Multiple binding sites for substrates and modulators of semicarbazide-sensitive amine oxidases: kinetic consequences

Andrew HOLT, David J. SMITH, Laura CENDRON, Giuseppe ZANOTTI, Adelio RIGO and Maria Luisa DI PAOLO

Department of Pharmacology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7 (A.H.)*

BioTie Therapies Corp., Tykistökatu 6, FIN-20520 Turku, Finland (D.J.S.)

Department of Chemistry, University of Padua, & ICTB Section of Padua, Via Marzolo 1, 35131 Padua, Italy (G.Z., L.C.)

Department of Biological Chemistry, University of Padua, Viale G. Colombo 3, 35121 Padua, Italy (A.R., M.L.D.P.)
Running title page

Multi-site regulation of copper amine oxidases

Corresponding author

Dr. Andrew Holt,

Department of Pharmacology, Faculty of Medicine & Dentistry, 9-70 Medical Sciences Building,

University of Alberta, Edmonton, AB, Canada, T6G 2H7

Telephone ++ (780) 492 8620 Fax ++ (780) 492 4325
e-mail aholt@pmcol.ualberta.ca

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Non-standard abbreviations

(+)-AM, (+)-amphetamine; 2-BFI, 2(2-benzofuranyl)-2-imidazoline; BPAO, bovine plasma amine oxidase; BZ, benzylamine; CLON, clonidine; GBZ, guanabenz; PEA, 2-phenylethylamine; hSSAO, human semicarbazide-sensitive amine oxidase; MA, methylamine; SPD, spermidine; TPP⁺, tetraphenylphosphonium; TPQ, topaquinone; (+)-TCP, (+)-tranylcypromine; VAP-1, vascular adhesion protein-1.
Abstract

Human semicarbazide-sensitive amine oxidase (SSAO) is a target for novel anti-inflammatory drugs which inhibit enzymatic activity. However, progress in developing such drugs has been hampered by an incomplete understanding of mechanisms involved in substrate turnover. We report here results of a comparative study of human and bovine SSAO enzymes which reveal binding of substrates and other ligands to at least two (human) and up to four (bovine) distinct sites on enzyme monomers. Anaerobic spectroscopy reveals binding of substrates (spermidine, benzylamine) and of an imidazoline site ligand (clonidine) to the reduced active site of bovine SSAO, while interactions with oxidised enzyme are evident in kinetic assays and crystallisation studies. Radioligand binding experiments with $[^3]$H]tetraphenylphosphonium, an inhibitor of bovine SSAO which binds to an anionic cavity outside the active site, reveal competition with spermidine, benzylamine and clonidine, indicating that these ligands also bind to this second anionic region. Kinetic models of bovine SSAO are consistent with one spermidine molecule straddling the active and secondary sites on both oxidised and reduced enzyme, while these sites are occupied by two individual molecules of smaller substrates such as benzylamine. Clonidine and other imidazoline site ligands enhance or inhibit activity as a result of differing affinities for both sites on oxidised and reduced enzyme. In contrast, while analyses of kinetic data obtained with human SSAO are also consistent with ligands binding to oxidised and reduced enzyme, we observed no apparent requirement for substrate or modulator binding to any secondary site in order to model enzyme behaviour.
Introduction

Human semicarbazide-sensitive amine oxidase (EC 1.4.3.6; hSSAO) is a glycosylated dimeric copper-containing enzyme found predominantly as a vascular ectoenzyme anchored to outer membranes of smooth muscle and endothelium, as well as adipocytes (Boomsma et al., 2005). The extracellular portion of hSSAO is cleaved to generate a soluble enzyme which circulates in the plasma (Schwelberger, 2007). Oxidation of circulating amines such as methylamine (MA) or aminoacetone, or protein-bound amines (Lyles, 1995), likely occurs by a ping-pong mechanism, similar to that followed by other copper-containing amine oxidases (Mure et al., 2002):

(i)  \( E_{\text{ox}} + RCH_2NH_3^+ + H_2O \leftrightarrow E_{\text{ox}}-\text{NH}^+\text{CH}_2R \rightarrow E_{\text{red}} + RCHO + H_2O \)

(ii)  \( E_{\text{red}} + O_2 \rightarrow E_{\text{ox}} + H_2O_2 + NH_4^+ \)

MA oxidation products such as formaldehyde and \( H_2O_2 \) contribute to vascular wall damage observed in conditions such as atherosclerosis and diabetes (Gubisne-Haberle et al., 2004). Accordingly, interest has existed for some time regarding potential therapeutic benefits of hSSAO inhibition; this interest has intensified with the observation that hSSAO on vascular endothelium acts as an adhesion molecule, termed vascular adhesion protein-1 (VAP-1), which is upregulated in some inflammatory conditions and which initiates rolling of lymphocytes (Salmi and Jalkanen, 2001). Inhibitors of hSSAO activity attenuate adhesion molecule expression and lymphocyte-endothelium interactions, and thus possess anti-inflammatory efficacy (Jalkanen et al., 2007; Wang et al., 2006).

Development of novel hSSAO inhibitors for the purposes of model and target validation and lead compound generation would be facilitated by a more complete understanding of the mechanisms by which substrates interact with hSSAO. Although molecular mechanisms have not been studied thoroughly, other closely-related copper-containing amine oxidases such as bovine plasma amine oxidase (BPAO) have been characterised extensively in mechanistic and kinetic experiments (Hartmann et al., 1993; Mure et al., 2002; Su and Klinman, 1998). Nevertheless, some puzzling observations remain to be explained satisfactorily. For example, the theory that allosteric communication between monomers may occur (Morpurgo et al., 1992) is
consistent with several reports documenting a reduction in initial velocity observed at higher concentrations of some substrates (Holt et al., 2007; Iigesti, 2003; Shepard and Dooley, 2006). However, the ping-pong nature of substrate oxidation by hSSAO and BPAO raises the possibility that substrates may bind to both oxidised and reduced enzyme forms, yielding hormetic kinetic plots, and recent kinetic analyses provide indirect evidence consistent with this possibility (Holt et al., 2007). Data from our laboratory also reveal an ability of several imidazoline binding site ligands to cause partial inhibition, or activation, of BPAO activity (Holt et al., 2004). Kinetic modelling implicated the involvement of up to four distinct sites through which these ligands exert their effects, although neither the locations of the putative sites nor their functional interrelationships could be deduced from the models.

The N1 and N10 atoms of the polyamine BPAO substrate, spermidine (SPD), interact electrostatically with BPAO via negatively-charged residues close to the topaquinone (TPQ) cofactor and in a secondary anionic cavity close to Asp445', on a hairpin loop extending from the opposing monomer, approximately 10Å from TPQ (Di Paolo et al., 2003). Tetraphenylphosphonium (TPP+), an inhibitor too large to access the BPAO active site, is able to inhibit oxidation of both SPD and benzylamine (BZ) through binding to this secondary site (Di Paolo et al., 2004). While polyamines are not hSSAO substrates, hSSAO also possesses a similar cavity, in a similar position to that on BPAO (Airenne et al., 2005), although the cavity appears smaller and more occluded than in BPAO.

In light of data supporting substrate binding to oxidised and reduced enzymes (Holt et al., 2007) and of the affinity of the N10 primary amine group of SPD for the secondary site on BPAO (Di Paolo et al., 2003), we hypothesised that substrates and modulators of BPAO may bind to the active and/or secondary sites on both oxidised and reduced BPAO, and that hSSAO may present similar targets. Direct spectroscopic evidence was obtained revealing binding of substrates and modulators to reduced BPAO, while radioisotopic experiments confirmed binding of ligands to both active and secondary sites. Crystallisation studies revealed the manner of clonidine binding, as well as major structural changes induced in the enzyme-inhibitor complex. Consequences of these interactions were modelled kinetically, with derived
equations allowing successful curve-fitting to data showing substrate inhibition and regulation by imidazoline ligands. Kinetic analyses of hSSAO did not reveal a parallel involvement of the secondary (imidazoline) site in ligand binding, although compounds bridging the active and imidazoline sites may offer enhanced potency and selectivity as hSSAO inhibitors.
Materials and Methods

Enzyme preparation

BPAO was purified as described previously (Di Paolo et al., 2007). The enzyme was stored at -80°C, at a concentration of approximately 8 mg ml⁻¹ in HEPES (10 mM) and NaCl (100 mM), pH 7.2. Full-length recombinant hSSAO was expressed in CHO cells and was purified as described previously (Airenne et al., 2005). The enzyme was stored at 4°C, at a concentration of approximately 120 µg ml⁻¹ in phosphate-buffered saline (20 mM), pH 7.4, containing Triton X-100 (0.1% v/v).

Assay of enzyme activities

The assay buffer used in all kinetic analyses was a modified Tyrode solution buffered with HEPES (Holt et al., 2007). HEPES was dissolved in pure water (Milli-Q) to a concentration of 100 mM. The following salts were then added to the HEPES solution to yield the indicated concentrations: KCl (10 mM), CaCl₂ (4 mM) and MgCl₂ (2.8 mM). The pH of the buffer was adjusted to 7.4 with NaOH (approximately 40 mM) and then NaCl was added such that the final sodium concentration in the buffer was 280 mM. Following two-fold dilution in assay wells, the concentrations of buffer components were thus half of those indicated above.

