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ASCORBATE BASED NOVEL HIGH AFFINITY ALTERNATE REDUCTANTS AND COMPETITIVE INHIBITORS OF DOPAMINE β-MONOOXYGENASE

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A series of ascorbate derivatives has been used to examine the specificity and the chemistry of the reduction site of dopamine β-monooxygenase (DβM). Replacement of the 6-OH group of ascorbic acid with either bromine or hydrogen does not alter the enzyme reduction efficiency significantly. Unexpectedly, the 6-OH modified ascorbate derivatives, 6-S-phenyl-6-thio-L-ascorbic acid and 6-O-phenyl-L-ascorbic acid were found to have much higher affinity for the enzyme than the most effective known electron donor, ascorbic acid ($AscH^-$). The affinity of 2-amino-6-S-phenyl-L- ascorbic acid was found to be similar to that of 2-amino-L-ascorbic acid. 6-Amino-6-deoxy-L-ascorbic acid is neither a substrate nor an inhibitor for the enzyme. Although glucoascorbic acid is an excellent substrate for the enzyme, imino glucoascorbic acid was found to be an extremely potent competitive inhibitor for the enzyme. The stereoelectronic properties and alternate binding modes of these molecules have been considered in explaining the observations.

Dopamine β-monooxygenase [DβM; E.C. 1.14.17.1], a copper-containing monooxygenase, catalyzes the conversion of dopamine to the neurotransmitter, norepinephrine, within the neurosecretory vesicles in the adrenal medullae and the large dense-cored synaptic vesicles of the sympathetic nervous system (1-2). The ability of $AscH^-$ to stimulate DβM activity *in vivo* and *in vitro* (1-2), the presence of high concentrations of $AscH^-$ (3-5), and an efficient cyt b_{561}-mediated ascorbate regeneration system in DβM-containing neurosecretory vesicles strongly suggest that $AscH^-$ is the physiological electron donor for the DβM (6) catalyzed monooxygenation reaction.

Although $AscH^-$ is the most efficient reductant known for the enzyme, glucoascorbic acid (1,7), isoascorbic acid (1) as well as $K_4Fe(CN)_6$ (8-9), dopamine (9), hydroquinone, and dichlorophenolindophenol (10) are also long known as DβM reductants with varying potencies. We have demonstrated that structurally vastly different N,N,N',N'-tetramethyl-1,4-phenylenediamine and N,N-dimethyl-1,4-phenylenediamine are also efficient chromophoric electron donors for the enzyme and proposed that either the reduction site of the enzyme is highly non-specific or the enzyme possesses multiple reduction sites with different specificities (11-12).

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Abbreviations: $AscH^-$, Ascorbic acid; DβM, Dopamine β-monooxygenase; red pigment, 2,2'-dinitrilodi-2(2')-deoxy-6(6')-dithiophenyl-L-ascorbic acid monoammonium salt.

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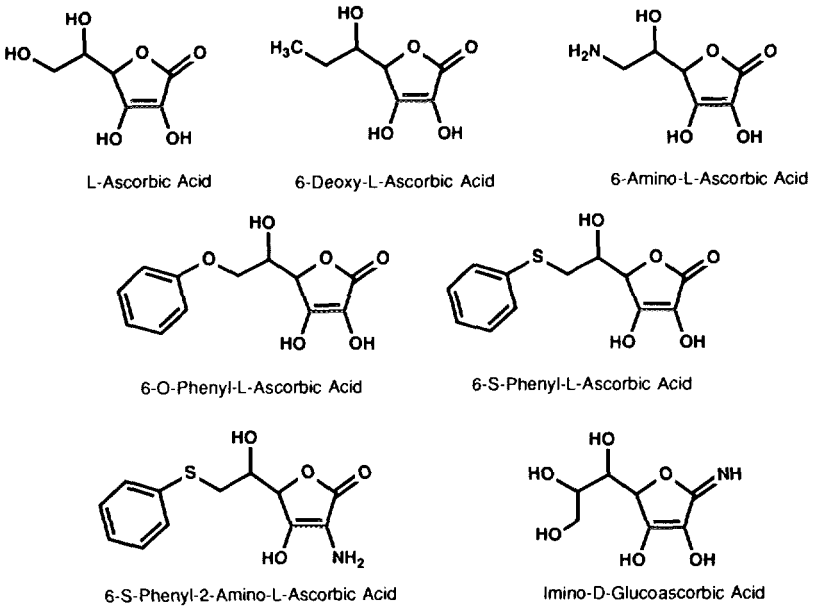

