (c) Chromatogram of the standard assay mixture containing 2.3 mM L-ascorbate incubated with $0.167 \mu\text{g/ml}$ PAM for 25 min. (d) Chromatogram of the standard assay mixture containing 5.1 mM DMPD incubated with $0.167 \mu\text{g/ml}$ PAM for 25 min. (e) Chromatogram of the assay mixture from (d) after further incubation with $5 \mu\text{l}$ of PGL ($0.4 \mu\text{g}$ of protein) for 6 min. (f) Chromatogram of the assay mixture from (d) after further incubation with $5 \mu\text{l}$ of boiled PGL ($0.4 \mu\text{g}$ of protein) for 6 min.

and that the product of DMPD-supported PAM catalysis is indeed the expected TNP-YV(OH)G. The results of these experiments are shown in Figure 1. It is clear from the h.p.l.c. traces that both the DMPD- and the ascorbate-supported PAM reactions give rise to the same single product, with retention time corresponding to that of authentic TNP-YV(OH)G. Furthermore, as shown in Figure 1(e), the TNP-YV(OH)G formed from DMPD-supported PAM turnover is completely converted into the final amide product, TNP-D-Tyr-Val-NH₂, by PGL. This represents unequivocal evidence that the TNP-YV(OH)G produced from DMPD-supported PAM turnover is indeed identical with that from the 'normal' ascorbate-supported reaction, as we have recently demonstrated the tandem stereospecificity of the amidation process; PGL reacts only with the (S)- α -hydroxyGly moiety, and PAM produces only α -hydroxyGly derivatives of the same (S) configuration [4]. Thus the results shown in Figure 1 demonstrate that, as expected,

Figure 2 Time course and kinetics of DMPD-supported PAM reaction

The enzyme reactions were carried out using the standard PAM discontinuous assay as described in the Experimental section. (a) Time course of PAM reaction. The concentrations of DMPD and TNP-YVG in 1 ml of enzyme reaction mixture were 10 mM and $71 \mu\text{M}$ respectively. A $100 \mu\text{l}$ sample was withdrawn from the reaction mixture at 0, 5, 10, 15, 20 and 30 min, and analysed quantitatively for TNP-YV(OH)G product by h.p.l.c. (b) Double-reciprocal plot with DMPD as the variable electron donor for PAM at a constant concentration of $60.2 \mu\text{M}$ TNP-YVG in each $250 \mu\text{l}$ of enzyme reaction mixture. (c) Double-reciprocal plot with TNP-YVG as the variable substrate for PAM at a constant concentration of 5.0 mM DMPD in each $250 \mu\text{l}$ of enzyme reaction mixture.

DMPD-supported PAM turnover produces only (S)-TNP-YV(OH)G.

Having established that DMPD functions as a viable electron donor for PAM-catalysed mono-oxygenation, we proceeded to examine the kinetics of the DMPD-supported PAM reaction. As shown in Figure 2(a), the time course of the DMPD-supported production of TNP-YV(OH)G measured by h.p.l.c. is linear over at least 30 min. Double-reciprocal plots for the rates of PAM-mediated TNP-YV(OH)G production, as measured by h.p.l.c., with either DMPD or TNP-YVG as the variable substrate are shown in Figures 2(b) and 2(c) respectively. It is evident that both of these Lineweaver-Burk plots are linear, indicating normal Michaelis-Menten kinetic behaviour for DMPD-supported PAM-catalysed mono-oxygenation of TNP-YVG.

The above results establish the suitability of DMPD as an

Figure 3 Kinetics of Ac-YVG for the DMPD-supported PAM reaction

The initial rates of the enzyme-mediated oxidation of DMPD were monitored spectrophotometrically at 515 nm with a constant 5.0 mM DMPD and various concentrations of Ac-YVG in 509 μ l of enzyme reaction mixture as described in the Experimental section. Inset: Ac-YVG concentration-dependence of initial rates of the enzyme-mediated oxidation of DMPD. The rates of enzyme-mediated absorbance change at 515 nm were 1.98, 2.31, 3.32, 5.64, 7.63 and 9.46 per h for Ac-YVG concentrations of 6.20, 9.30, 14.0, 20.9, 41.8 and 83.7 μ M respectively. Under the same assay conditions, the rate of absorbance change caused by DMPD auto-oxidation was 1.58 per h.

