Anion- and pH-dependent activation of the soluble form of dopamine β -hydroxylase

Ole TERLAND and Torgeir FLATMARK¹

Department of Biochemistry and Molecular Biology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway

The present study presents evidence for the conclusion that the catalytic activity of dopamine β -hydroxylase (DBH; dopamine β -mono-oxygenase, EC 1.14.17.1) is regulated independently by pH and by anions. In the absence of activating anions (i.e. in 50 mM Mes buffer) the activity was essentially zero at low pH (5.1–5.3) when assayed with the artificial electron donors ferrocyanide (0.25 mM), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD, 2 mM) or *N,N*-dimethyl-*p*-phenylenediamine (1 mM) and tyramine (8 mM) as the substrate to be hydroxylated. However, in the presence of activating anions (e.g. 0.05–0.6 M Cl[−] in 50 mM Mes buffer, 0.1 M phosphate buffer or 0.2 M acetate buffer) a high catalytic activity was observed at pH 5.1–5.3. The pronounced effect of anions at this pH may be related to the postulated anion-induced conformational change of DBH [Syvertsen, Melø and Ljones (1987) *Biochim. Biophys. Acta* **914**, 6–18] resulting in a facilitated access of the substrates to the active site(s). The anion-activated DBH was inhibited when assayed with ferrocyanide and activated when assayed with

TMPD as electron donors by increasing the pH (5.1 to 6.0). By contrast, in the absence of anions the activity increased from essentially zero at pH 5.1–5.3 to high values at pH 6.0, irrespective of the electron donor used. The data suggest that the conformational change induced by anion activation exposes a negatively charged group at or near the electron-donor-binding site(s) imposing an electrostatic repulsion towards ferrocyanide (four negative charges) and an electrostatic attraction towards the positively charged TMPD, thus explaining the different pH-activity curves obtained for the two electron donors. In contrast to the artificial electron donors, the physiological donor ascorbate [Terland and Flatmark (1975) *FEBS Lett.* **59**, 52–56] supports hydroxylation of tyramine at low pH also in the absence of Cl[−], acetate or phosphate, confirming that ascorbate also functions as an anion activator.

Key words: ascorbate, catecholamine, chromaffin granule, mono-oxygenase, tyramine.

INTRODUCTION

The copper enzyme dopamine β -hydroxylase (DBH; dopamine β -mono-oxygenase, EC 1.14.17.1) is a mixed-function oxidase catalysing the last step in the biosynthesis of noradrenaline [1], i.e. the hydroxylation of the β -carbon in dopamine in the presence of dioxygen and ascorbate as the physiological electron donor [2]. The enzyme exists as a homodimer (subunits linked by disulphide bridges) at pH $\approx$ 5 and as a tetramer (dimers of dimers linked by non-covalent forces) at pH $\approx$ 6 [3]. The formation of tetramers may be related to the observed change in the intrinsic tryptophan fluorescence, compatible with a global or local conformational change in the protein, with an apparent pK of 5.7 for the transition [4]. Experimental evidence has been presented in support of one catalytic copper per active site [5], and in addition to ascorbate [2] the non-physiological single-electron donors ferrocyanide [6,7] and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) [8] function as electron donors in the coupled hydroxylation reactions. Furthermore, DBH accepts a broad spectrum of substrates, electron donors and inhibitors, suggestive of an open active-site crevice structure with high steric and electronic tolerance [9].

It was observed several decades ago that DBH isolated from the bovine adrenal medulla revealed a pH-activity curve with a sharp optimum at around pH 6.0 and that various anions (acetate, phosphate, chloride and the dicarboxylic acid fumarate) activated the enzyme in a concentration- and saturation-dependent manner [10,11]. The molecular mechanisms of these effects have since been the subject of thorough investigations and

discussions [4,11–13] and it has been suggested that fumarate exerts its activating effect by increasing the pK_a of an active-site residue essential for catalysis [12]. However, these steady-state kinetic studies [12] were performed in the presence of acetate/phosphate, which *per se* strongly activate DBH [10,11]. Moreover, fumarate has also been reported to affect the apparent catalytic mechanism of DBH [14]. In the present study, therefore, we selected a zwitterionic buffer [15], i.e. 50 mM Mes, in order to study the effects of pH (from 5.1 to 6.7) and anions in an independent and specific manner. Since Cl[−] is a physiological anion of the catecholamine storage granules [16], the anion activation experiments were preferentially performed with chloride. The activation of DBH by pH and anions is also biologically interesting, since the information can be used to predict the activity of DBH in different compartments, such as in the catecholamine storage granules and in the interstitium or other body fluids after its exocytotic release, as well as to define the optimum assay conditions for DBH in clinical chemistry.

