Kinetic parameters for dimeric and tetrameric forms of bovine dopamine β-monooxygenase and their relationship to non-Michaelis-Menten behavior

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Received 5 April 1999; received in revised form 26 May 1999

Abstract Bovine dopamine β-monooxygenase has been assayed over a 10 000-fold range in protein concentration, to approximate conditions where the enzyme was shown to be a dimer or tetramer. Michaelis-Menten kinetics are observed with k_{cat} and k_{cat}/K_m for dissociated enzyme reduced 30% and 200–300% relative to tetramer. Addition of chloride ions to very dilute enzyme or the use of intermediate enzyme concentrations causes non-Michaelis-Menten behavior, attributed to an equilibration between dimer and tetramer. This is not expected to contribute to activity within the chromaffin vesicle, where enzyme and chloride ions are at high levels.

Key words: Dopamine β-monooxygenase; Bovine adrenal chromaffin granule; Anion activation; Dimeric and tetrameric enzyme form

1. Introduction

Dopamine β-monooxygenase (DβM) and peptidylglycine α-amidating enzyme (PAM) constitute a unique class of copper containing enzymes in higher eukaryotes that catalyze the oxidative production of hormones and neurotransmitters within their respective subcellular organelles [1,2]. The hydroxylating domain of the bifunctional PAM (referred to as peptidylglycine α-hydroxylating enzyme, PHM) shows a considerable sequence homology to positions 190–490 in DβM [3,4]. A recent crystal structure for PHM indicates the position of the two active site coppers and their respective ligands, together with the location of conserved active site tyrosine residues [5]. While the active site structure of DβM is assumed to be highly homologous to that of PHM, little information is currently available regarding the structure and function of the remaining ca. 50% of DβM.

Significant differences between DβM and PAM include their oligomeric structure (DβM is a dimer of dimers [1] while PAM is a bifunctional monomer [2]) and their sensitivity to regulation (only DβM has shown to be activated by exogenous effectors, e.g. fumarate [6], and to give rise to apparent cooperativity in response to changing concentrations of ascorbate [7] and amine substrates [8,9]). The apparent cooperative behavior of bovine DβM as a function of the amine concentration was earlier proposed to arise from very large kinetic differences between the dimeric and tetrameric forms of protein and to play a role in the in vivo functioning of this enzyme [8,9]. In this report, we investigate the kinetic behavior of bovine DβM over a 10 000-fold range of protein concentration, in order to characterize directly the behavior of the enzyme in its dissociated and associated forms. The resulting kinetic parameters, though not greatly different, are sufficient to generate deviations from Michaelis-Menten kinetics at intermediate levels of protein. While the addition of chloride ions to dimeric enzyme produces a kinetic pattern analogous to intermediate protein levels, this does not occur when the enzyme is in its associated form. A model is proposed in which chloride ions induce association of DβM. In the light of the high levels of both DβM [10–12] and chloride ions [13] in bovine adrenal chromaffin vesicles, DβM is expected to be functional in its associated, tetrameric form. The absence of detectable fumarate ions within the chromaffin granules argues against a role for this anion in the modulation of DβM activity.

2. Materials and methods

2.1. Materials

Soluble and membranous DβM was isolated as previously described [14]. All other materials were of reagent grade.

2.2. Assay of enzyme activity

Unless otherwise noted, all assay mixtures contained 50 mM 2-(*N*-morpholino) ethane sulfonic acid (MES) buffer, pH 6, 10 mM ascorbate, 100 μg/ml catalase and tyramine hydrochloride as a substrate source. No effects of added cupric ions were observed and it was omitted from assay mixtures. At intermediate protein concentrations, a continuous oxygen electrode assay was used, as previously described [14]. Although the reaction was initiated by the addition of enzyme, identical velocities were obtained upon pre-incubation of DβM with the desired tyramine hydrochloride concentration and initiation of the reaction with ascorbate. pH determinations of mock assay mixtures indicated a stable pH over the range of tyramine concentrations examined.

In order to assay the enzyme at very high (1 mg/ml) or very low (0.1 μg/ml) enzyme concentrations, a discontinuous HPLC/optical detection assay was employed [14]. We have previously shown our ability to assay mg/ml levels of DβM by a hand mixing device that allows data to be collected on a second time scale [15]. In control experiments, the continuous and discontinuous assay systems were shown to give similar results. Limiting kinetic parameters were obtained by non-linear fitting as described in [23].