Three distinct approaches were used to measure initial rates of amine oxidation by SSAO enzymes. Most kinetic assays were done following one of two alternative continuous peroxidase-coupled platereader-based protocols. A fluorescence protocol, with Amplex Red as fluorogen (Holt et al., 2007), or an absorbance protocol, generating a quinoneimine dye from 4-aminoantipyrene and 2,4-dichlorophenol (Holt and Palcic, 2006), were done as described previously. Briefly, fluorescence assays were made in black polystyrene 96-well plates (Whatman), in a volume of 200 µl, containing amine substrate (50 µl in water) or water in blank wells, Amplex Red (50 µl in modified Tyrode-HEPES buffer containing 4 U ml⁻¹ type II horseradish peroxidase, to give a final assay concentration of Amplex Red of 20 µM), test compound (50 µl in water) or water in control and blank assay wells, and the reaction was started by the addition of enzyme (50 µl in modified Tyrode-HEPES buffer). The increase in fluorescence associated with generation of resorufin was
Absorbance assays were made in clear polystyrene 96-well flat bottom plates (Corning), in a volume of 300 µl, containing amine substrate (75 µl in water) or water in blank wells, chromogenic solution (75 µl, containing type II horseradish peroxidase (4 U ml⁻¹), 4-aminoantipyrine (500 µM) and 2,4-dichlorophenol (1 mM) in modified Tyrode-HEPES buffer), test compound (75 µl in water) or water in control and blank assay wells, and the reaction was started by the addition of enzyme (75 µl in modified Tyrode-HEPES buffer). The increase in absorbance associated with generation of the quinoneimine dye was monitored in a SPECTRAmax 190 platereader (λₐₗ₉ 510 nm). All kinetic assays were done at 30°C or 37°C, with 3 - 6 replicates per experiment, and initial rates were obtained from product versus time data with the linear regression facility of SoftMax Pro (version 4.8).

Substrates used in kinetic assays were, with BPAO, SPD and BZ, and with hSSAO, MA, BZ and 2-phenylethylamine (PEA) (Holt et al., 2007). The reversible compounds assessed as modifiers of enzyme activity are shown in Figure 1, and were, for BPAO, TPP⁺ (Di Paolo et al., 2004), clonidine (CLON) (Holt et al., 2004), guanabenz (GBZ) (Holt et al., 2004; Ozaita et al., 1997), (+)tranylcypromine ((+)TCP) (Carpéné et al., 1995) and 2(2-benzofuranyl)-2-imidazoline (2-BFI) (Holt et al., 2004), and for hSSAO, (+)TCP, GBZ and (+)amphetamine ((+)AM) (Lyles, 1984). Although fluorescence quenching or inhibition of peroxidase have been observed with several amine oxidase substrates and inhibitors (Holt and Palcic, 2006; Sayre et al., 1996), preliminary screening assays confirmed that none of the modulators used interfered with chromogenic or fluorogenic reagents. Modifier ligands were shown in a dilution assay to bind reversibly to the enzymes (data not shown), reversibility being an important requirement of the kinetic analytical approach adopted.

**Kinetic model development**

Based on observations from previous kinetic experiments (Holt et al., 2007), reaction schemes were drawn which might account for the ligand-enzyme interactions observed, and corresponding rate equations were then formulated. These were entered as user-defined equations within the nonlinear regression facility
of GraphPad Prism (version 5.00; GraphPad Software Inc., San Diego, CA). Equations were applied to multiple data sets, composed of several $v$ versus $[S]$ curves generated at different inhibitor/modulator concentrations, with the global curve-fitting facility of Prism allowing data from all curves to contribute to generation of universal kinetic constants. A consistently good fit (usually $r^2 > 0.90$ for every data set) obtained by global curve-fitting, with no obvious pattern of digression of fitted curves from data points, was viewed as supportive of the proposed model. A failure in this regard resulted in the scheme being considered inconsistent with the observed results.

Previous observations have suggested that substrates may bind to two sites on each SSAO monomer (either BPAO or hSSAO), with the most likely explanation being that amines bind to the active site in both oxidised and reduced forms of SSAO enzymes (Holt et al., 2007). In addition, a second site within the substrate entrance channel of BPAO has been identified, to which inhibitors such as TPP$^+$ and the amine group at the 10-position of spermidine likely bind (Di Paolo et al., 2004; Di Paolo et al., 2003). The reaction schemes developed thus allowed for the possible interactions of substrates and inhibitors with one or both of the active and secondary sites, on both the oxidised and reduced forms of the enzymes.

The method used to generate equations from reaction schemes was a “pseudoquantitative” rapid equilibrium approach, developed recently in our laboratory (Holt et al., 2007). The non-sequential (ping-pong) nature of the SSAO reaction, with the existence of enzyme in both oxidised and reduced forms, precludes rapid equilibrium analyses and demands that a steady-state approach be used (Segel, 1993). However, the complexity of the proposed models, coupled with a lack of suitable software to facilitate equation derivation, demanded that an alternative means of generating equations be found. To permit use of a rapid equilibrium approach, reaction schemes were drawn in which “ES” refers both to the complex between oxidised SSAO and substrate, as well as to the reduced enzyme following release of aldehyde. Thus, “ESS” refers to a complex between a single substrate molecule and reduced enzyme; the apparent presence of a single enzyme species (E) allows derivation of equations by a rapid equilibrium approach (Segel, 1993). Theoretical modelling studies indicate that while this “pseudoquantitative” approach
generates equations yielding dissociation and rate constants which differ to varying degrees from true values, global curve-fitting to multiple data sets nevertheless provides a valid indication of whether or not a proposed reaction scheme might explain experimental kinetic data obtained (Holt et al., 2007).

**Radioligand binding**

In order to assess directly the binding characteristics of the interaction between TPP⁺ and BPAO, and to determine whether substrates and inhibitors might compete with TPP⁺ for binding to the imidazoline site on BPAO, a novel radioligand binding procedure was devised. Preliminary experiments (not shown) indicated that BPAO was not retained on a Whatman GF/B fibreglass filter, while non-specific binding of TPP⁺ to filters, as well as to plastics such as pipette tips, resulted in reduced ligand concentration and very high background counts. Therefore, we adapted a method used previously for quantifying binding of ⁴⁵Ca²⁺ to proteins (Menguy et al., 1998).

In experiments to determine binding constants for TPP⁺, BPAO (250 µl; ≈ 40 nM in modified Tyrode-HEPES buffer), [³H]TPP⁺ (100 µl; nominally 0.5 nCi pmol⁻¹; prepared at initial concentrations of 0.25 - 8.75 µM), TPP⁺ (50 µl; 10 mM: to define non-specific binding) or water (50 µl) to define total binding, and water (100 µl), were pipetted into the sample reservoir of a Microcon YM-10 centrifugal filter device (MWCO 10 kDa; Millipore, Billerica, MA). Each reservoir therefore contained ≈ 20 pmol of BPAO subunits. The concentration of BPAO subunits was determined accurately by titrating an aliquot of BPAO solution with p-nitrophenylhydrazine and monitoring both the absorbance increase at 455 nm as a result of formation of TPQ-nitrophenylhydrazone adduct, as well as the corresponding loss of enzymatic activity (Holt et al., 1998). Samples were incubated at 30⁰ C for 15 minutes prior to centrifugation (25⁰ C, 14,000 x g) for 40 minutes. Centrifugation resulted in a removal of more than 99% of unbound [³H]TPP⁺, without reducing the ligand concentration and thus without altering the proportion of BPAO bound by ligand. The sample reservoir containing the BPAO-[³H]TPP⁺ complex was detached from the filtrate vial, and the entire reservoir was placed in a 6 ml plastic scintillation vial with EcoLite(+) scintillation fluid (4 ml; MP
Biomedicals, Solon, OH) and was allowed to soak for 18 hours, prior to counting for 5 minutes in a Beckman LS6500 liquid scintillation counter.

To account for ligand depletion due both to the high affinity of [3H]TPP+ for plastics and also to binding to BPAO in assays where low concentrations of TPP+ were not in significant excess over that of BPAO, the concentration of unbound TPP+ at equilibrium was determined experimentally. An aliquant of the filtrate from each assay (100 µl) was counted for radioactivity in EcoLite(+) (2 ml) (V1). The remaining solution in each filtrate vial was transferred to a second scintillation vial (V2) and was counted for radioactivity, as above. To a third scintillation vial (V3) were added the empty filtrate vial, along with the pipette tip used to transfer samples of retentate to Vials 1 and 2, and the contents were counted for radioactivity in 4 ml EcoLite(+), after vigorous shaking and soaking for 18 hours. The free ligand concentration at equilibrium, which was assumed to be equal to the free ligand concentration in the filtrate following centrifugation, was calculated from Equation 1, where V1dpm, for example, refers to the measured radioactivity in scintillation vial V1.