MATERIALS AND METHODS

Preparation of chromaffin granule matrix phase

Bovine adrenal medulla chromaffin granules (in 1.6 M sucrose) isolated by sucrose-gradient centrifugation [17] were subjected to hypo-osmotic lysis (2.0 ml of 7.5 mM Hepes buffer, pH 7.0, for each pellet). The combined lysates (usually 16 ml) were centrifuged (125 000 *g*, 30 min) to remove membranes. The supernatant was dialysed overnight against a 100 $\times$ vol. (with four changes) in a cellulose dialysis bag (Spectropor) with a molecular-mass cut

Abbreviations used: DBH, dopamine β -hydroxylase; DMPD, *N,N*-dimethyl-*p*-phenylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

¹ To whom correspondence should be addressed (e-mail torgeir.flatmark@ibmb.uib.no).

off of 30000 Da. The dialysed matrix phase was concentrated by ultrafiltration (Centriplus 30) to a protein concentration of about 5–10 mg of protein/ml. All procedures were carried out at 0–4 °C.

Isolation of DBH

The dialysed and concentrated matrix phase (see above) was added to NaCl to a final concentration of 0.2 M and passed through a pre-equilibrated column (0.2 M NaCl in 7.5 mM Mes buffer, pH 6.5) of concanavalin A–Sephacrose; the resulting effluent had no DBH activity. The column was washed extensively with 0.2 M NaCl/7.5 mM Mes buffer, pH 6.5, until the light absorption at 280 nm in the effluent was essentially zero. DBH was eluted from the concanavalin A–Sephacrose by 5 ml of buffer (as above) containing 0.5 M methyl- α -D-mannopyranoside [18]. This elution was repeated five to seven times, the eluates were pooled, dialysed against a 20–26 $\times$ vol. of 7.5 mM Mes buffer (pH 6.5) and finally concentrated by ultrafiltration (Centriplus 30) to a protein concentration of 1.5–2.0 mg/ml. On SDS/PAGE our affinity purified DBH preparation revealed a purity of at least 90% and a specific activity among the highest reported [18]. All procedures were carried out at 0–8 °C.

Assay of DBH activity

In the standard assay DBH activity was measured with tyramine (8 mM, final concentration) as the substrate to be hydroxylated and ascorbate as the electron donor in the presence of 8×10^3 units/ml of catalase. The octopamine formed was measured by HPLC [19]. Alternatively, the activity was measured by the oxidation of ferrocyanide, *N,N*-dimethyl-*p*-phenylenediamine (DMPD; catalase present) or TMPD (catalase present) in the presence of tyramine (8 mM, final concentration) and followed spectrophotometrically [6,8,20]. In separate experiments we found a 2:1 stoichiometry for the oxidation of ferrocyanide (followed spectrophotometrically [6]) relative to the formation of octopamine (determined by HPLC [19]). The ferrocyanide/tyramine assay is thus a reliable and truly coupled assay of DBH activity. The assays were started by the addition of tyramine (or dopamine, see the Discussion) and run at 25 °C unless otherwise stated. DBH was also assayed under conditions exactly as described by Stewart and Klinman [21], i.e. in 50 mM Na⁺-phosphate buffer, pH 6.0, in the presence of catalase, 10 mM fumarate and 4 μ M CuSO₄ at 35 °C with either 2 mM ferrocyanide or 8 mM ascorbate as the electron donors and dopamine (8 mM) as the substrate. This reaction was stopped after 10 min by adding an equal volume of ice-cold ethanol containing acetic acid to pH 4.0, and the noradrenaline formed was determined by HPLC using a slight modification of our standard method [22] by which a complete separation of noradrenaline from dopamine was obtained. When assayed in Mes buffer, the addition of copper to the assay medium had little (< 20% increase) effect on DBH activity, as also observed by others [23,24]. For this reason we did not include copper in our assays unless specifically indicated.