Figure 4 PAM concentration-dependence of DMPD oxidation rate

The rates of enzyme-mediated oxidation of DMPD were measured with various concentrations of PAM in the presence of 0.18 mM Ac-YVG and 5 mM DMPD under standard PAM continuous assay conditions in 150 mM Mes, pH 5.5. The total volume of each assay mixture was 510 μ l. The enzyme-mediated formation of the DMPD cation radical was determined at 515 nm and its concentration was calculated as described in the Experimental section.

Figure 5 Spectra of oxidized DMPD

PAM-mediated oxidation of DMPD was carried out in Mes buffer, pH 5.5, containing copper, TNP-YVG, DMPD and purified PAM as described in the Experimental section. The two spectra are, from top to bottom, DMPD oxidized by PAM for 3 min, DMPD with the buffer mixture containing no PAM. The concentration of enzymically generated DMPD cation radical is 127 μ M according to the absorbance at 515 nm.

electron donor for PAM catalysis. Our findings parallel those of Wimalasena and co-workers [18], who recently demonstrated that DMPD supports the activity of dopamine β -mono-oxygenase leading to a spectrophotometric assay for that enzyme. We therefore proceeded to develop a continuous spectrophotometric assay for PAM based on monitoring the increase in absorbance at 515 nm which accompanies DMPD oxidation [18–20]. By incubation of DMPD, copper, Ac-YVG and purified PAM in Mes buffers of different pH, the optimum pH for the DMPD-supported PAM reaction was found to be about 5.5, which is about the same as for the ascorbate-supported PAM reaction [6]. Therefore we decided to use Mes buffer at pH 5.5 for the DMPD-supported PAM continuous assay. Figure 3 shows a Lineweaver–Burk plot for the DMPD-supported reaction of PAM with Ac-YVG, a substrate whose reaction cannot be monitored by any of the previously reported PAM assay methods because of its lack of a radiolabel or chromophore. The reaction was monitored spectrophotometrically at 515 nm; as shown in the inset of Figure 3, the enzyme-mediated absorbance increases with time at 515 nm are linear at all substrate concentrations examined. In addition, the data in Figure 4 confirm that the initial rate of the DMPD-supported reaction with Ac-YVG, monitored by this spectrophotometric method, is directly proportional to the PAM concentration.

On the basis of previous studies on the redox chemistry of DMPD and related aromatic diamines [19,20], we expected that the oxidation product formed from DMPD as a consequence of PAM turnover would be the DMPD cation radical. Figure 5 shows the spectra of DMPD before and after PAM turnover of TNP-D-Tyr-Val-Gly at pH 5.5. The spectrum obtained after PAM turnover exhibits absorption maxima at 515 nm and 552 nm. The spectrum in Figure 5 corresponds to that previously reported for the DMPD cation radical [19,20], except that at pH 6.0, the 552 nm band undergoes a slight shift to 560 nm. The pK_a and molar absorption coefficients for both the protonated and unprotonated forms of the DMPD cation radical have been

Table 1 Stoichiometry of DMPD-supported PAM reaction

The concentration of DMPD cation radical formed was determined spectrophotometrically. The concentration of TNP-YV(OH)G was determined by h.p.l.c.

pH	DMPD cation radical formed (μ M)	TNP-YV(OH)G formed (μ M)	[DMPD oxidized]/[product formed]
5.0	16.9	8.40	2.01
5.5	37.2	18.2	2.04
6.0	35.1	17.5	2.01

reported to be as follows: pK_a , 6.1; protonated, 7300 (515 nm), 6300 (560 nm); unprotonated, 5200 (515 nm), 5500 (560 nm). From these values, we calculate an expected A_{515}/A_{560} ratio of 1.12 at pH 5.5, which is in excellent agreement with the measured ratio of 1.08 obtained from the spectrum in Figure 5. Thus this quantitative comparison confirms that the DMPD cation radical is indeed the product formed during PAM turnover.