[3H]TPP+ was prepared at the desired concentrations in silanised microcentrifuge tubes, by diluting a stock solution of [3H]TPP+ (nominally 0.5 nCi pmol⁻¹) with water. The actual specific activity of this stock solution was determined directly by counting an aliquot of the solution, along with the pipette tip used to remove the aliquot, in EcoLite(+) (3 ml), since a portion of the ligand in the high activity manufacturer’s stock solution was lost through binding to the pipette tip used to remove the initial small volume from the manufacturer’s stock vial. Standard curves prepared thereafter from all prepared [3H]TPP+ solutions were linear (r² > 0.999) but crossed the abscissa below zero (not shown), suggesting a consistent amount of [3H]TPP+ lost to binding to plastic from all samples, even when silanised plasticware was used.

Specific binding was calculated as (total binding - nonspecific binding) and was plotted versus calculated concentrations for free [3H]TPP+. Hyperbolic curves were fitted to data with the nonlinear regression facility of GraphPad Prism.
In competition binding experiments to assess the abilities of other SSAO ligands to compete with TPP+ for binding to the imidazoline site, binding experiments were done as described above, but in the presence of a single concentration of [³H]TPP+ (nominally 150 nM, 0.5 nCi pmol⁻¹ in the assay), and with water (100 µl) replaced by 100 µl of the competing ligand, dissolved in water. Ligands examined were TPP+ (30 nM - 10 µM), BZ (1 mM - 300 mM), SPD (100 µM - 30 mM) and CLON (30 µM - 10 mM). The specific activity and free concentration of [³H]TPP+ were determined as described above. Specific binding, expressed as a percentage of that in control experiments in which competitive ligands were replaced by water, was plotted versus log₁₀[competitor], and sigmoidal curves were fitted with the nonlinear regression facility of GraphPad Prism.

**Anaerobic spectroscopy**

Benzylamine-induced reduction of BPAO was achieved following a modification of published procedures (Hartmann et al., 1993; Shepard and Dooley, 2006). High purity argon (grade 5, ≥ 99.9997%; Matheson) was passed through a pur-gas oxygen purifier (Matheson) and was then bubbled through pure water in a sealed plastic conical tube to hydrate the gas. An argon flow rate of approximately 8 ml min⁻¹ was maintained. The copper line carrying oxygen-free hydrated argon from the conical tube was spliced to supply both a sample preparation cuvette, as well as the cuvette chamber of a Beckman DU640 spectrophotometer. The line carrying the argon to the spectrophotometer entered the cuvette chamber via an access port normally used for circulating warm water to regulate cuvette temperature, and complete exclusion of light was thus maintained.

Modified Tyrode-HEPES buffer was degassed under house vacuum and was then bubbled overnight with hydrated argon, in a sealed plastic conical tube with a PTFE/silicone septum glued over an access hole drilled in the plastic lid. This oxygen-free buffer was used in all subsequent anaerobic studies. BPAO was prepared at 10.3 µM in oxygen-free buffer containing catalase (50 U ml⁻¹) and D-glucose (30 mM), in a sealed septum-cap vial flushed with argon. The solution was then flushed gently with hydrated argon via a 25G ⁵/₈” needle inserted through the PTFE/silicone septum, with a second needle allowing argon to exit the
cuvette. After flushing for 90 minutes on ice, the vial was allowed to warm to 22°C and 20 µl glucose oxidase were added to the vial contents via injection with a gas-tight syringe, to yield a glucose oxidase concentration of 50 U ml⁻¹. The vial was flushed for a further 30 minutes with hydrated argon and was then replaced on ice. An aliquant of this solution (390 µl) was injected through a septum into an argon-flushed 0.6 ml quartz (far-UV) glass anaerobic cuvette (pathlength 1 cm; Spectrocell, Orelan, PA) with a gas-tight syringe. The cuvette was placed in the spectrophotometer and the contents were flushed gently with hydrated argon via a needle penetrating the septum, as described above, for a further 5 minutes. This steady flow of argon into the anaerobic cuvette was maintained for the duration of the experiments, ensuring no slow leakage of air into the cuvette. A blank spectrum (240 - 700 nm) was then measured for this solution.

A solution of benzylamine (2 mM) was prepared in oxygen-free buffer in a sealed septum-cap vial flushed with argon, and 10 µl were injected with a gas-tight syringe into the cuvette containing BPAO. The benzylamine concentration (50 µM) was thus five times that of the BPAO present (10 µM). Following rapid mixing with a second gas-tight syringe primed with argon, reduction of BPAO was observed by obtaining difference spectra (240 - 700 nm) at several time points following injection of benzylamine, until spectra remained stable. At this point, a blank spectrum was again obtained for the cuvette contents.

Solutions of BZ (800 mM), SPD (16 mM) and CLON (3.2 mM) were prepared in oxygen-free buffer and were flushed with argon in sealed septum-cap vials. To assess whether or not these ligands interact with the active site of reduced BPAO, ligands were injected cumulatively into the anaerobic cuvette with a gas-tight syringe, in volumes of 2.5, 5 or 10 µl, to generate a range of concentrations of the ligands. Difference spectra (240 - 700 nm) were obtained at several time points following each injection, until spectra remained stable. A fresh preparation of reduced BPAO was used to examine each of the three ligands of interest. Corrections were made to measured absorbance values to account for enzyme dilution on addition of ligands.

TPP⁺ chloride (2.5 mM) was prepared in oxygen-free buffer and was flushed with argon in a sealed septum-cap vial. To determine the degree to which ligands binding to the imidazoline site influenced the
spectra obtained for reduced cofactor, 10 µl of this solution were injected after the final ligand aliquant had been added to the anaerobic cuvette and spectra had stabilised. The resulting concentration of TPP⁺ (around 50 µM) was sufficiently high to displace other ligands binding to the imidazoline site. Difference spectra (240 - 700 nm) were obtained at several time points following addition of TPP⁺, until spectra remained stable.

Spectral data from all scans were captured and transferred to GraphPad Prism, and absorbance readings were then corrected by applying the Beer-Lambert equation to account for protein dilution on addition of ligand, before data were plotted.

**Crystallography studies of the BPAO-clonidine complex**

BPAO was crystallised as previously reported (Calderone et al., 2003). Briefly, after purification, the enzyme was deglycosylated with a glycoprotein deglycosylation kit (Calbiochem, San Diego, CA), dialysed in 10 mM HEPES (pH 7.2) containing 10 mM NaCl and concentrated to 2 - 2.4 mg ml⁻¹. The BPAO sample was incubated in the presence of CLON (2 mM) at 25°C for 2 hours. Thereafter, crystals were grown by the vapour-diffusion technique at 25°C, by mixing equivalent volumes of the BPAO-CLON sample and a standard precipitant solution containing KH₂PO₄ (0.2 M, pH 7.4) and PEG 3350 (20%, w/v), and the enzyme was left to equilibrate against the precipitant. The crystals grew within 3 weeks.

The X-ray data were collected at the BM16 beamline of the European Synchrotron Radiation Facility (Grenoble, France). The crystals were mounted directly under a nitrogen flux (-173°C), without addition of cryoprotectant. The data were processed using the MOSFLM program (Leslie, 1992). Crystals appeared isomorphous to the apoenzyme structure (PDB code 1TU5) determined previously (Lunelli et al., 2005). One dimer, corresponding to an independent biological unit, was present in the asymmetric unit (Supplementary Table S1). The structure of the apoenzyme (1TU5) was used as the starting model, including the TPQ₄₋₇₀ modified tyrosine residue. After several cycles of rigid body and restrained refinement with Refmac software (Murshudov et al., 1997), the two copper cations bound within the active sites could be identified. Some residual electron density in the same pocket was also observed, which could be
attributed only to the CLON molecules bound to the enzyme. In particular, the two chloride atoms of each CLON molecule were visible in the Fourier difference map at more than 3 sigma. Both active sites appeared similar in this regard, with minor differences likely attributable to incomplete occupancy of the active sites by CLON, due to the low affinity of CLON for that site.

The inhibitor molecule was constructed with PRODRG software (Schuttelkopf and van Aalten, 2004) and was aligned to fit within the residual electron density. Non-crystallographic symmetries were introduced to account for the binary axis relating the two molecules of the dimer present in the asymmetric unit. However, all the residues belonging to the active site cavity and the more flexible regions were excluded. In further refinement steps (Murshudov et al., 1997; Sheldrick and Schneider, 1997), 342 water molecules were added to the structure, resulting in a final R-factor of 23.7 % and an R-free of 25.2 %. Data processing and refinement statistics are shown in Supplementary Table S1.

Coordinates have been deposited at the Protein Data Bank of the Research Collaboratory for Structural Bioinformatics (http://www.rcsb.org/pdb) with accession number 2PNC.