Other analytical methods

Protein was determined using BSA as a standard [25]. KOH was used to adjust the pH of the Mes buffer measured with a PHM 84 pH-meter (Radiometer, Copenhagen, Denmark) at room temperature (20 °C).

Chemicals

Catalase (4.18×10^6 units/ml as a suspension in water containing 0.1% thymol), dopamine/HCl, fumarate (di-sodium salt),

DMPD, Mes, noradrenaline/HCl, octopamine/HCl and tyramine/HCl were from Sigma. Ascorbate (free acid) and ferrocyanide [$K_4[Fe(CN)_6] \cdot 3H_2O$] were from Merck. Concanavalin A–Sephacrose was from Amersham Biosciences. TMPD was from Kodak. Methyl- α -D-mannopyranoside was from Koch-Light. All other chemicals (e.g. KCl) were of reagent grade.

RESULTS

pH-dependent activity of DBH in the absence of anion activation

When assayed at pH 5.1–5.3 in 50 mM Mes buffer the activity of DBH was found to be essentially zero with 0.25 mM ferrocyanide (Figure 1A and Table 1), 2 mM TMPD (Figure 1A) or 1 mM DMPD (Table 1) as the electron donor. Only with the physiological electron donor ascorbate did DBH reveal a measurable (Figure 2) and concentration-dependent (Figure 3) activity at this low pH (5.1–5.2). The DBH activity increased at pH > 5.3, as assayed with all four electron donors, reaching an optimum at around pH 6.0 (Figures 1A and 2; data for DMPD not shown).

Anion activation of DBH at pH 5.2

In the presence of Cl[−] DBH revealed high catalytic activity at around pH 5.1–5.3 with all four electron donors (Figures 1B, 2 and 3, Table 1). At pH 5.2, maximum activation was obtained at around 0.15 M Cl[−] for ferrocyanide (Figure 4) and at around

Figure 1 The charge of the artificial electron donor profoundly affects the pH-activity relationship of DBH in the presence (0.6 M Cl[−]) but not in the absence of anions

The effect of pH on the catalytic activity of DBH (2–5 μ g/ml) at 25 °C in 50 mM Mes buffer (A) or 50 mM Mes buffer with 0.6 M Cl[−] added (B) was determined by the artificial electron donors 0.25 mM ferrocyanide (Δ) or 2 mM TMPD ($\circ$) in the presence of 8 mM tyramine and catalase (TMPD only). The DBH activity was assayed spectrophotometrically (ferrocyanide at 420 nm and TMPD at 565 nm). Each data point represents the mean of three to five determinations. The S.D. for each data point was less than 10% of the mean. For this reason bars showing S.D. were omitted.

Table 1 DBH requires anion activation to be catalytically active at pH 5.2

DBH (4 $\mu\text{g/ml}$) activity was assayed at 25 °C in 50 mM Mes buffer, 0.1 M phosphate buffer (KP_i) or 0.2 M acetate using the artificial electron donors ferrocyanide or DMPD (with catalase present) and 8 mM tyramine as the substrate to be hydroxylated. The effects of 0.6 M Cl⁻ or 10 mM fumarate were also determined. Oxidation of the electron donors was followed spectrophotometrically. The specific activities represent the means $\pm$ S.D. from three to five determinations. ND, not determined; Zero, no measurable activity, even at 20 μg of DBH/ml.