2.3. Assay for fumarate ions in chromaffin granules

Chromaffin vesicles were purified through a 1.6 M sucrose density gradient and stored as aliquots in 0.32 M sucrose at −78°C, as described [16]. An aliquot containing 1.2 ml of 12 mg/ml chromaffin granules (ca. 14 mg total protein) was thawed and suspended in 9 ml of 5 mM MES buffer, pH 6.0. After four freeze/thaw cycles, the sample was centrifuged at 14 000 rpm. An equivalent volume of 95% ethanol/10 mM H_3PO_4, pH 3.3, was added to the supernatant.

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0014-5793/99/$20.00
PII: S0014-5793(99)00761-9

After 1 h at 4°C, the precipitated soluble protein was removed by centrifugation at 17000 rpm for 30 min. The supernatant was concentrated in vacuo to a volume of 2 ml, neutralized to pH 6.0 with NaOH and applied to a column (3 ml bed volume) of Dowex AG50WX (H^+ form, equilibrated in 10 mM phosphate, pH 6.0) for the removal of catecholamines. Components eluting in the void volume which contained an optical density >0.5 were pooled and concentrated to 0.65 ml. Analysis of later fractions from the Dowex resin did not reveal the presence of any fumarate. Aliquots of this extract were injected onto a Pharmacia Mono-Q anion exchange FPLC column equilibrated in a running buffer of 20 mM piperazine, pH 5.0. A Shimadzu LC-6A/SPD-6A chromatograph/detector ($\lambda = 254$ nm) and a C-R3A integrator system were employed for quantification of ascorbate and fumarate.

3. Results and discussion

3.1. Assay for fumarate in bovine adrenal chromaffin granules

The fumarate ion has been reported to be the most potent in vitro anion activator of DβM [17]. In order to understand the impact of anions on the in vivo behavior of DβM, the level of fumarate in chromaffin vesicles was estimated. As described in Section 2, non-cationic, small molecule components from chromaffin granules were isolated and analyzed by FPLC. Shown in Fig. 1A are standard injections of ascorbate (5 nmol) and fumarate (5 nmol), where ascorbate elutes within 10 min after injection of the buffered sample and fumarate elutes 13.5 min after the beginning of a salt gradient. The ascorbate peak can provide an internal control during analysis of our chromaffin granule extracts, which were estimated in a separate experiment to contain 1.5 mM ascorbate. At the level of granular extract used in Fig. 1B, the first peak corresponds to ca. 75 nmol ascorbate. As can be seen, two new peaks are detected relative to ascorbate, which correspond to retention times for ADP (8 min) and ATP (37 min). Importantly, no fumarate peak was detected at its anticipated position of 13.5 min after the initiation of the salt gradient (40.5 min for the chromatogram in Fig. 1B). As a control, we were able to show that spiking diluted lysates with 100 μM fumarate, followed by precipitation of soluble proteins, removal of catecholamines and FPLC separation led to essentially full recovery of fumarate. From an estimated detection limit of ca. 0.2 nmol in a 50 μl injection and a granular water content of 3 μl/mg [18,19], intravesicular fumarate is estimated to be $<<60$ μM. Although early studies of DβM activity indicated that 10 mM fumarate was required for full activation of the enzyme, a subsequent re-appraisal has led to values in the range of 100 μM (pH 5.5)–200 μM (pH 5.2) [7]. Thus, our level of detection for fumarate lies below the lowest levels yet reported for fumarate saturation, arguing against a role for fumarate in the in vivo activation of DβM.