Materials

[Phenyl-\(^3\)H]tetraphenylphosphonium bromide (29.0 Ci mmol\(^{-1}\), 1.0 mCi ml\(^{-1}\)) was purchased from Moravek Biochemicals Inc., Brea, CA. Tetraphenylphosphonium chloride, methyltriphenylphosphonium iodide, dimethyltriphenylphosphonium iodide, trimethylphenylphosphonium iodide, clonidine hydrochloride, guanabenz, catalase, glucose oxidase, horseradish peroxidase (Type II), 4-aminoantipyrene, 2,4-dichlorophenol and amine substrates (as their hydrochloride salts) were from Sigma-Aldrich (Oakville, ON). Amplex Red was obtained from Invitrogen/Molecular Probes (Burlington, ON). 2-BFI was purchased from Tocris (Ellisville, MO). (+) and (-)TCP and (+)amphetamine were generous gifts from Dr. Glen Baker, University of Alberta. All other materials were of molecular biology grade and were purchased from Fluka-Sigma-Aldrich or EMD/Calbiochem (La Jolla, CA).
Results

Binding of [³H]TPP+ to BPAO

Figure 2 shows specific binding of [³H]TPP+ to 17.4 pmol of BPAO subunits. Non-specific binding (not shown) was linear, and passed through the origin. Thus, the failure to reach a plateau is due to a low affinity component of the specific binding, rather than incomplete determination of the non-specific contribution to total binding. Data are fitted to an equation for two-site hyperbolic binding (Prism 5.0). While nonlinear regression failed to yield meaningful values for $K_d$ or $B_{\text{max}}$ for the low affinity component of specific binding, likely due to the low affinity $K_d$ lying beyond the highest TPP+ concentration used in these experiments, the high affinity component had a $K_d$ of 94 nM and a $B_{\text{max}}$ of 32 pmol sites per assay well. This $B_{\text{max}}$ value is double that expected, based on the known concentration of BPAO subunits, and indicates binding of two TPP+ molecules per BPAO subunit. While it is hypothesised that one of these sites is the secondary site within the substrate entrance channel, the location of the other site is not known, although binding to the active site can be discounted based upon kinetic observations. Nevertheless, the two sites appear to bind TPP+ with similar affinities, since trihyperbolic curve-fitting failed to separate two distinct high affinity binding components without restricting some of the variables (not shown). In this regard, an affinity of TPP+ for the secondary site of around 100 nM is consistent with the inhibitor dissociation constant of around 140 - 200 nM for TPP+ determined in kinetic experiments (see Tables 1 and S2).

Competition with [³H]TPP+ for binding to BPAO

The abilities of TPP+, SPD, BZ and CLON to compete with specific binding of [³H]TPP+ were assessed. At the concentration of [³H]TPP+ used (calculated at around 106 nM), the contribution of low affinity binding was presumed to be negligible. Figure 3 shows competition curves for ligands of interest, revealing complete inhibition of binding by TPP+ but inhibition of only around 50% of binding by SPD and BZ. A two-site competition equation applied to data for TPP+ failed to differentiate between two high affinity binding sites (not shown). CLON also failed to inhibit completely the binding of [³H]TPP+, although results from nonlinear regression suggested that more than 50% of binding may be inhibited, and thus that CLON
may also compete for binding at the second high affinity TPP⁺ binding site. Dissociation constants were estimated from the Cheng-Prusoff equation, making the assumptions that TPP⁺ was present at a concentration similar to its $K_d$ value at the secondary site and that binding of all ligands within the concentration ranges used was uniphasic. Dissociation constants thereby estimated were 234 nM (TPP⁺), 296 µM (SPD), ≈ 1.2 mM (CLON) and 13.5 mM (BZ); values therefore shared the same rank order of affinities as constants determined kinetically ($K_M$, $K_{imid}$ and $K_{sec}$ values in Tables 1 and S2), although $K_d$ values for the lower affinity ligands (CLON and BZ) differed quite significantly from constants determined kinetically. These observations confirm that a single SPD molecule probably binds both to the active site and to the secondary anionic site within the substrate entrance channel. Furthermore, small cationic substrates and inhibitors may bind simultaneously at the active and secondary sites, with the latter action potentially influencing substrate turnover kinetics at the active site.

Under assay conditions used to assess interactions of ligands with oxidised BPAO, some oxidation of SPD and BZ would be expected during incubation with BPAO in the presence of [³H]TPP⁺, resulting in depletion of these substrates and generation of oxidation products which might influence TPP⁺ binding in some way. Although the estimated depletion of SPD ($≥ 12\%$) was rather higher than that for BZ ($≥ 0.5\%$), analysis of data obtained in the presence of SPD (Figure 3) revealed a monophasic curve with Hill slope not significantly different from unity and indicative of a $K_d$ similar to $K_M$ and $K_i$ values obtained in kinetic analyses. Thus, there is no outward indication of any unexpected effect due, for example, to interactions of SPD oxidation products with the enzyme.

The second high affinity TPP⁺ binding site, for which BZ and SPD do not compete, could be the secondary external cation binding site present on each subunit that was found to coordinate Ca²⁺ and other cations (Lunelli et al., 2005).

**Confirmation of substrate and drug binding to reduced BPAO**

Following reduction of anaerobic BPAO with a 5-fold excess of BZ, further addition of SPD (99 µM - 2.1 mM), BZ (5 - 104 mM) or CLON (78 - 640 µM) resulted in concentration-dependent spectral changes...
associated with binding of ligands to the reduced enzyme (Figure 4) and, presumably, perturbation of the absorbance characteristics of reduced cofactor (Hartmann et al., 1993). Specifically, after consideration of the blank spectra of ligands alone (not shown), binding of SPD resulted in the appearance of peaks at around 251 and 299-306 nm, BZ binding resulted in the appearance of a peak at around 297 nm, and CLON binding resulted in the appearance of peaks at around 246 nm and 310 nm. Since the peaks appearing in each case in the region of 297 - 310 nm may result from perturbation of species similar to that described previously as being associated with the complex between reduced BPAO and BZ (Hartmann et al., 1993), peak heights were plotted versus ligand concentrations (Supplementary Figure S1). Based on the assumption that observed spectral changes were a result of interactions of ligands at the active site, rather than at the secondary site, data were fitted by nonlinear regression to a four-parameter logistic equation (Prism 5.0) to estimate the affinities of ligands for the active site of the reduced enzyme. The approximations for $K_d$ values obtained were $\approx 265$ µM (SPD) and $\approx 59$ mM (BZ). No point of inflexion, and thus no estimate of $K_d$, was obtained from data from experiments with CLON. The rank order of potency of these estimates is similar to that for binding of these substrates to the active site of the reduced enzyme obtained by kinetic analyses, of around 520 µM (SPD) and around 4.21 mM (BZ). ($K_i$ values in Tables 1 and S2).

Addition of TPP+ ($\approx 50$ µM) to anaerobic cuvettes containing reduced BPAO and SPD, BZ or CLON failed to reverse spectral changes caused by these ligands (data not shown). Since this concentration of TPP+ should be sufficiently high to displace ligands almost completely, these observations suggest that spectral changes on addition of SPD, BZ or CLON result predominantly from ligands binding at the BPAO active site, rather than at the secondary imidazoline site. Addition of TPP+ alone to reduced BPAO induced only minor spectral differences (not shown).

**Crystal structure of the BPAO-clonidine complex**

The dimeric architecture and quaternary structure of the enzyme in complex with CLON are well conserved, compared with the apoenzyme (Lunelli et al., 2005). Superpositioning of Ca atoms shows an
overall r.m.s.d. value of 0.35 Å, with major changes involving the residues defining the catalytic pocket and the substrate entrance channel (see Supplementary Table S3). Loops Tyr230-His240 and Met467-Asp471 exhibit the highest Cα displacements. Asn469 rotates approximately 180° away from the internal pocket to face the mouth of the entrance channel, thereby permitting the docking of CLON. In addition, several residues (Gly580-Ala581, Ala610-Pro613 and Val696-Gly701) forming an internal channel present at the interface between monomers show small but significant displacements with respect to native BPAO. This channel may represent a conduit through which molecular oxygen can reach the active site and H2O2 could exit (Lunelli et al., 2005). Some displacement was also evident in the residues forming a hairpin loop (Val430-Val460), protruding from each monomer to wrap around the other subunit. In this case the residues between His442 and Gly452 were not visible at all in the electron density maps, and some residues just before and after that fragment show a strong displacement and high temperature factors (Supplementary Table S3). However, this region is also highly flexible in the apoenzyme structure, thereby indicating a somewhat flexible nature for this protein arm.

One molecule of CLON binds similarly in each of the two active sites of the BPAO dimer. CLON extends as far as the bottom of the funnel shaped, solvent accessible, catalytic cavity next to TPQ470. Binding of CLON causes a displacement of TPQ, inducing a rotation of the TPQ side chain to the “on-copper” non-productive orientation, in which the hydroxyl group of TPQ coordinates the copper ion, together with three histidine residues (His519, His521 and His683). The phenyl ring of CLON is observed to lie almost perpendicular to the imidazolidine portion of the molecule, according to the more stable conformation optimized by Remko (Remko et al., 2001), and it is clearly trapped by stacking interactions in a “sandwich like” orientation within the aromatic side chains of TPQ470 and Tyr472 residues (Figure 5).