Electron donor	DBH activity (nmol oxidized/min)			
	Buffer	Buffer only	+10 mM Fumarate	+0.6 M KCl
Ferrocyanide (0.25 mM)	50 mM Mes	Zero	ND	507 $\pm$ 30
Ferrocyanide (0.25 mM)	0.1 M KP _i	333 $\pm$ 13	ND	440 $\pm$ 35
Ferrocyanide (0.25 mM)	0.2 M Acetate	1400 $\pm$ 133	ND	ND
DMPD (1 mM)	50 mM Mes	Zero	ND	6600 $\pm$ 300
DMPD (1 mM)	0.1 M KP _i	4600 $\pm$ 230	4000 $\pm$ 170	ND

Figure 2 The pH-activity relationship as determined with ascorbate in the absence and presence of anions (0.6 M Cl⁻)

The effect of pH on the catalytic activity of DBH (2–5 $\mu\text{g/ml}$) in 50 mM Mes buffer (25 °C) in the absence (○) or presence (●) of 0.6 M Cl⁻ was determined using 8 mM ascorbate and 8 mM tyramine as the substrates (catalase present). The formation of octopamine was assayed by HPLC as described in the Materials and methods section. Each data point represents the mean of three to five determinations. The S.D. for each data point was less than 10% of the mean. For this reason bars showing S.D. were omitted. The specific activities in the absence and presence of 0.6 M Cl⁻ were statistically significant ($P < 0.05$) only at pH < 5.5 .

0.5 M Cl⁻ for TMPD (Figure 4) as electron donors. A high catalytic activity was also observed at pH 5.2 in 0.1 M phosphate buffer when assayed with ferrocyanide or DMPD as electron donors as well as in 0.2 M acetate buffer with ferrocyanide as the electron donor (Table 1). In phosphate buffer at pH 5.2, the addition of 0.6 M Cl⁻ with ferrocyanide or 10 mM fumarate with DMPD as the electron donors did not further increase the catalytic activity (Table 1).

Anion activation of DBH as a function of pH and its dependence on the electron donor

In the presence of the activating anion Cl⁻ the pH-activity curve was strongly dependent on the electron donor used (Figures 1B and 2). In the presence of 0.6 M Cl⁻ the highest activity was observed at around pH 5.2 with ferrocyanide (Figure 1B) or ascorbate (Figure 2) as the electron donors. The activity approached zero at pH 6.5 with ferrocyanide as the electron donor (Figure 1B). By contrast, when assayed with TMPD as the

Figure 3 The effect of ascorbate concentration on DBH activity at pH 5.2 in the absence and presence of different degrees of anion activation

The effect of the ascorbate concentration on DBH (2–5 $\mu\text{g/ml}$) activity in the absence (○) or presence of Cl⁻ (□, 0.05 M; ●, 0.6 M) in 50 mM Mes buffer, pH 5.2, at 25 °C was assayed as described in the legend to Figure 2. Each data point represents the mean of three to five determinations. The S.D. for each data point was less than 10% of the mean. For this reason bars showing S.D. were omitted.

Figure 4 The effect of Cl⁻ concentration on the DBH activity at pH 5.2 with artificial electron donors of different charge

The effect of [Cl⁻] on DBH (2–5 $\mu\text{g/ml}$) activity in 50 mM Mes buffer, pH 5.2, at 25 °C was determined using 0.25 mM ferrocyanide (○) or 2 mM TMPD (●) as electron donors in the presence of 8 mM tyramine. The oxidation of ferrocyanide and TMPD were measured as described in the legend to Figure 1. Each data point represents the mean of three determinations. The S.D. for each data point was less than 10% of the mean. For this reason bars showing S.D. were omitted.

electron donor the activity increased 2–3-fold by increasing the pH from 5.1 to 6.0 (Figure 1B). In separate experiments (results not shown) we found that the apparent concentration for half-maximal activity ($[S]_{0.5}$) value for TMPD decreased from ≈ 40 mM in the absence of Cl⁻ to ≈ 2 mM in the presence of 0.6 M Cl⁻ at pH 6.0 with no change in V_{max} (≈ 12.5 μmol of TMPD oxidized/min per mg). As ferrocyanide imposes 'substrate inhibition' [7], determination of $[S]_{0.5}$ values for ferrocyanide was not possible. However, it appears (Figure 5) that 0.6 M Cl⁻ increases the optimum concentration of ferrocyanide from 0.25 mM (no anions) to 2 mM.