Scheme 1. **Structures of Alternate Reductants and Inhibitors of DβM.**

In this present work we have examined the specificity and the chemistry of the reduction site of DβM further by using a series of $AscH^-$ derivatives (see Scheme 1).

MATERIALS AND METHODS

Tyramine hydrochloride was from Sigma, disodium fumarate, N,N-dimethyl phenylene diamine and $AscH^-$ were from Aldrich. Beef liver catalase (65,000 units/mg of protein) was from Boehringer-Mannheim. Soluble DβM was purified (sp. act. 16-20 units/mg) according to the procedure of Ljones et al. (13) with minor modifications using freshly prepared bovine adrenal chromaffin granules (14-15). All spectrophotometric measurements were carried out on a HP 8452 diode array spectrophotometer equipped with temperature-regulated cell compartments. Initial rates of steady-state oxygen consumptions were measured using a Yellow Springs model YSI 5300 polarographic oxygen monitor. ^1H and ^{13}C NMR spectra were recorded on a Varian 300 (300 MHz) instrument using 3-(tetramethylsilyl)-1-propane sulfonic acid sodium salt as the internal standard. Kinetic constants for the various substrates were determined by the computer fit of data to the hyperbolic form of the Michaelis-Menton equation using the ENZFIT program. Inhibition constants were determined by direct fit of the data to the Clealand programs (16).

Spectrophotometric assays of Dopamine β-monooxygenase: The standard assay solution contained 10 mM fumarate, 100 μg/mL crystalline catalase, and 0.5 μM $CuSO_4$ in 125 mM acetate buffer at pH 5.2 in a total volume of 1.0 mL. DβM concentrations were kept at a constant of 1.6 μg protein per assay. The enzymatic reactions were usually initiated with the variable substrate. The rate of increase in absorbance at 386 nm due to the enzyme-mediated formation of the red pigment at 37 °C was measured against a reference identical to the enzymatic reaction mixture but without the enzyme (12).

Standard Oxygen Monitor Assay of Dopamine β-monooxygenase: All enzymatic reactions were carried out under standard assay conditions in a total volume of 2.6 mL at 37°C. DβM concentrations were kept at 3.0 μg protein per assay. Enzymatic reactions were initiated with an appropriate concentration of the variable substrate, and initial rates were measured as the rate of oxygen consumption minus the small background rates due to auto-oxidation. When

$AscH^-$ derivatives were the variable substrate, tyramine concentration was kept at a constant saturating concentration of 10 mM.

Syntheses: The $AscH^-$ derivatives, 6-bromo-6-deoxy-L-ascorbic acid (17), 6-deoxy-L-ascorbic acid, 6-S-phenyl-6-thio-L-ascorbic acid, 6-O-phenyl-L-ascorbic acid, and 6-amino-6-deoxy-L-ascorbic acid (18), 2-amino-2-deoxy-L-ascorbic acid (19) and D-imino glucoascorbic acid (20) were synthesized according to literature procedures.

6-Bromo-6-Deoxy-L-Ascorbic acid : Yield 48%, mp. 172-173 °C [lit. 170-172 °C (17)], 97.5% pure by I_2 titration. ^{1}H-NMR(D_2O): δ 3.59(1H, dd, ABX, J=7.6Hz, 10.7Hz), 3.68(1H, dd, ABX, J=5.8, 10.7Hz), 4.24(1H, ddd, J=2.1, 5.8, 7.7Hz), 5.10(1H, d, j=2.1Hz); ^{13}C-NMR(D_2O): δ 35.2(C6), 71.5(C5), 79.3(C4), 120.5(C2), 158.1(C3), 175.7(C1).