Two of the nitrogen atoms (N2 and N3) in the imidazolidine moiety are at a suitable orientation and distance to interact through hydrogen bonding with Asp385, the general catalytic base (Hartmann et al., 1993; Mure et al., 2002). The N3 nitrogen in the heterocyclic ring can also strengthen the inhibitor-enzyme interaction through a NH-π interaction with Tyr383 (Figure 5).
Kinetic analyses of BPAO-ligand interactions

With SPD as substrate, kinetic analyses of the effects of TPP⁺ on BPAO activity (Figure 6) were consistent with a model (Scheme 1) in which a single SPD molecule could bridge the active and secondary sites on a monomer, and in which TPP⁺ could inhibit binding and oxidation of SPD through a competitive interaction at a single site. However, although Scheme 1 is described by Equation 2, data were fitted with a modification of Equation 2, in which the terms $K_{act}$, $\sigma$ and $f$ were replaced with the terms $K_{imid}$, $\gamma$ and $d$, respectively, to reflect the fact that single site binding of TPP⁺ occurs to the imidazoline site rather than to the active site with that particular ligand. Kinetic constants were determined and are listed in Table 1. In contrast, when BZ was used as substrate, TPP⁺ was significantly less potent as an inhibitor (Figure 7), and data could be fitted with similar success to models in which BZ bound only to the active site, or to both the active and secondary sites (Scheme 2), on both oxidised and reduced enzyme, with binding of TPP⁺ again restricted to the secondary site. Since radioligand binding studies had suggested that BZ binds both to the active and secondary sites, kinetic constants (Supplementary Table S2) were thus obtained following fitting of data to an appropriate equation (Supplementary Equation S1) based on scheme 2.

While TPP⁺ is too large to enter the active site cavity of BPAO, the other modulators of enzyme activity examined (CLON, GBZ, 2-BFI and (+)TCP) are sufficiently small that access might be gained both to the active and to the secondary sites. With SPD as substrate, kinetic analyses of the effects of CLON on BPAO activity (Figure 8) were consistent with a model (Scheme 3) in which CLON could compete with SPD binding through interactions at both the active and secondary sites, on both forms of the enzyme. This contention was supported by data from radioligand binding and crystallisation studies (vide supra). Data were fitted with an appropriate equation (Supplementary Equation S2) from which kinetic constants were determined (Table 1). The effects of GBZ, 2-BFI and (+)TCP on SPD oxidation could also be described successfully with this equation, and kinetic constants thereby derived are listed in Table 1. (-)Tranylcypromine was largely devoid of inhibitory potency (not shown).
When BZ was used as substrate, acceptable fits could not be obtained with equations derived from models based upon an interaction of CLON with a single site, whether for BZ interacting only with the active site, or with both the active and secondary sites. While better fits were attainable with models requiring binding of CLON to both sites, the number of kinetic and rate constants (variables) in the associated equations (14 for BZ binding to the active site and 23 for BZ binding to the active and secondary sites, on both oxidised and reduced enzyme) made difficult global curve-fitting without constraining several variables. Nevertheless, based on deductions from results of other experiments described here, the latter model (Scheme 4) and associated equation (Supplementary Equation S3) were used to fit curves to data showing effects of CLON upon BZ oxidation by BPAO (Figure 9). Several variables were initially constrained to equal those determined in experiments with CLON in which SPD was the substrate used, to facilitate preliminary regression analyses. Calculated variables obtained thereafter are listed in Supplementary Table S2. Data from experiments with 2-BFI and GBZ were also fitted with Supplementary Equation S3, in the manner described above (Supplementary Table S2). In contrast with CLON, which enhanced the oxidation rate of BZ significantly at all concentrations of CLON used, only inhibition of BZ oxidation was observed in the presence of GBZ, 2-BFI or (+)TCP. Analyses of model data (not shown) suggested that marked enhancement of substrate turnover can only occur when enzyme reoxidises faster with drug bound to E$_{\text{red}}$ than with substrate bound, regardless of relative affinities of the ligands for E$_{\text{ox}}$ and E$_{\text{red}}$.

While data presented here demonstrate clearly the abilities of several imidazoline binding site ligands such as CLON, 2-BFI and GBZ to interact with BPAO and influence enzyme activity, the ligand specificity of the imidazoline site identified on BPAO, as well as the affinities of these ligands for this site, indicate that the present site does not display characteristics typical of other imidazoline sites identified and characterised thus far (Head and Mayorov, 2006).
Kinetic analyses of hSSAO-ligand interactions

As is the case with BPAO, an anionic binding cavity can be seen at the mouth of the substrate entrance channel on a hSSAO monomer (Airenne et al., 2005). However, in hSSAO, the cavity appears smaller and is more occluded from bulk solvent. While we have not observed any ability of TPP⁺ to inhibit hSSAO activity, replacement of TPP⁺ with the smaller mono-, di- and tri-methylphenylphosphonium analogues also failed to cause any hSSAO inhibition, although potency versus BPAO was still evident (Supplementary Figure S2). In the absence of a hSSAO inhibitor specifically targeting the secondary site, subsequent kinetic experiments focussed upon compounds presumed to act primarily through binding to the active site, with the possibility that simultaneous binding of a second ligand molecule to the secondary site may also occur.

Oxidation of all three hSSAO substrates examined (MA, BZ and PEA) was regulated by each of the three reversible ligands used (GBZ, (+)TCP and (+)AM) in a manner which could be accounted for adequately by the model shown in Scheme 1, described by Equation 2. Data obtained with GBZ are shown in Figure 10, with kinetic constants obtained by global nonlinear regression listed in Table 2, along with those from experiments with (+)TCP and (+)AM.

We found no evidence supporting the view that any of the substrates or modulators examined interact with the secondary site on hSSAO in a manner that influences substrate kinetics. Accordingly, slight improvements in goodness-of-fit obtained with more complex kinetic models implicating binding at the secondary site (not shown) were thought likely to be due to the increased number of variables, and thus to the increased flexibility, in the associated equations.

Both the validity and the usefulness of the pseudoquantitative rapid equilibrium approach used to generate rate equations for reactions catalysed by both BPAO and hSSAO are supported by the reproducibility of kinetic constants obtained by global analyses of data for a single substrate in the presence of different modulators (mean ± SE values in Tables 1, 2 and S2).

The combinations of enzyme-ligand interactions proposed to occur based on results from the kinetic, spectral, radioligand binding and crystallographic studies described here are illustrated in Figure 11.
Discussion

We have examined interactions of substrates and modulators with BPAO, an enzyme for which extensive structural and mechanistic data are available (Hartmann et al., 1993; Janes et al., 1990; Lunelli et al., 2005; Su and Klinman, 1998), and which has been purified in amounts sufficient to facilitate spectroscopic and radioligand binding studies. Kinetic models based upon information obtained from experiments with BPAO were applied to recombinant hSSAO, available in amounts precluding spectroscopic analyses, in order to define the roles played by substrates and modulators binding to active and imidazoline binding sites in regulating substrate catalysis.

Extended kinetic curves for copper-containing amine oxidases show clearly that initial velocities fall at higher substrate concentrations (Holt et al., 2007; Ignesti, 2003; McEwen, 1965; Shepard and Dooley, 2006). With both BPAO and hSSAO, oxidation proceeds by a ping-pong bi-ter process. While methodologies are available which could generate substrate inhibition equations in such systems, these involve complex mathematics (Bardsley et al., 1980; Segel, 1993) which become unmanageable when terms describing regulators acting at multiple sites are introduced.

We therefore adopted a “pseudoquantitative” rapid equilibrium approach, in which several intermediate reactions are grouped together under one “pseudo” rate constant. In designating ES to represent both E_{ox-NH^+CH_2R} and E_{red}, we imply the existence of only one enzyme species (E), with formation of Schiff’s base intermediates, release of aldehyde and generation of an aminoquinol (Mure et al., 2002), occurring instantaneously. The rate-limiting contribution of this reductive half-reaction (Farnum et al., 1986) indicates that this implication is incorrect. We have nevertheless found these equations useful for fitting multiple data sets with the global nonlinear regression facility of GraphPad Prism, since acceptable global fits are generally obtained only when underlying models are correct.

Subsequently, we have found that oxidation of SPD by BPAO is consistent with a model describing partial uncompetitive inhibition by substrate (Holt et al., 2007). Although kinetic analyses could also be interpreted as substrate binding to a separate allosteric site, or as cooperativity between subunits,
spectroscopic data support the view that substrates can bind both to the active site of resting enzyme and to the aminoquinol (reduced) active site following release of aldehyde. Addition of BZ to anaerobic BPAO resulted in characteristic absorbance changes, with peaks at 300-310 nm and 480 nm associated with reduced TPQ cofactor (Hartmann et al., 1993). Addition of SPD to reduced BPAO resulted in a concentration-dependent increase in the 300-310 nm peak height, suggesting that SPD binds sufficiently close to reduced TPQ to cause perturbation of the cofactor absorbance spectrum.

With this two-site model used to describe oxidation of BZ by BPAO, addition of steps to account for effects of CLON and other modulators on BZ metabolism were unsuccessful. Previous kinetic analyses indicated that CLON and other imidazoline ligands bind to four sites on BPAO (Holt et al., 2004). We therefore hypothesised that two of these sites correspond to the active site on an oxidised and reduced subunit, and that the remaining two sites correspond to the TPP+/imidazoline site present on an oxidised and reduced subunit. Through use of a novel radioligand binding assay procedure allowing quantification of modest affinity binding of $[^3]HTPP^+$ to pure soluble BPAO, we found that CLON, as well as SPD and BZ, could compete with $[^3]HTPP^+$ for specific binding. Furthermore, while examination of BPAO crystals grown in the presence of CLON did not reveal low affinity binding at the imidazoline site, marked distortion of residues Glu432-Val458, at the mouth of the substrate entrance channel and within the hairpin loop region was nevertheless apparent. Significantly, binding of CLON was confirmed within the active site cavity, with the aromatic ring of CLON $\pi$-stacked between the rings of TPQ$_{470}$ and Tyr$_{472}$, forcing the TPQ into an inactive “on-copper” conformation. Taken together, these data indicate that while compounds such as SPD are able to bridge the active and imidazoline sites of BPAO, smaller cationic species such as BZ or CLON may bind at one or both of the active and imidazoline sites on both an oxidised and a reduced subunit.