Effect of ascorbate concentration on DBH activity

In the presence of 0.6 M Cl⁻ DBH revealed saturation kinetics with respect to the concentration of ascorbate with an apparent

Figure 5 Anions (0.6 M Cl^-) affect the optimum concentration of ferrocyanide as electron donor at pH 6.0

The effect of the ferrocyanide concentration on DBH activity was determined in the absence (●) or presence (○) of 0.6 M Cl^- in 50 mM Mes buffer, pH 6.0 (25°C), using 8 mM tyramine as the second substrate (catalase not present). The oxidation of ferrocyanide was assayed as described in Figure 1. Each data point represents the mean of three determinations. The S.D. for each data point was less than 10% of the mean. For this reason bars showing S.D. were omitted.

$[S]_{0.5}$ value for ascorbate of $\approx 0.8\text{ mM}$ (Figure 3, confirmed in several other DBH preparations). In the absence of anion activation (i.e. no Cl^- added) there was no saturation kinetics for ascorbate at concentrations $< 20\text{ mM}$ (Figure 3).

DISCUSSION

In spite of several previous studies [3,11–13], the relative contribution of pH and anions to the catalytic activity of DBH has been confounded because the pH-dependency of DBH activity has been assayed in buffers (e.g. acetate/phosphate) that themselves activate DBH. In this study the enzyme was found to have essentially no catalytic activity at pH 5.1–5.3 in 50 mM Mes buffer when assayed with the artificial electron donors ferrocyanide, DMPD or TMPD (Figure 1A, Table 1), which is in apparent conflict with another report (see Figure 2 in [13]). On the other hand, in the presence of anions ($0.05\text{--}0.6\text{ M Cl}^-$ in 50 mM Mes, 0.1 M phosphate or 0.2 M acetate) DBH revealed high catalytic activity at pH 5.1–5.3 when assayed with the same electron donors (Figure 1, Table 1). Interestingly, when the activity was assayed in the presence of phosphate or acetate, no or marginal additional activating effect by adding Cl^- or fumarate was observed (Table 1 and [11]).

In the absence of anion activation, the DBH activity was increased (Figures 1A and 3) by raising the pH from 5.1–5.3 (essentially zero activity) to 6.0 (pH optimum) followed by a decline at pH > 6 . It should be noted that the same pattern was found both for the artificial electron donors (Figure 1B) and for the physiological electron donor ascorbate (Figure 2). The pH–activity relationship in the absence of anion activation (Figures 1A and 2) fits with the model proposed by Ahn and Klinman [12] that catalysis is associated with the ionization of a functional group with a pK value around pH 5.7. It is interesting that increasing the pH from 5 to 6 leads to a measurable shift in the intrinsic tryptophan fluorescence [4] and to a tetramerization of the protein [3], both of which have transitions around pH 5.7 [3,4]. Our studies (Figures 1A and 2) support the conclusion that, in the absence of anion activation, the dimeric form of DBH has zero catalytic activity whereas the tetramer has a high activity.

The catalytic activity measured in the presence of 0.6 M Cl^- decreased with the negatively charged electron donor ferrocyanide (Figure 1B), but increased with the positively charged TMPD (Figure 1B), on increasing the pH from 5.1 to 6.0. A marked decrease in the apparent $[S]_{0.5}$ value for TMPD from $\approx 40\text{ mM}$ in the absence of anions to $\approx 2\text{ mM}$ in the presence of 0.6 M Cl^- , together with no significant change in V_{max} (see the Results section), explains the different specific activities observed in the absence ($\approx 0.45\text{ }\mu\text{mol/min per mg}$) and presence ($\approx 4\text{ }\mu\text{mol/min per mg}$) of 0.6 M Cl^- , as assayed by a constant [TMPD] of 2 mM at pH 6.0. In contrast to the positively charged TMPD [13], the highly negatively charged ferrocyanide was a poor electron donor for the anion-activated enzyme at pH 6.0 (Figure 1B). This finding is contradictory to the claim [21] that ferrocyanide is an equally efficient electron donor as ascorbate at pH 6.0 (in the presence of anion activation). To further elucidate this point, we compared ferrocyanide and ascorbate as electron donors at experimental conditions as described by Stewart and Klinman [21]. We found a specific activity of $58 \pm 4\text{ nmol of noradrenaline/min per mg}$ ($n = 4$) using 2 mM ferrocyanide as compared with $3200 \pm 130\text{ nmol of noradrenaline/min per mg}$ ($n = 4$) using 8 mM ascorbate as an electron donor. Hence, ferrocyanide is a very poor electron donor for the anion-activated DBH at pH 6.0. Due to ‘substrate inhibition’ ([7] and Figure 5), it was not possible to determine an accurate $[S]_{0.5}$ value for ferrocyanide. The increase in the optimum concentration of ferrocyanide from $\approx 0.25\text{ mM}$ in the absence of, to $\approx 2\text{ mM}$ in the presence of, 0.6 M Cl^- (Figure 5), however, indicates a markedly reduced affinity for ferrocyanide in the presence of anion activation.