In order to ascertain the electron/product stoichiometry for DMPD-supported PAM turnover, the amount of TNP-YV(OH)G produced, determined by h.p.l.c., was compared with the amount of DMPD cation radical formed, determined spectrophotometrically as described in the Experimental section. Table 1 shows the amounts of TNP-YV(OH)G and DMPD cation

radical produced by PAM turnover at three different pH values. It is clear that in all cases 2 mol of DMPD are oxidized per mol of α -hydroxyGly product formed. This stoichiometry of two electrons required per mol of product formed is diagnostic for mono-oxygenase-catalysed reactions, thus confirming that DMPD-supported PAM catalysis proceeds by the normal reductive oxygenation pathway of mono-oxygenase reactions.

In order to compare the kinetic mechanism of DMPD-supported PAM turnover with that of the ascorbate-supported reaction, steady-state kinetic data were obtained by measuring the rates for a series of peptide substrate concentrations at different fixed concentrations of each electron donor. As shown in Figures 6(a) and 6(b), the double-reciprocal plots of the rates of PAM-mediated TNP-YV(OH)G production at various concentrations of TNP-YVG and different fixed concentrations of either DMPD or ascorbate reveal a series of parallel lines. Thus both electron donors give rise to apparent 'Ping Pong' kinetics for PAM mono-oxygenation. Murthy et al. [11] have also reported apparent 'Ping Pong' steady-state kinetics for the ascorbate-supported PAM reaction with a tripeptide substrate. As an additional probe for analogous behaviour of DMPD and ascorbate as reductants for PAM catalysis, the inhibition of DMPD-supported PAM turnover by mimosine was examined, as this compound has been shown to exhibit competitive inhibition with respect to ascorbate [22]. We find that mimosine is indeed a well-behaved competitive inhibitor of PAM when DMPD is used as the electron donor, exhibiting a K_i of 9 μ M. Taken together, these kinetic results provide strong support for

Figure 6 Double-reciprocal plot of velocity (v) versus substrate concentration (TNP-YVG) at various concentrations of electron donor [DMPD (a); ascorbate (b)]

(a) DMPD concentrations were 0.690 (●), 1.25 (□), 2.50 (■) and 5.00 (○) mM. The total volume of each assay was 505 μ l. Inset: secondary plot of the intercepts against $1/[DMPD]$ concentrations. (b) Ascorbate concentrations were 1.20 (●), 1.54 (□), 3.00 (■) and 6.00 (○) mM. The total volume of each assay was 500 μ l. Inset: secondary plot of the intercepts against $1/[ascorbate]$ concentrations.

Table 2 Kinetic parameters of PAM-mediated reaction

K_m (TNP-YVG), K_m (electron donor) and V_{max} (app.) were calculated from the data in Figure 6 [23].

Electron donor	K_m (TNP-YVG) (μ M)	K_m (electron donor) (mM)	V_{max} (app.) (nmol/ μ g per min)
DMPD	6.79 ± 1.12	1.35 ± 0.27	25.9 ± 1.9
Ascorbate	3.07 ± 0.36	0.789 ± 0.078	28.7 ± 0.7

the conclusion that our substitution of DMPD for ascorbate as the reductant does not alter the steady-state kinetic pathway of PAM catalysis.

The kinetic parameters for the DMPD and ascorbate reactions obtained from secondary replots (insets in Figures 6a and 6b) are listed in Table 2. The steady-state kinetic measurements were carried out at atmospheric O_2 concentration, and the kinetic parameters are thus 'apparent' values. Under these conditions, V_{max} (app.) differs from the 'true' V_{max} by the factor $1 + (K_{O_2}/[O_2])$. It is clear that the apparent V_{max} values obtained under atmospheric conditions with either electron donor are very similar. The apparent K_m values for ascorbate-supported PAM turnover are lower than those measured with DMPD by about a factor of 2.

The above results clearly demonstrate the validity of utilizing DMPD as a reductant for PAM, thereby providing a new continuous spectrophotometric method for monitoring this enzyme reaction. In contrast with all previous assay methods for PAM, our assay does not require preparation of ^{125}I -labelled or chromophorically modified substrates. This is a crucial advantage in carrying out comparative kinetic evaluations of a series of substrates or turnover-dependent inactivators, as derivatization of potential substrates (and subsequent purification) is often highly problematical.