6-Deoxy-L-Ascorbic Acid: Yield 20%, mp. 163-164 °C [lit. 162-163 °C (18)], 99.5% pure by I_2 titration. ^{1}H-NMR(D_2O): δ 1.35(3H, d, J=6.6Hz), 4.18(1H, qd, J=6.6Hz, 2.2Hz), 4.78(1H, d, J=2.2Hz); ^{13}C-NMR(D_2O): δ 20.8(C6), 67.7(C5), 82.1(C4), 120.5(C2), 158.6(C3), 176.0(C1).

6-S-Phenyl-6-Thio-L-Ascorbic Acid: Yield 36%, mp. 100-102 °C [lit. 99-100 °C (18)], 99.7% pure by I_2 titration. ^{1}H-NMR(DMSO): δ 3.09(1H, dd, ABX, J=7.2, 13.8Hz)), 3.16(1H, dd, ABX, J=6.8, 13.7Hz) 3.85(1H, dt, J=1.4, 6.8Hz), 4.79(1H, d, J=1.7Hz), 7.16-7.23(1H, m), 7.29-7.39(4H, m); ^{13}C-NMR(DMSO): δ 35.7(C6), 66.5(C5), 75.9(C4), 118.1(C2), 125.7(Ar), 128.0(Ar), 129.1(Ar), 135.9(Ar), 152.5(C3), 170.3(C1).

6-O-Phenyl-L-Ascorbic acid: Yield 26% , mp. 160-161 °C [lit. 160-161 °C (18)]. ^{1}H-NMR(D_2O): δ 4.20-4.35(2H, m), 4.35-4.45(1H, m), 4.68(1H, d, J=2.1Hz), 7.05-7.15(3H, m), 7.47-7.50(2H, m); ^{13}C-NMR(D_2O): δ 70.5(C6), 71.9(C5), 81.2(C4), 117.9(C2, Ar), 124.6(Ar), 132.8(Ar), 160.9(C3), 176.8(C1).

6-Amino-6-Deoxy-L-Ascorbic acid: Yield 85%, mp. 210 °C(dec.) [lit. 210 °C (dec.) (18)], 93% pure by I_2 titration. ^{1}H-NMR (D_2O): δ 3.22(1H, dd, J=13.3Hz, 9.2Hz), 3.34(1H, dd, ABX, J=13.4Hz, 3.5Hz), 4.22(1H, td, J=2.7Hz, 8.3Hz), 4.48(1H, d, J=2.1Hz); ^{13}C-NMR (D2O): δ 44.9(C6), 68.5(C5), 81.6(C4), 115.9(C2), 176.9(C3), 179.7(C1).

2-Amino-2-Deoxy-6-S-Phenyl-6-Thio-L-Ascorbic Acid: 2-Amino-2-deoxy-L-ascorbic acid (20) (2.0 g,11 mmol) was stirred with 10.0 mL of 30% HBr-HOAc for 15 h at room temperature. Water (50.0 mL) was added to this mixture and stirred for another 2 h at room temperature. The solution was evaporated *in vacuo* at 30 °C. The resultant semi-crystalline product was triturated with $CHCl_3$ and refrigerated overnight. The crude solid product formed was collected by filtration and recrystallized from EtOAc to obtain white crystalline 2-amino-6-bromo-L-ascorbic acid which was subsequently used for the following step. Thiophenol (0.27 ml, 4.6 mmol) and 2-amino-6-bromo-L-ascorbic acid, (1.09 g, 4.6 mmol) were added sequentially to a solution of Na_2CO_3 (2.16 g, 17.4 mmol) in 3:1 water:methanol (20 mL) and stirred for 90 min. The reaction mixture was acidified to pH 1 with 6M HCl and extracted with EtOAc (4 x 50 mL). The organic layer was dried with anhydrous Na_2SO_4 and evaporated *in vacuo* . The resultant solid was recrystallized from EtOH and water to yield 0.8 g (29%) of the expected product. Mp. 157-160 °C (dec.). ^{1}H-NMR(D_2O): δ 3.24(1H, dd, ABX, J=7.8, 14.1Hz), 3.33(1H, dd, ABX, J=6.3, 14.1Hz), 4.08(1H, ddd, J=1.8, 6.3, 7.9Hz)), 4.72(1H, d, J=1.9Hz), 7.32-7.53(5H, m); ^{13}C-NMR(D_2O): δ 39.2(C6), 70.8(C5), 84.2(C4), 90.5(C2), 129.9(Ar), 132.3(Ar), 132.7(Ar), 137.2(Ar), 177.8(C3), 186.1(C1).