The different pH–activity relationship observed for DBH when assayed by the ferrocyanide and the TMPD method, in the absence and presence of 0.6 M Cl^- , may be accounted for by the presence of a negatively charged group at or near the copper reduction site in the anion-activated enzyme. The increased degree of ionization of this putative functional group by raising the pH from 5.1 to 6 imposes an increasing degree of repulsive interaction with ferrocyanide (four negative charges) and an increasing attractive interaction with the positively charged TMPD. As ferrocyanide has four negative charges, it is subject to a stronger electrostatic repulsion than ascorbate, thus explaining the different catalytic efficiency of the enzyme with the two electron donors for the Cl^- -activated enzyme at pH 6.0.

In conclusion, at low pH, i.e. pH < 5.5 , DBH is extremely sensitive to anions (Figure 4), and anion activation is obligate for activity at pH 5.1–5.3 (Figures 1–4, Table 1). This activation may be related to an anion-induced conformational change [4] which (i) controls the access of the substrates to the active site and (ii) changes the pK value of a group essential for catalysis [12] in a direction that allows catalysis to occur at a low pH value. Our data are compatible with a model in which the anion-induced conformational change exposes a negatively charged group at or near the copper reduction site. By analogy to the functionally closely related copper enzyme peptidylglycine α -hydroxylating mono-oxygenase [26], at least one salt bridge may be present at or near the active site of DBH. It has been shown that, e.g., 0.6 M Cl^- changes the pK_a value of ionizable groups, especially if they participate in salt bridges [27].

Relevance for DBH activity in the catecholamine storage granules and after its exocytotic release

At the intragranular pH of 5.3–5.5 [28] the catalytic activity of DBH is very low in the absence of anion activation when assayed with the artificial electron donors ferrocyanide and TMPD

(Figure 1A). By contrast, the physiological electron donor ascorbate [2] supports some DBH activity at this low pH in the absence of added anions (Figures 2 and 3), since ascorbate itself activates the enzyme [29,30] (Figure 3). However, even with ascorbate as the electron donor, anion activation is required for maximum activity at pH 5.2 (Figure 3), and 50–150 mM Cl^- gives full activation when using a negatively charged electron donor (Figure 4). Thus the combined effect of the intragranular concentration of Cl^- (i.e. ≈ 50 mM) [16] and ascorbate (i.e. ≈ 10 mM) [2] activates DBH to its full activity at pH 5.3–5.5 in the chromaffin granules. In this context, it should be noted that we have presented evidence that only the membrane-bound form of DBH is catalytically active in the mature chromaffin granules because the soluble form is part of the condensed granule matrix and subject to strong inhibition by the abundant chromogranin proteins [31]. As the soluble and membrane-bound forms of the enzyme are encoded by the same mRNA [32] and differ only at the N-terminal end (by a signal sequence in membrane-bound DBH), we assume that the pH and anion activation demonstrated for soluble DBH (this study) also holds for membrane-bound DBH.