Accordingly, control BZ data were fitted to a model allowing BZ to bind to the active and imidazoline sites on BPAO, binding to the latter influencing substrate access and/or substrate kinetics at the active site, to some degree. Nevertheless, inhibition remained partial for both BZ and SPD, suggesting that enzyme can
still be reoxidised in the presence of substrate at a concentration sufficient to saturate the reduced active site, albeit at a rate lower than that at which enzyme reoxidation normally occurs. It seems unlikely that substrate binds directly to the reduced cofactor. Rather, electrostatic and hydrophobic interactions, including aromatic stacking similar to that observed with CLON, may facilitate substrate binding within the reduced active site. Binding of substrate in this manner may result in steric hindrance of O$_2$ access to, or of H$_2$O$_2$ diffusion from, the active site copper-quinone complex, thereby reducing catalytic rate constants. Such a mechanism may be supported by the observation that binding of CLON to BPAO resulted in some displacement of residues forming a channel from the large internal cavity of the dimer via which O$_2$ and H$_2$O$_2$ may enter and exit the active site (Lunelli et al., 2005). Alternatively, it is also possible that substrate binding stabilises the aminoquinol enzyme in a non-productive “on-copper” conformation.

Our analyses support the view that activation of BPAO by imidazoline ligands results from disinhibition of the enzyme by competition with substrate for binding to E$_{red}$, leading to a drug-E$_{red}$ complex which reoxidises more rapidly than a substrate-E$_{red}$ complex, as well as an apparent reduction in $K_M$ typical of an ordered uncompetitive mechanism (Segel, 1993). The substrate-dependence of modulation may thus be attributed to the relative affinities of the substrate and modulator for both enzyme forms, and to the relative rates of reoxidation of the complexes formed between E$_{red}$ and the respective ligands. Enzyme activation with CLON, for example, may then reflect a faster off-rate for CLON, compared with that for substrate, with reoxidation occurring while dissociated CLON occludes the substrate entrance channel. It is interesting that substrate inhibition is attenuated as the concentration of buffer cations increases (Holt et al., 2007). Alkali metal cations may bind close to an on-copper iminoquinone in *H. polymorpha* copper amine oxidase (Plastino et al., 1999), and it seems plausible that cation binding close to an aminoquinol may repel binding of cationic substrate without hindering enzyme reoxidation.

Kinetic analyses of the effects of several reversible BPAO modulators support binding of ligands to both the active and imidazoline sites on oxidised and reduced BPAO, with modulation occurring in a substrate-specific manner. While CLON enhanced SPD oxidation only at high substrate concentrations, BZ oxidation
was always enhanced, relative to controls. GBZ and (+)TCP caused only inhibition at all substrate concentrations. BZ oxidation was always inhibited by 2-BFI, while SPD metabolism was enhanced at higher substrate concentrations. Effects versus SPD occurred at 2-BFI concentrations 10-fold lower than those versus BZ, indicating a preference for binding of 2-BFI to the imidazoline site. TPP⁺, which only binds to the imidazoline site, was far more potent as an inhibitor of SPD oxidation, consistent with previous observations (Di Paolo et al., 2004).

When mechanisms deduced for BPAO were applied to kinetic data for hSSAO, curves could be fitted successfully with two-site equations describing ligands binding only at the active site, even in the case of ligands which bound to both active and imidazoline sites on BPAO. SPD is neither a substrate nor a competitive inhibitor of hSSAO (A. Holt, unpublished), perhaps because the Leu₄₆₉ active site gate (Airenne et al., 2005) would require SPD to adopt a sharply-angled conformation in order to bind. While a hSSAO substrate which bridges the active and imidazoline sites is not presently available, neither TPP⁺ nor its analogues were able to inhibit turnover of the smaller amine substrates used and binding of TPP⁺ analogues to the imidazoline site on hSSAO without affecting enzyme activity may not be ruled out.

Although none of the compounds tested enhanced substrate turnover, substrate inhibition of hSSAO was nevertheless evident, and it is presumed that the mechanism by which this occurs is similar to that with BPAO. Thus, enhancement of hSSAO activity by a suitable modulator seems entirely feasible. In this regard, an endogenous substrate such as methylamine might either inhibit or enhance the oxidation of other substrates, including protein-bound substrates, thereby offering a novel mechanism for regulating the physiological function of hSSAO. Furthermore, the substrate selectivity which is clearly evident for inhibitors/modulators of hSSAO (and BPAO) may offer therapeutic benefits, since physiological and pathophysiological roles for hSSAO may involve several distinct substrates. Thus, it may be possible to inhibit a deleterious action of hSSAO while leaving physiological functioning of the enzyme largely unaltered.
Our data thus offer a feasible explanation for several anomalous kinetic observations made previously with copper-containing amine oxidases. Drugs which show selectivity for oxidised or reduced hSSAO, or which bridge active and imidazoline sites, may possess unique therapeutic potential as regulators of hSSAO in inflammatory cardiovascular conditions.
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Footnotes

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Address reprint requests to:

Dr. Andrew Holt, Department of Pharmacology, Faculty of Medicine and Dentistry, 9-70 Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7 *

e-mail aholt@pmcol.ualberta.ca

* Primary laboratory of origin
Legends for figures and schemes

**Figure 1. Structures of SSAO ligands used in these studies.** (a) clonidine; (b) 2-BFI; (c) guanabenz; (d) S-(+)-amphetamine; (e) 1S,2R-(+)-tranylcypromine; (f) Tetraphenylphosphonium.

**Figure 2. Specific binding of [3H]TPP+ to BPAO.** To quantify total binding, BPAO (17.4 pmol subunits) and [3H]TPP+ were added to the sample reservoir of a Microcon YM-10 centrifugal filter device. Following incubation (15 min, 30° C), devices were centrifuged to remove more than 99% of unbound [3H]TPP+, and reservoirs were then soaked in scintillation fluid and counted for radioactivity. Non-specific binding was quantified in parallel experiments by including TPP+ (1 mM) in the reservoirs. Calculated values for specific binding (total binding minus non-specific binding) were plotted and data fitted by nonlinear regression to an equation for two-site hyperbolic binding. The high affinity \( B_{\text{max}} \) of 32 pmol per 17.4 pmol subunits indicates binding of two [3H]TPP+ molecules to two high affinity sites per subunit, with similar \( K_d \) values of around 94 nM. Replicate readings were not made, since final free ligand concentrations could only be determined following centrifugation.

**Figure 3. Competition binding studies at the imidazoline binding site on BPAO.** Specific binding of [3H]TPP+ (106 nM) to BPAO was assessed in the presence of TPP+ (●), SPD (○), clonidine (▲) and BZ (△). Data were fitted to uniphasic sigmoidal curves with a 4-parameter logistic equation. While TPP+ could displace all bound [3H]TPP+ in a uniphasic manner, SPD and BZ displaced approximately 49% and 59% at calculated plateaus, respectively, while curve-fitting to clonidine data failed to indicate the position of a plateau. Competitor \( K_d \) values were calculated from IC\( _{50} \) values (see text). Issues relating to solubility and effects on non-specific binding precluded the use of higher concentrations of competitor ligands. Data are the mean ± SEM of 3 replicate determinations.

**Figure 4. BPAO difference spectra following anaerobic binding of SPD (a), BZ (b) and CLON (c) to the reduced enzyme.** Ligands were added cumulatively to BPAO (10 µM) which had been reduced under anaerobic conditions with BZ (50 µM) and readings were recorded after spectra had stabilised (30 s - 17 min).
Figure 5. **Molecule of CLON bound within a BPAO active site.** CLON occupies a hydrophobic pocket next to the Cu²⁺ binding site, forcing the cofactor (TPQ₄⁷₀) into an “on-copper” inactive orientation. The aromatic ring of CLON is seen clearly to undergo π-stacking between the rings of TPQ₄⁷₀ and Tyr₄⁷₂. Hydrogen bonding of CLON nitrogens N₂ and N₃ to Tyr₃₈₃ and Asp₃₈₅ are indicated by hatched lines.

Figure 6. **Effects of TPP⁺ on oxidation of SPD by BPAO.** SPD (10 µM - 4 mM) was incubated with BPAO in the absence (controls; ●) or presence of TPP⁺ at concentrations of 50 (○), 120 (▲), 250 (△) and 500 nM (▼). Initial velocities were measured by a peroxidase-coupled absorbance assay. Data were fitted with Equation 2 by the global curve-fitting nonlinear regression facility of GraphPad Prism v. 5.00. Kinetic constants thereby derived are listed in Table 1. Data are the mean ± SEM of 5 replicate observations.