Imino D-Glucoascorbic Acid: Yield 17%, mp. 210 °C (dec.) [lit. 220 °C (dec.) (21)], pure by HPLC. ^{1}H-NMR(D_2O): δ 3.60(4H, m), 4.68(1H, d, J=1.7Hz); ^{13}C-NMR(D_2O): δ 66.4(t), 73.6(d), 74.6(d), 75.4(d), 82.8(s), 126.2(s), 184.0(s).

All the compounds listed above are stable for a long period of time as dry solids. However, in aqueous solutions they tend to slowly auto-oxidize similar to ascorbic acid. While the 2-amino and 6-amino derivatives were soluble in water like $AscH^-$, all the other compounds were less soluble than $AscH^-$, especially 6-S-phenyl and 2-amino-6-S-phenyl derivatives. Both of them were sparingly soluble in water and freely soluble in polar organic solvents such as methanol, ethanol, or DMSO. The concentrated stock solutions of these two compounds were prepared in ethanol and used in all kinetic studies.

RESULTS AND DISCUSSION

It is evident from the data presented in Table 1 that 6-deoxy-L-ascorbic acid is an efficient electron donor for the enzyme with kinetic parameters similar to that of the most efficient electron donor, $AscH^-$, at the range of concentrations tested. It is also noteworthy that although the affinity of this molecule for the enzyme was about one half, the turnover number is almost identical to that of $AscH^-$ under identical experimental conditions suggesting that the 6'-OH of $AscH^-$ has no significant effect on the electron donor efficiency, but may provide a minor contribution for the efficient interaction with the enzyme. This possibility is further supported by the observation that 6-bromo-6-deoxy- as well as 5,6-isopropylidene $AscH^-$ derivatives are also good reductants for the enzyme (data not shown) similar to that of 6-deoxy-L-ascorbic acid. However, the modification of the 3-OH group of $AscH^-$ either with a methyl, an alkyl or acetyl group completely abolishes the DβM electron donor capability (data not shown) demonstrating the crucial role of the 3-OH functionality in the DβM reduction activity.

The observation that 6-amino-6-deoxy-L-ascorbic acid is not a substrate for the enzyme (Table 1) together with the lack of significant competitive inhibition of the enzyme with respect to either $AscH^-$ or tyramine even at mM concentrations strongly suggest that this molecule is not interacting efficiently with the enzyme. Since the replacement of the 6-OH functionality with an amino group is not expected to affect the reduction potential of the molecule significantly the unexpected behavior of this molecule could only be attributed to its altered structural properties. Although it is possible that the positively charged (under experimental conditions) 6-amino group of the molecule may prevent the efficient interaction with the enzyme, previous observations that structurally similar amino compounds such as dopamine are efficient electron donors for the enzyme (9) argue against such a possibility. On the other hand, it is quite clear from the inspection of the structure of the molecule that it should exist as a zwitterion under the experimental conditions

TABLE 1

Initial Rate Kinetic Parameters of Various Ascorbic Acid Derivatives[a]