After its exocytotic release, soluble DBH is found in the interstitium and in the blood [33] where the pH is ≈ 7.4 . If the pH in the assay of, e.g., serum DBH is as low as 5.0, anion activation is obligatory for activity (Figures 1 and 2), and was actually used in a most sensitive assay with 0.5 M acetate buffer at pH 5.0 [34]. When assayed at pH 6.0, anion activation is not obligatory for activity [19] (Figures 1 and 2). The finding that DBH prefers an electron donor with a positive charge at pH > 6 (Figure 1) points to the possibility that soluble DBH may have some catalytic activity after its exocytotic release. During high adrenergic activity, as in chronic heart failure, soluble DBH may theoretically support extravesicular noradrenaline biosynthesis with dopamine as the electron donor, since it supports a fairly high DBH activity at pH 7.0 in the presence of anion activation [11].

The expert technical work by Sissel V. Berge is greatly appreciated. This work was supported by the Norwegian Council of Cardiovascular Diseases, the Research Council of Norway and Solvay Pharma Norway (travel grant to O.T.).

REFERENCES

- Flatmark, T. (2000) Catecholamine biosynthesis and physiological regulation in neuroendocrine cells. *Acta Physiol. Scand.* **168**, 1–17
- Terland, O. and Flatmark, T. (1975) Ascorbate as a natural constituent of chromaffin granules from the bovine adrenal medulla. *FEBS Lett.* **59**, 52–56
- Saxena, A., Hensley, P., Osborne, J. C. and Fleming, P. J. (1985) The pH-dependent subunit dissociation and catalytic activity of bovine dopamine β -hydroxylase. *J. Biol. Chem.* **260**, 3386–3392
- Syvrtsen, C., Mølø, T. B. and Ljones, T. (1987) Fluorescence studies on dopamine β -monooxygenase: effects of salts, pH changes, metal-chelating agents and Cu^{2+} . *Biochim. Biophys. Acta* **914**, 6–18
- Abudu, N., Banjaw, M. Y. and Ljones, T. (1998) Kinetic studies on the activation of dopamine β -monooxygenase by copper and vanadium ions. *Eur. J. Biochem.* **257**, 622–629
- Ljones, T. and Flatmark, T. (1974) Dopamine β -hydroxylase: Evidence against a ping-pong mechanism. *FEBS Lett.* **49**, 49–52
- Rosenberg, R. C., Gimble, J. M. and Lovenberg, W. (1980) Inhibition of dopamine- β -hydroxylase by alternative electron donors. *Biochim. Biophys. Acta* **613**, 62–72
- Wimalasena, K. and Wimalasena, D. S. (1991) N,N,N',N'-Tetramethyl-1,4-phenylenediamine: a facile electron donor and chromophoric substrate for dopamine β -monooxygenase. *Biochem. Biophys. Res. Commun.* **175**, 920–927
- Wimalasena, K., Wimalasena, D. S., Dharmasena, S., Haines, D. C. and Alliston, K. R. (1997) Chiral multisubstrate inhibitors of dopamine β -monooxygenase: evidence for dual modes of interaction. *Biochemistry* **36**, 7144–7153
- Goldstein, M., Joh, T. H. and Garvey, T. Q. (1968) Kinetic studies of the enzymatic dopamine β -hydroxylation reaction. *Biochemistry* **7**, 2724–2730
- Craine, J. E., Daniels, G. H. and Kaufman, S. (1973) Dopamine- β -hydroxylase. The subunit structure and anion activation of the bovine adrenal enzyme. *J. Biol. Chem.* **248**, 7838–7844
- Ahn, N. and Klinman, J. P. (1983) Mechanism of modulation of dopamine β -monooxygenase by pH and fumarate as deduced from initial rate and primary deuterium isotope effect studies. *Biochemistry* **22**, 3096–3106
- Wimalasena, K., Dharmasena, S., Wimalasena, D. S. and Hughbanks-Wheaton, D. K. (1996) Reduction of dopamine β -monooxygenase. A unified model for apparent negative cooperativity and fumarate activation. *J. Biol. Chem.* **271**, 26032–26043
- Palatini, P. (1985) Fumarate is the cause of the apparent ping-pong kinetics of dopamine β -hydroxylase. *Biochem. Int.* **11**, 565–572