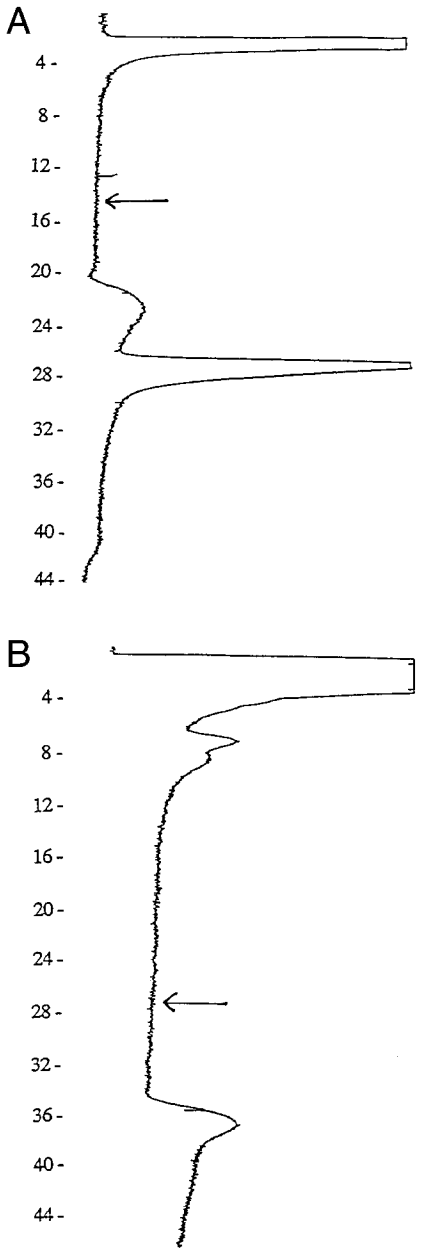

Fig. 1. Assay for intravesicular fumarate. (A) Standard injection of ascorbate (5 nmol) and fumarate (5 nmol) to a Mono-Q anion exchange FPLC column equilibrated in 20 mM piperazine, pH 5.0. A gradient containing 50 mM chloride was initiated at 13 min, with the fumarate peak eluting 13.5 min after the start of the gradient. The arrow indicates the beginning of the gradient. (B) 50 μl of the concentrated extract from the bovine adrenal chromaffin granules (see Section 2) was subjected to FPLC analysis under the conditions of A. The salt gradient begun at 27 min. No fumarate peak was detected at 40.5 min. The arrow indicates the beginning of the gradient.

3.2. Kinetic properties of DβM as a function of the protein concentration in the absence of fumarate

An assay of DβM at changing levels of the substrate tyramine is shown to give rise to non-Michaelis-Menten kinetics at intermediate protein concentrations (5 μg/ml), Fig. 2 (+). This type of deviation from linearity in Eadie-Hofstee plots has been seen previously and attributed to an equilibrating mixture of dimeric and tetrameric forms of DβM [8,9]. If correct, an assay of enzyme in its pure dimeric and tetrameric forms is predicted to lead to normal Michaelis-Menten kinetics. We estimated that we could measure the steady state kinetic behavior of DβM over a 10000-fold change of protein concentrations (in the limits of 0.1 μg/ml and 1 mg/ml). These concentrations are expected to lead to enzyme solutions that are ca. 85% dimer and tetramer, respectively (using $K_d = 10^{-7}$ M ($K_d = [E_2]^2/[E_4]$), from the ultracentrifugation experiments by Dhawan et al. [9]).

As seen in Fig. 3, the assay of enzyme at 1 mg/ml produces, within the experimental error, a linear pattern and results in the values of k_{cat} and k_{cat}/K_m summarized in Table 1. Not

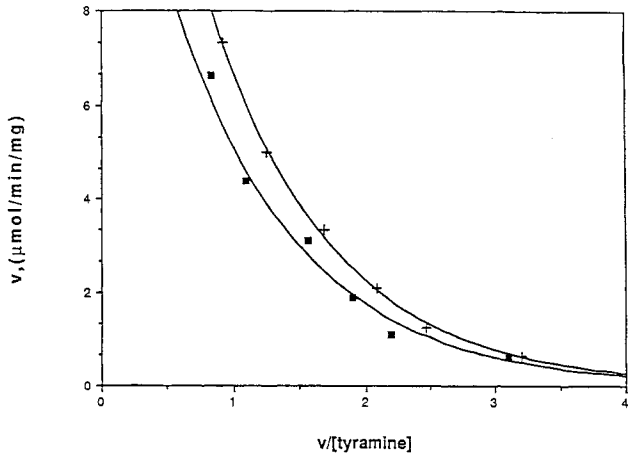

Fig. 2. Non-Michaelis-Menten kinetic behavior for DβM. Velocities were determined in MES buffer, pH 6, with 0.2–8 mM tyramine at 5 μg/ml of enzyme (+). In a second plot (■), the enzyme concentration was 0.1 μg/ml and the chloride ion concentration was 50 mM.

unexpectedly, addition of high concentrations of chloride ions (150 mM) leads to a marked decrease in the K_m for substrate while retaining the linear character of the Eadie-Hofstee plot. This is due to the ability of high levels of monovalent ions to mimic the behavior of the fumarate ion, which has been shown to exert its primary effect on the reduction of K_m for amine substrate [6].