Figure 7. **Effects of TPP⁺ on oxidation of BZ by BPAO.** BZ (200 µM - 48 mM) was incubated with BPAO in the absence (controls; ●) or presence of TPP⁺ at concentrations of 150 nM (○), 300 nM (▲), 700 nM (△), 1.5 µM (▼), 3 µM (▼) and 10 µM (◆). Initial velocities were measured by a peroxidase-coupled absorbance assay. Data were fitted with Supplementary Equation S1 by the global curve-fitting nonlinear regression facility of GraphPad Prism v. 5.00. Kinetic constants thereby derived are listed in Supplementary Table S2. Data are the mean ±SEM of 4 replicate observations.

Figure 8. **Effects of CLON on oxidation of SPD by BPAO.** SPD (10 µM - 4 mM) was incubated with BPAO in the absence (controls; ●) or presence of CLON at concentrations of 50 µM (○), 150 µM (▲), 400 µM (△) and 1 mM (▼). Initial velocities were measured by a peroxidase-coupled absorbance assay. Data were fitted with Supplementary Equation S2 by the global curve-fitting nonlinear regression facility of GraphPad Prism v. 5.00. Kinetic constants thereby derived are listed in Table 1. Data are the mean ±SEM of 5 replicate observations.

Figure 9. **Effects of CLON on oxidation of BZ by BPAO.** BZ (200 µM - 48 mM) was incubated with BPAO in the absence (controls; ●) or presence of CLON at concentrations of 30 µM (○), 100 µM (▲), 300 µM (△), 1 mM (▼), 3 mM (▼) and 10 mM (◆). Initial velocities were measured by a peroxidase-coupled absorbance assay. Data were fitted with Supplementary Equation S3 by the global curve-fitting nonlinear regression facility of GraphPad Prism v. 5.00. Kinetic constants thereby derived are listed in Table 1. Data are the mean ±SEM of 5 replicate observations.
nonlinear regression facility of GraphPad Prism v. 5.00. Kinetic constants thereby derived are listed in Supplementary Table S2. The curve fitted to control data is shown as a hatched line to emphasise the stimulatory effects of CLON with BZ as substrate. Data are the mean ±SEM of 4 replicate observations.

**Figure 10. Effects of GBZ on oxidation of BZ (a), MA (b) and PEA (c) by hSSAO.** Substrates (BZ, 30 \(\mu\)M - 14 mM; MA, 250 \(\mu\)M - 100 mM; PEA, 500 \(\mu\)M - 100 mM) were incubated with hSSAO in the absence (controls; ●) or presence of GBZ at concentrations of 6 (○), 12 (▲), 20 (△), 30 (▼), 40 (◇), 52 (◆), 65 (◇), 80 (□) and 100 \(\mu\)M (□) (with BZ as substrate) or at concentrations of 3 (○), 7 (▲), 12 (△), 18 (▼), 25 (▽), 35 (◆), 45 (◇), 60 (□) and 75 \(\mu\)M (□) (with MA or PEA as substrate). Initial velocities were measured by a peroxidase-coupled fluorescence assay. Data were fitted with Equation 2 by the global curve-fitting nonlinear regression facility of GraphPad Prism v. 5.00. Kinetic constants thereby derived are listed in Table 2. Data are the mean ±SEM of 3 replicate observations.

**Figure 11. Cartoon illustrating the catalytic cycles of BPAO and hSSAO and the combinations of ligand-protein equilibria affecting reaction velocities.** Enzymes contain anionic cavities close to the mouth of the entrance channel (the imidazoline site) or deep within the protein (the active site). Molecules shown as light structures on a dark background reflect binding only to BPAO and not to hSSAO. Substrates may bind to the active site on the oxidised \((K_M)\) or reduced \((K_i)\) enzyme forms. Binding of SPD bridges both the active and imidazoline sites of BPAO; in contrast, binding of smaller substrates such as BZ to the active site does not preclude binding of a second substrate molecule to the imidazoline site on the oxidised \((K_{sec})\) or reduced \((\alpha K_{sec})\) forms of BPAO. Drug ligands may compete with substrates for binding to the active site on the oxidised \((K_{act})\) and reduced \((\sigma K_{act})\) enzymes, and/or in the case of BPAO, for binding to the imidazoline site on the oxidised \((K_{imid})\) and reduced \((\gamma K_{imid})\) forms of BPAO. Binding of substrates to the active site of the oxidised enzyme results in catalysis, while binding to the active site of the reduced enzyme results in substrate inhibition. The specific effects of a modulator on enzyme activity depend largely upon the relative degrees to which catalysis and substrate inhibition are influenced by the compound of interest. Although the current kinetic studies revealed no requirement for involvement of an imidazoline binding site...
on hSSAO in order to model data successfully, the potential nevertheless exists for future noncompetitive hSSAO inhibitors to target this anionic cavity, and novel hSSAO ligands which bind solely, or in a bridging manner, to the imidazoline site may offer significant improvements in selectivity, potency and side-effect profile.

**Scheme 1. Reaction scheme for interactions of a single substrate molecule or a single inhibitor molecule with the active site on oxidised and reduced SSAO enzyme forms.** To facilitate equation derivation by a “pseudoquantitative” rapid equilibrium approach (see text), ES is used to refer both to oxidised enzyme with substrate bound and to reduced enzyme following release of aldehyde product. As such, when substrate or inhibitor bind to the reduced enzyme, the resulting species are indicated as ESS or ESI, respectively. The equation describing this mechanism is Equation 2. If binding of substrate and inhibitor are exclusive processes, the value of factor $f$ should approximate zero. While scheme 1 (and equation 2) illustrates interactions of substrate and inhibitor with the SSAO active site, binding of an exclusive imidazoline site-directed BPAO inhibitor, such as TPP*, and subsequent competitive inhibition of oxidation of a bridging substrate such as SPD, may also be described by this scheme if $K_{act}$, $\sigma$ and $f$ are replaced with $K_{imid}$, $\gamma$ and $d$, respectively (see text).

**Scheme 2. Reaction scheme for interactions of small substrate molecule(s) with active and/or secondary (imidazoline) sites, as well as interactions of a single molecule of TPP+ with the secondary site, on oxidised and reduced SSAO enzyme forms.** The equation describing this mechanism is Supplementary Equation S1. Notations used are as indicated in the legend to Scheme 1. In addition, binding of a ligand to the SSAO active site is indicated by inclusion of a suffix to E, while binding to the secondary site is indicated by inclusion of a prefix to E. For example, IESS refers to reduced SSAO (ES) with a single substrate molecule (suffix S) bound to the active site and TPP+ (prefix I) bound to the secondary site.

**Scheme 3. Reaction scheme for interactions of a single substrate molecule with the active site, as well as interactions of inhibitor/modulator molecule(s) with the active and/or secondary sites, on oxidised
and reduced SSAO enzyme forms. The equation describing this mechanism is Supplementary Equation S2. Notations used are as indicated in the legends to Schemes 1 and 2.

Scheme 4. Reaction scheme for interactions of small substrate molecule(s) with the active and/or secondary sites, as well as interactions of inhibitor/modulator molecule(s) with the active and/or secondary sites, on oxidised and reduced SSAO enzyme forms. The equation describing this mechanism is Supplementary Equation S3. Notations used are as indicated in the legends to Schemes 1 and 2.
## Tables

### Table 1.

Kinetic constants determined for metabolism of SPD by BPAO in the presence of modulatory ligands, measured in an absorbance assay. Data were obtained from global nonlinear regression of five data sets (curves) composed of 18 points per curve, with five replicate determinations made for each point. Curves were fitted with the equations indicated.

| Modulator | Eq | $V_{\text{max}}$ (mOD/min) | $K_M$ (µM) | $K_i$ (µM) | $K_{\text{inact}}$ (µM) | $\gamma$ | $\delta$ | $\sigma$ | $\epsilon$ | $\pi$ | $a$ | $d$ | $e$ | $f$ | $g$ | $r^2$ | $n$ |
|-----------|----|-----------------|----------|---------|-----------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| (Control) | PUS $^a$ | 5.56 (0.33)$^c$ | 40.2 (5.5) | 456 (137) | - | - | - | - | - | - | - | 0.448 (0.023) | - | - | - | - | 0.620 | 25 |
| TPP$^+$ | 2$^b$ | 5.91 (0.21) | 41.0 (3.6) | 543 (77) | 0.141 (0.018) | 2.57 (0.56) | - | - | - | - | - | 0.387 (0.012) | 0.00233 | - | - | - | - | 0.935 | 5 |
| CLON S2 | 5.17 (0.30) | 35.5 (5.2) | 466 (163) | 42.4 (+) | 23.4 (+) | 13.2 (+) | 0.932 (+) | 2.81 (+) | 0.825 (+) | 3.31 (+) | 0.506 (0.026) | 4.25 (+) | 0.776 (0.165) | 1.02 (+) | $\leq$ 0 | 0.956 | 5 |
| GBZ S2 | 6.31 (0.24) | 38.2 (3.8) | 782 (180) | 1.40 (+) | 0.518 (+) | 1.50 (+) | 40.9 (+) | 4.89 (+) | 1.34 (+) | 1.49 (+) | 0.417 (0.020) | 0.194 (+) | $\leq$ 0 | 0.0572 (+) | $\leq$ 0 | 0.962 | 5 |
| 2-BFI S2 | 5.55 (0.37) | 36.4 (5.6) | 310 (109) | 8.70 (+) | 118 (+) | 5.07 (+) | 88.5 (+) | 2.70 (+) | 0.316 (+) | 2.05 (+) | 0.528 (0.03) | 1.01 (+) | $\leq$ 0 | 1.92 (+) | $\leq$ 0 | 0.963 | 5 |
| (+)TCP S2 | 6.96 (0.34) | 40.8 (5.0) | 565 (148) | 63.5 (+) | 4.56 (+) | 2.90 (+) | 1.097 (+) | 23.9 (+) | 0.795 (+) | 0.291 (+) | 0.458 (0.021) | 0.431 (+) | $\leq$ 0 | 0.629 (+) | $\leq$ 0 | 0.940 | 5 |
| Mean±SE $^d$ | - | - | 38.7 ± 1.0 | 520 ± 64 | - | - | - | - | - | - | - | 0.457 ± 0.022 | - | - | - | - | - | - |

**a.** Partial uncompetitive inhibition by substrate - see Holt et al. (2007). Control data were those obtained from experiments with modulators where [modulator] = 0.