- Good, N. E., Winget, D., Winter, W., Connolly, T. N., Izawa, S. and Singh, R. M. M. (1966) Hydrogen ion buffers for biological research. *Biochemistry* **5**, 467–477
- Ornberg, R. L., Kuijpers, G. A. J. and Leapman, R. D. (1988) Electron probe microanalysis of the subcellular compartments of bovine adrenal chromaffin cells. *J. Biol. Chem.* **263**, 1488–1493
- Terland, O. and Flatmark, T. (1980) Oxidoreductase activities of chromaffin granule ghosts isolated from the bovine adrenal medulla. *Biochim. Biophys. Acta* **597**, 318–330
- Ljones, T., Skotland, T. and Flatmark, T. (1976) Purification and characterization of dopamine β -hydroxylase from bovine adrenal medulla. *Eur. J. Biochem.* **61**, 525–533
- Flatmark, T., Skotland, T., Ljones, T. and Ingebrechtsen, O. C. (1978) Fluorimetric detection of octopamine in high-performance liquid chromatography and its application to the assay of dopamine β -monooxygenase in human serum. *J. Chromatogr.* **146**, 433–438
- Wimalasena, K. and Wimalasena, D. S. (1991) Continuous spectrophotometric assays for dopamine β -monooxygenase based on two novel electron donors: *N,N*-dimethyl-1,4-phenylenediamine and 2-aminoascorbic acid. *Anal. Biochem.* **197**, 353–361
- Stewart, L. C. and Klinman, J. P. (1987) Characterization of alternate reductant binding and electron transfer in the dopamine β -monooxygenase reaction. *Biochemistry* **26**, 5302–5309
- Terland, O., Flatmark, T. and Kryvi, H. (1979) Isolation and characterization of noradrenalin storage granules of bovine adrenal medulla. *Biochim. Biophys. Acta* **553**, 460–468
- Terland, O., Slinde, E., Skotland, T. and Flatmark, T. (1977) Inhibition of dopamine β -mono-oxygenase by non-ionic detergents of the Triton X-series. *FEBS Lett.* **76**, 86–90
- Stewart, L. and Klinman, J. P. (1999) Kinetic parameters for dimeric and tetrameric forms of bovine dopamine β -monooxygenase and their relationship to non-Michaelis-Menten behavior. *FEBS Lett.* **454**, 229–232
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- Prigge, S. T., Mains, R. E., Eipper, B. A. and Amzel, L. M. (2000) New insights into copper monooxygenases and peptide amidation: structure, mechanism and function. *CMLS Cell. Mol. Life Sci.* **57**, 1236–1259
- Oliveberg, M., Arcus, V. L. and Fersht, A. R. (1995) pK_a values of carboxyl groups in the native and denatured states of barnase: the pK_a values of the denatured state are on average 0.4 units lower than those of model compounds. *Biochemistry* **34**, 9424–9433
- Njus, D., Kelley, P. M. and Harnadek, G. J. (1986) Bioenergetics of secretory vesicles. *Biochim. Biophys. Acta* **853**, 237–265
- Stewart, L. C. and Klinman, J. P. (1991) Cooperativity in the dopamine β -monooxygenase reaction. Evidence for ascorbate regulation of enzyme activity. *J. Biol. Chem.* **266**, 11537–11543
- Levine, M., Hartzell, W. and Bdoah, A. (1988) Ascorbic acid and Mg-ATP co-regulate dopamine β -monooxygenase activity in intact chromaffin granules. *J. Biol. Chem.* **263**, 19353–19362
- Terland, O. and Flatmark, T. (2000) The condensed matrix of mature chromaffin granules. The soluble form of dopamine β -hydroxylase is catalytically inactive. *Adv. Exp. Med. Biol.* **482**, 55–67
- Lewis, E. J. and Asnani, L. P. (1992) Soluble and membrane-bound forms of dopamine β -hydroxylase are encoded by the same mRNA. *J. Biol. Chem.* **267**, 494–500
- Weinshilboum, R. and Axelrod, J. (1971) Serum dopamine- β -hydroxylase activity. *Circ. Res.* **28**, 307–315
- Fujita, K., Nagatsu, T., Maruta, K., Teradaira, R., Beppu, H., Tsuji, Y. and Kato, T. (1977) Fluorescence assay for dopamine β -hydroxylase activity in human serum by high-performance liquid chromatography. *Anal. Biochem.* **82**, 130–140