We have routinely used the DMPD assay in our laboratory for a variety of both peptide and non-peptide PAM substrates. For example, the reactions of PAM with hippuric acids, which we have previously shown to be the simplest synthetic substrates for PAM [21], are very conveniently monitored using the DMPD assay. Typical values obtained for kinetic parameters [K_m (mM) and V_{max} (nmol/ μ g/min) respectively] are as follows: hippuric acid: 2.70 ± 0.51 , 31.5 ± 2.4 ; 4-nitrohippuric acid: 1.48 ± 0.28 , 17.5 ± 1.4 ; 4-methoxyhippuric acid: 3.00 ± 0.56 , 21.1 ± 1.7 . Likewise, the kinetic parameters measured for the peptide substrate, Ac-YVG, were $K_m = 0.0475 \pm 0.0073$ mM and $V_{max} =$

58.9 ± 4.7 nmol/ μ g per min. As mentioned above, Ac-YVG represents a peptide substrate whose reaction cannot be monitored by any of the previously reported PAM assay methods because of its lack of a radiolabel or chromophore. Clearly, the DMPD assay now allows systematic kinetic evaluations of series of peptide PAM substrates to proceed in a straightforward manner. Finally, evaluation of the potency of potential PAM inhibitors is also easily accomplished using this assay; competitive inhibition with $K_i = 0.16$ mM was measured for [(4-methoxybenzoyl)oxy]acetic acid, in good agreement with our previous results (0.48 mM) [21].

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REFERENCES

- Douglass, J., Civelli, O. and Herbert, E. (1984) *Annu. Rev. Biochem.* **53**, 665–715
- 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
- Murthy, A. S. N., Manis, R. E. and Eipper, B. A. (1986) *J. Biol. Chem.* **261**, 1815–1822
- Mizuno, K., Sakata, J., Kojima, M., Kangawa, K. and Matsuo, H. (1986) *Biochem. Biophys. Res. Commun.* **137**, 984–991
- Gilligan, J. P., Lovato, S. J., Mehta, N. M., Bertelsen, A. H., Jeng, A. Y. and Tamburini, P. P. (1989) *Endocrinology* **124**, 2729–2736
- Oldham, C. D., Li, C., Girard, P. R., Nerem, R. M. and May, S. W. (1992) *Biochem. Biophys. Res. Commun.* **184**, 323–329
- Murthy, A. S. N., Keutmann, H. T. and Eipper, B. A. (1987) *Mol. Endocrinol.* **1**, 290–299
- Bradbury, A. F. and Smyth, D. G. (1985) in *Biogenetics of Neurohormonal Peptides* (Hakanson, R. and Thorell, J., eds.), pp. 171–186, Academic Press, London
- Bradbury, A. F., Finnie, M. D. A. and Smyth, D. G. (1982) *Nature (London)* **298**, 686–688
- Katopodis, A. G. and May, S. W. (1988) *Biochem. Biophys. Res. Commun.* **151**, 499–505
- Jones, B. N., Tamburini, P. P., Consalvo, A. P., Young, S. D., Lovato, S. J., Gilligan, J. P., Jeng, A. Y. and Wennogle, L. P. (1988) *Anal. Biochem.* **168**, 272–279
- Chikuma, T., Hanaoka, K., Loh, Y. P., Kato, T. and Ishii, Y. (1991) *Anal. Biochem.* **198**, 263–267
- Miyazaki, N. and Uemura, T. (1991) *Anal. Biochem.* **197**, 108–112
- Wimalasena, K. and Wimalasena, D. S. (1991) *Anal. Biochem.* **197**, 353–361
- Rao, P. S. and Hayon, E. (1975) *J. Phys. Chem.* **79**, 1063–1066
- Michaelis, L., Schubert, M. P. and Granick, S. (1939) *J. Am. Chem. Soc.* **61**, 1981–1992
- Katopodis, A. G. and May, S. W. (1990) *Biochemistry* **29**, 4541–4548
- Miller, D. A., Sayad, K. U., Kulathila, R., Beaudry, G. A., Merkler, D. J. and Bertelsen, A. H. (1992) *Arch. Biochem. Biophys.* **298**, 380–388
- Segel, I. H. (1975) in *Enzyme Kinetics*, pp. 608–610, John Wiley, New York