In an analogous manner, enzyme was assayed at the very low concentration (0.1 μg/ml), leading to the linear pattern of Fig. 4 (■) and the k_{cat} and k_{cat}/K_m values of Table 1. It can be seen that k_{cat} is reduced 30% and the k_{cat}/K_m is decreased 2–3-fold relative to tetramer. The trends in the measured parameters (Table 1) are very different from values reported previously for dimeric and tetrameric bovine adrenal DβM [8]. In the earlier study, kinetic parameters for extreme enzyme forms were derived from curve fitting of the non-Michaelis-Menten behavior of DβM at intermediate protein concentrations. In this previous study, k_{cat} values were assumed to be identical and K_m was calculated to differ by 50-fold (0.082 mM versus 4.06 mM for dimer and tetramer).

What can be the origin of the large difference between the computed K_m values of Saxena et al. [8] and the direct analyses presented herein (Table 1)? The values of Saxena et al. were derived from a model in which the fraction of tetrameric

Table 1
Limiting kinetic parameters for dimeric and tetrameric DβM[a]

Enzyme	k_{cat}, s^{-1}	k_{cat}/K_m, s^{-1} M^{-1}
Dimer	8.6 (±1.0)	1.8 (±0.5)×10^3
Tetramer	12.3 (±1.0) (13.8)[b]	4.4 (±1.1)×10^3 (6.9×10^3)[b]

[a]Rate constants were calculated from initial velocities using a subunit molecular mass for enzyme of 75 000 g/mol.
[b]These values were used in the fitting of Eq. 1 to the data for 5 μg/ml DβM (+) in Fig. 2.

enzyme was obtained from ultracentrifugation experiments and then assumed to exist in a fixed proportion at all substrate concentrations. However, as long as dimer and tetramer are characterized by greatly different differing K_m values, the ratio of dimer to tetramer is expected to change with increasing substrate levels. In order to reproduce the data in this paper, we have used a model that assumes distinct values for the k_{cat} and k_{cat}/K_m for dimer and tetramer (Table 1) and then allows the fractional tetramer concentration (F_T) to vary with an increasing tyramine concentration:

$$v = (V_{m,T}[S]/K_{m,T} + S)\,F_T + (V_{m,D}[S]/K_{m,D} + [S])(1-F_T) \quad (1)$$

According to Eq. 1, $V_{m,T}$ and $K_{m,T}$ are the maximal velocity and Michaelis constant for tetramer and $V_{m,D}$ and $K_{m,D}$ are the corresponding constants for dimer. We find that a very slight adjustment of the measured kinetic parameters for tetramer and dimer (cf. Table 1) can reproduce exactly the relationship between the substrate concentration and measured velocities at a protein concentration of 5 μg/ml (Fig. 2 (+)) and for tyramine concentrations above 0.5 mM. Deviations are observed below 0.5 mM substrate and are tentatively attributed to the fact that experimentally determined velocities at tyramine <0.2 mM were at the detection limit of the oxygen electrode. Importantly, relatively small changes in k_{cat} and k_{cat}/K_m for dimer and tetramer are sufficient to produce the non-Michaelis-Menten behavior observed with bovine adrenal DβM at an intermediate protein concentration.