Compound	$K_m(mM)$	$k_{cat}(s^{-1})$	$k_{cat}/K_m(s^{-1}/mM)$	$K_i(\mu M)$
L-Ascorbic acid	0.34±0.01	11.6±0.2	33.2	-
6-Deoxy-L-ascorbic acid	0.76±0.08	11.7±0.3	15.4	-
6-S-Phenyl-L-ascorbic acid	0.02±0.001	10.3±0.1	513.5	-
6-O-Phenyl-L-ascorbic acid	0.05±0.004	11.8±0.2	235.6	-
2-Amino-6-S-phenyl-L-ascorbic acid[b]	1.63±0.06	13.5±0.3	8.3	-
6-Amino-L-ascorbic acid	-[c]	-[c]	-	
Imino D-glucoascorbic acid	-[d]	-[d]	-	26.4±5.2

[a]All kinetic parameters are apparent and determined at a single concentration of oxygen (256 μM) using DβM of 16-20 units/mg specific activity under standard assay conditions as detailed in Materials and Methods. [b]The initial steady-state rates of the enzymatic reactions were measured spectrophotometrically by following the increase in absorbance at 386 nm (ε = 16,600 M^{-1} cm^{-1}) due to the formation of the red pigment. [c]Very poor substrate activity by oxygen monitor assay. [d]No detectable substrate activity.

where the primary 6-amino group of the molecule is positively charged and the 3-OH group is deprotonated and negatively charged. Therefore, the electrostatic interaction of the two charged groups of the molecule may alter the conformation in such a way that it could not interact with the enzyme efficiently. Thus, it can be concluded that although the 5-OH and/or 6-OH groups of the ascorbate molecule are not essential for the efficient interaction with the enzyme, positively charged groups are intolerable at the six position.

In order to examine the steric tolerance of the reduction site of the enzyme towards the 6-OH modified $AscH^-$ derivatives, we have synthesized and characterized 6-O-phenyl and 6-S-phenyl derivatives of $AscH^-$ and tested them with the enzyme. Both these compounds were found to be well behaved excellent reductants for the enzyme. Careful steady state kinetic analysis of the two compounds revealed that while the K_m values for both compounds were much lower, the k_{cat} values were almost identical to that of $AscH^-$ resulting in specificity constants (k_{cat}/K_m) which were close to an order of magnitude greater for both compounds in comparison to $AscH^-$ under identical reaction conditions (Table 1, Figure 1). These results strongly suggest that the bulky hydrophobic phenyl substituents at the 6-position of these molecules considerably enhanced their

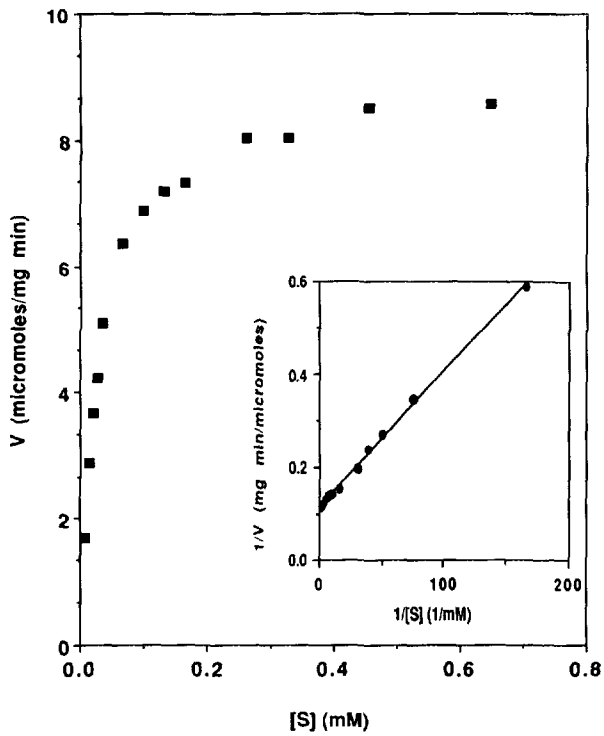

Figure 1. Initial Rate Steady State Kinetics of 6-S-Phenyl-L-Ascorbic Acid/Tyramine/DβM Reaction.