**b.** Data from experiments with TPP$^+$ are fitted with a modified version of Equation 2 (see text).

**c.** Values in parentheses indicate SEM from nonlinear regression analyses. (+) indicates SEM significantly greater than the estimated value for the parameter in question.

**d.** Mean values are shown for substrate-dependent kinetic constants expected to remain unaltered in the presence of modulators. For abbreviations, see text.
Table 2. Kinetic constants determined for metabolism of amines by hSSAO in the presence of modulatory ligands, measured in a fluorescence assay. Data were obtained from global nonlinear regression of ten data sets (curves) composed of 18 points per curve, with three replicate determinations made for each point.

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Eq</th>
<th>$V_{\text{max}}$ (RFU/s)</th>
<th>$K_M$ (mM)</th>
<th>$K_I$ (mM)</th>
<th>$K_{act}$ (mM)</th>
<th>$\sigma$</th>
<th>a</th>
<th>f</th>
<th>$r^2$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BZ as substrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Control)</td>
<td>PUS</td>
<td>4.769 (0.385)</td>
<td>0.194 (0.043)</td>
<td>11.5 (14.7)</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>(+)TCP</td>
<td>2</td>
<td>5.395 (0.054)</td>
<td>0.162 (0.006)</td>
<td>19.3 (30.3)</td>
<td>0.731 (0.044)</td>
<td>17.7 (2.9)</td>
<td>0.283 (0.084)</td>
<td>≈ 0</td>
<td>0.982</td>
<td>3</td>
</tr>
<tr>
<td>GBZ</td>
<td>2</td>
<td>4.529 (0.086)</td>
<td>0.184 (0.011)</td>
<td>18.7 (3.9)</td>
<td>0.011 (0.001)</td>
<td>2.41 (0.29)</td>
<td>0.166 (0.075)</td>
<td>0.154 (0.013)</td>
<td>0.970</td>
<td>3</td>
</tr>
<tr>
<td>(+)AM</td>
<td>2</td>
<td>4.073 (0.103)</td>
<td>0.181 (0.013)</td>
<td>13.1 (3.7)</td>
<td>0.121 (0.200)</td>
<td>0.260 (0.077)</td>
<td>≈ 0</td>
<td>0.899</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>-</td>
<td>-</td>
<td>0.180 ± 0.007</td>
<td>15.7 ± 2.0</td>
<td>-</td>
<td>0.260 ± 0.035</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>MA as substrate</strong></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Control)</td>
<td>PUS</td>
<td>5.691 (0.418)</td>
<td>0.576 (0.166)</td>
<td>237 (+)</td>
<td>-</td>
<td>-</td>
<td>0.294 (+)</td>
<td>-</td>
<td>0.584</td>
<td>3</td>
</tr>
<tr>
<td>(+)TCP</td>
<td>2</td>
<td>6.599 (0.043)</td>
<td>0.458 (0.016)</td>
<td>406 (+)</td>
<td>0.542 (0.053)</td>
<td>24.0 (17.1)</td>
<td>≈ 0</td>
<td>≈ 0</td>
<td>0.973</td>
<td>3</td>
</tr>
<tr>
<td>GBZ</td>
<td>2</td>
<td>6.400 (0.031)</td>
<td>0.461 (0.011)</td>
<td>398 (+)</td>
<td>0.0142 (0.0006)</td>
<td>2.14 (0.11)</td>
<td>≈ 0</td>
<td>0.136 (0.008)</td>
<td>0.992</td>
<td>3</td>
</tr>
<tr>
<td>(+)AM</td>
<td>2</td>
<td>4.599 (0.048)</td>
<td>0.642 (0.024)</td>
<td>96.1 (11.9)</td>
<td>4.93 (2.12)</td>
<td>0.255 (0.102)</td>
<td>0.515 (0.018)</td>
<td>0.015 (0.053)</td>
<td>0.966</td>
<td>3</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>-</td>
<td>-</td>
<td>0.534 ± 0.045</td>
<td>284 ± 74</td>
<td>-</td>
<td>0.202 ± 0.125</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>PEA as substrate</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Control)</td>
<td>PUS</td>
<td>2.741 (0.370)</td>
<td>3.78 (1.09)</td>
<td>70.8 (73.4)</td>
<td>-</td>
<td>-</td>
<td>0.040 (+)</td>
<td>-</td>
<td>0.8035</td>
<td>3</td>
</tr>
<tr>
<td>(+)TCP</td>
<td>2</td>
<td>3.157 (0.067)</td>
<td>3.45 (0.18)</td>
<td>63.8 (8.2)</td>
<td>0.564 (0.035)</td>
<td>5.07 (0.65)</td>
<td>0.116 (0.033)</td>
<td>≈ 0</td>
<td>0.980</td>
<td>3</td>
</tr>
<tr>
<td>GBZ</td>
<td>2</td>
<td>2.928 (0.119)</td>
<td>3.81 (0.336)</td>
<td>43.7 (8.4)</td>
<td>0.011 (0.001)</td>
<td>5.94 (1.32)</td>
<td>0.178 (0.033)</td>
<td>0.157 (0.047)</td>
<td>0.961</td>
<td>3</td>
</tr>
<tr>
<td>(+)AM</td>
<td>2</td>
<td>2.405 (0.116)</td>
<td>4.54 (0.42)</td>
<td>39.7 (7.7)</td>
<td>3.68 (1.35)</td>
<td>0.710 (0.333)</td>
<td>0.127 (0.037)</td>
<td>0.377 (0.089)</td>
<td>0.922</td>
<td>3</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>-</td>
<td>-</td>
<td>3.90 ± 0.23</td>
<td>54.5 ± 7.6</td>
<td>-</td>
<td>0.115 ± 0.028</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations and other details are as indicated for Table 1.
MOL #40964 Scheme 1

\[
\begin{align*}
EI & \underset{K_{act}}{\rightleftharpoons} I \\
+ & \underset{K_M}{\rightleftharpoons} E+S
\end{align*}
\]

\[
\begin{align*}
ES & \underset{\sigma K_{act}}{\rightleftharpoons} EI+P \\
\end{align*}
\]

\[
\begin{align*}
ESI & \underset{fK_p}{\rightarrow} EI+P \\
ES & \underset{k_p}{\rightarrow} E+P \\
ESS & \underset{ak_p}{\rightarrow} E+P
\end{align*}
\]
\[
\begin{align*}
\text{IE} + S & \xrightleftharpoons{\gamma K_M} \text{IES} + S \\
& \xrightarrow{K_{\text{imid}}} \text{I} \\
\text{EI} + P & \xrightarrow{d k_p} \text{IE} + P \\
\text{ES} + S & \xrightarrow{\delta K_i} \text{ESS} \\
& \xrightarrow{K_{\text{imid}}} \text{E} + P \\
& \xrightarrow{\gamma K_{\text{imid}}} \text{IE} + P \\
\text{SE} + S & \xrightleftharpoons{\alpha K_M} \text{SES} + S \\
& \xrightarrow{K_{\text{sec}}} \text{S} \\
\text{SE} + S & \xrightarrow{\beta K_i} \text{SESS} \\
& \xrightarrow{K_{\text{imid}}} \text{S} \\
& \xrightarrow{\alpha K_{\text{sec}}} \text{ES} + S \\
\text{SE} + P & \xrightarrow{b k_p} \text{SE} + P \\
\text{SE} + P & \xrightarrow{c k_p} \text{SE} + P
\end{align*}
\]
Equation 1

\[
[TPP^+]_{\text{free}} \text{ (µM)} = \frac{n \text{ (nmol)}}{\text{Volume (ml)}} = \frac{\left( \frac{V_1 \text{dpm} + V_2 \text{dpm} + V_3 \text{dpm}}{\text{specific activity (dpm nmol}^{-1})} \right)}{\left( \frac{V_1 \text{dpm} + V_2 \text{dpm} + V_3 \text{dpm}}{V_1 \text{dpm}} \right) \times 0.1 \text{ ml}}
\]
Equation 2

\[ v = \frac{V_{\text{max}} [S]}{K_M \left( 1 + \frac{[I]}{K_{\text{act}}} \right) + [S] \left( 1 + \frac{[S]}{K_i} + \frac{[I]}{\sigma K_{\text{act}}} \right) + [S] \left( 1 + \frac{[S]}{K_i} + \frac{[I]}{\sigma K_{\text{act}}} \right) } \]