3.3. *Effect of chloride ions at a low protein concentration*

At 1 mg/ml protein, a high chloride ion concentration changes the K_m for tyramine without influencing the linearity of Eadie-Hofstee plots, Fig. 3. By contrast, the addition of

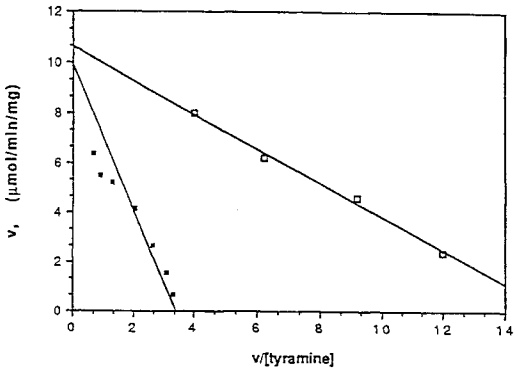

Fig. 3. Michaelis-Menten kinetics at a high enzyme concentration. Velocities were measured at 1 mg/ml of DβM. Initial rates determined in MES buffer, pH 6, with 0.2–8 mM tyramine (■) and with added chloride at 150 mM (□).

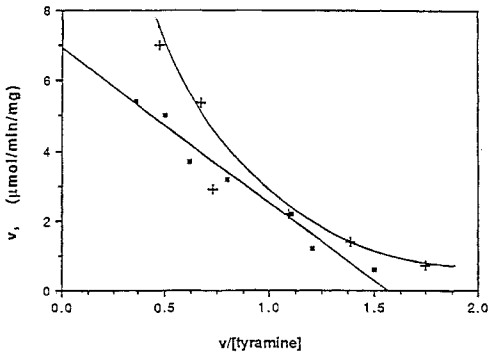

Fig. 4. Kinetic behavior at a low enzyme concentration. Velocities measured at 0.1 μg/ml of DβM. Initial rates determined in MES buffer, pH 6, with 0.4–15 mM tyramine (■) and in the presence of 15 mM chloride (+).

15 mM chloride ion to enzyme at 0.1 μg/ml converts a linear Eadie-Hofstee plot to one that shows deviation from Michaelis-Menten behavior (Fig. 4 (+)). The simplest explanation for the differential effects of chloride ions on the kinetic behavior at very high versus very low protein concentrations is a chloride-mediated association of dimer into tetrameric enzyme (such that the 0.1 μg/ml DβM sample in the presence of chloride ions can no longer be approximated by a single enzyme form). In fact, we can reproduce the behavior seen with 5 μg/ml enzyme at 0.1 μg/ml by adding 50 mM chloride ions to the sample (Fig. 2 (■)). While the data presented in Figs. 2–4 are for soluble DβM, similar results were seen when these experiments were repeated using the membrane-attached form of enzyme (data not shown).

3.4. Relevance to the in vivo behavior of DβM

The nature of the regulation of DβM in vivo has come up previously in the context of the in vitro activating effects of fumarate [6] and ascorbate [7]. The finding from the present study of little or no fumarate within bovine adrenal chromaffin granules argues that this effect is not relevant to the in vivo functioning of DβM. In the case of ascorbate, previous studies in the absence of fumarate have shown the phenomenological properties of negative cooperativity, with 3–4-fold increases in apparent k_{cat} and K_m values of ca. 0.05–0.10 mM (ascorbate < 1 mM) and $K_m > 10$ mM (very high ascorbate) [7]. The most likely explanation for this behavior is the ability of ascorbate to reduce both free enzyme (low ascorbate) and the enzyme product complex (high ascorbate) [7]. From a physiological perspective, levels of ascorbate are generally assumed to be high (ca. 20 mM) and fairly constant within chromaffin granules [20–22].

The data presented herein address the physiological relevance of the non-Michaelis-Menten behavior observed with regard to variation in the concentration of amine substrate ([8,9] and Fig. 1). The two key observations from the present study are (i) the demonstration of normal Michaelis-Menten behavior at very high protein levels (Fig. 2) and (ii) the proposed conversion of dimeric to tetrameric DβM in the presence of added chloride (Figs. 2 and 4). Given previous estimates of total soluble DβM within chromaffin granules of ca. 1–2 mg/ml [10–12], together with estimates of the intragranular chloride content of ca. 50 mM [13], we conclude that DβM will exist in an associated state within mature chromaffin granules and, thus, will follow normal Michaelis-Menten kinetics with high activity. This situation is likely to change upon the depletion of soluble intragranular components that accompanies exocytosis [1]. In this condition, the combined loss of anions, ascorbate and soluble DβM from the chromaffin granule is expected to convert DβM into a much less active form as the chromaffin granule undergoes recycling to the Golgi apparatus for re-packaging.

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