The initial rate of the DβM reaction was monitored by measuring the rate of oxygen consumption under standard assay conditions using 6-S-phenyl-L-ascorbic acid as the reductant. Tyramine concentrations were kept constant at 10 mM. *Insert:* double reciprocal plot of the data.

affinity towards the enzyme without significantly altering the enzyme reduction rate under the experimental conditions. However, in contrast the 6-S-phenyl-2-aminoascorbic acid was found to have a similar K_m to that of 2-aminoascorbic acid. The unexpected, unparalleled behavior of these two series of compounds suggested that they may interact with the enzyme by two different modes. In this regard one important difference between these two series of compounds is the pKa values of the 3-OH group. In the case of $AscH^-$ and its 6-OH substituted derivatives the pK_a of the 3-OH group is 4.2 (22), and we estimate that under the experimental conditions it should be mostly negatively charged and the negatively charged species may interact with the enzyme preferentially. On the other hand, the pK_a of the 3-OH of 2-aminoascorbic acid and its 6-OH substituted derivatives is about 6.8 (19) and mostly uncharged under the reaction conditions and the uncharged species may interact with the enzyme by an altered mode.

It has long been thought that the structure of $AscH^-$ is optimal for its function as the most efficient DβM reductant. However, the present results demonstrate that the modification of the 6-OH functionality of $AscH^-$ with a bulky hydrophobic phenyl group increases the affinity for the enzyme significantly. The high affinity of these molecules towards the enzyme could simply be due to the hydrophobic interactions of the phenyl group of the molecule with some hydrophobic amino acid residues in the reduction site of the enzyme. On the other hand, since the enzyme reduction and substrate hydroxylation sites of DβM appear to be distinct (23) and non overlapping, it could be hypothesized that the unexpected high affinity of 6-S-phenyl and 6-O-phenyl ascorbic acid toward the reduction site of the enzyme may be due to their ability to behave as bisubstrate analogs for the enzyme mimicking both $AscH^-$ and the phenylethylamine substrates. The additional experimentation necessary for the clarification of these possibilities is currently underway in our laboratory.

Although glucoascorbic acid is an excellent electron donor for DβM (7), imino glucoascorbic acid is not a substrate for the enzyme even at high concentrations. However, the data presented in Table 1 clearly show that this compound is an extremely potent, well behaved, reversible inhibitor for the enzyme. The inhibition was found to be competitive with respect to $AscH^-$ and uncompetitive with respect to tyramine demonstrating its preferential interaction with the oxidized form of the enzyme as expected. Although this kinetic behavior is consistent with the kinetic behavior of reversible inhibitors that are designed to mimic phenylethylamine and to interact exclusively with the oxidized form of the enzyme, the obvious structural similarity between imino glucoascorbic acid and $AscH^-$ strongly suggests that it must interact exclusively with the reduction site of the oxidized enzyme mimicking the $AscH^-$ binding mode. Therefore, the inability of imino glucoascorbic acid to act as a reductant must be due to the altered reduction potential of the molecule due to the replacement of the carbonyl group by an imino group.

Further experimental evidence is obviously necessary to fully understand the interaction of the various structurally different DβM reductants with the enzyme. However, it is quite clear that the reduction site of the enzyme is not as specific as previously thought and even several reductant binding modes may exist. The $AscH^-$ analogs described above may eventually be valuable tools in the detailed understanding of the chemistry and the specificity of the reduction site of the enzyme.

In addition, these analogs may also be valuable tools in the study of the proposed role of $AscH^-$ as a modulator in norepinephrine biosynthesis (24) and in the study of the intricate $AscH^-$ regenerating system in chromaffin granules.

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