Chlorella Virus PBCV-1 encodes an unusual arginine decarboxylase that is a close homolog of eukaryotic ornithine decarboxylases.

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Abbreviations. PBCV-1, *Paramecium bursaria* chlorella virus; PBCV-1 DC, PBCV-1 decarboxylase; ODC, ornithine decarboxylase; LDC, lysine decarboxylase; DAPDC, diaminopimelic acid decarboxylase; ADC, arginine decarboxylase; PLP, pyridoxal-5'-phosphate; DFMO, α-difluoromethylornithine; DFMA, α-difluoromethylarginine
*Paramecium bursaria* chlorella virus (PBCV-1) is a large double-stranded DNA virus that infects chlorella-like green algae. The virus encodes a homolog of eukaryotic ornithine decarboxylase (ODC) that was previously demonstrated to be capable of decarboxylating L-ornithine. However, the active site of this enzyme contains a key amino acid substitution (Glu for Asp) in a residue that forms an interaction with the δ-amino group of ornithine-analogs in the X-ray structures of ODC. In order to determine if this active site change affects substrate specificity, kinetic analysis of the PBCV-1 decarboxylase (PBCV-1 DC) on three basic amino acids was undertaken. The \( k_{cat}/K_m \) for L-arginine is 550-fold higher than for either L-ornithine or L-lysine, which are decarboxylated with similar efficiency. In addition, α-difluoromethylarginine (DFMA) is a more potent inhibitor of the enzyme than α-difluormethylornithine (DFMO). Mass spec analysis demonstrated that inactivation is consistent with the formation of a covalent adduct on Cys347. These data demonstrate that PBCV-1 DC should be reclassified as an arginine decarboxylase (ADC). The eukaryotic ODCs, as well as PBCV-1 DC, are only distantly related to the bacterial and plant ADCs from their common β/α fold class, thus the finding that PBCV-1 DC prefers L-arginine to L-ornithine was unexpected based on evolutionary analysis. Mutational analysis was carried out to determine if the Glu for Asp substitution at position 296 (position 332 in *Trypanosoma brucei* ODC) conferred the change in substrate specificity. This residue was found to be an important determinant of substrate binding for both L-arginine and L-ornithine, but it is not sufficient to encode the change in substrate preference.
Introduction.

The *Paramecium bursaria* chlorella virus (PBCV-1) is a large, icosahedral, plaque-forming double-stranded DNA virus that replicates in certain unicellular, eukaryotic chlorella-like green algae (1,2). Its 330-kb genome contains ~375 protein-encoding genes and 11 tRNA genes. Many of the gene products are unexpected for a virus, including enzymes in the pathways for hyaluronan, fucose and polyamine biosynthesis. Recently, a 372 codon open-reading frame (A207R) in the PBCV-1 genome was found to encode a homolog of eukaryotic ornithine decarboxylases (ODC) (3).

ODC is a pyridoxal-5'-phosphate (PLP) dependent enzyme that catalyzes the initial step in the polyamine biosynthetic pathway, the decarboxylation of L-ornithine to produce putrescine (4). The polyamines, putrescine, spermidine and spermine are ubiquitous compounds that are essential for cell growth and differentiation in many organisms. They have demonstrated roles in cell cycle, apoptosis, cancer, embryonic development, immune system functions and neurochemistry (5-9). Overexpression of polyamine biosynthetic enzymes leads to mammalian cell transformation (10), or to growth perturbations in plants (11), while knockout of the polyamine biosynthetic enzymes causes polyamine auxotrophy [e.g. yeast (12), *Trypanosoma brucei* (13), *Leishmania donovani* (14,15)] and is lethal at early embryonic stages in mice (16,17). Inhibitors of polyamine biosynthetic enzymes have anti-proliferative effects and have been utilized as anti-cancer and anti-parasitic agents (18,19).

Two distinct structural classes have evolved within the PLP dependent enzymes to catalyze the decarboxylation of basic amino acids (20,21). One family contains only bacterial decarboxylases, and these are structural homologs of aspartate aminotransferase. These include the
constitutive and inducible bacterial ODCs and lysine decarboxylases (LDC), as well as the biosynthetic (constitutive) arginine decarboxylase (ADC). An X-ray structure of a bacterial ODC from this class has been solved (22). The second family contains enzymes from both eukaryotic and prokaryotic organisms that are structurally related to alanine racemase. These include the eukaryotic ODCs, plant ADC, and a number of bacterial enzymes that encompass a wide range of substrate specificities [arginine, ornithine, lysine, diaminopimelic acid and carboxynorspermidine decarboxylase (20)]. The X-ray structures of several eukaryotic ODCs [mouse, *T. brucei* and human (23-27)] and of bacterial diaminopimelate decarboxylase (DAPDC) have been solved (28). The N-terminal domain forms a β/α barrel, and the C-terminal domain is folded into a β-barrel structure. The active sites are formed at the dimer interface between the N-terminal domain of one subunit and the C-terminal domain of the other (Fig. 1A). While all enzymes in the family share a number of essential active site residues (24,29-32), eukaryotic ODCs are very distantly related to ADC and DAPDC, and share only ~15% overall sequence identity based on pair-wise comparisons. However, a bacterial enzyme from this fold type has been described that has activity on both lysine and ornithine (Lys/OrnDC) and shares extensive sequence similarity (~35%) with eukaryotic ODCs (33).

The decarboxylase identified in chlorella virus (PBCV-1 DC) contains nearly 40% sequence identity with the family of eukaryotic ODCs (3). Phylogenetic analysis indicates that PBCV-1 DC branches with the eukaryotic ODCs and with the bacterial Lys/OrnDC enzymes (Fig. 2). Consistent with this grouping, the recombinant PBCV-1 enzyme was characterized and found to have activity on L-ornithine as a substrate. However it was inhibited more strongly by α-difluoromethylarginine (DFMA) than by α-difluoromethylornithine (DFMO), suggesting it might
have unusual substrate specificity.

Alignment of the primary amino acid sequence of this enzyme with the X-ray structures for mammalian and trypanosome ODCs reveals a key amino acid substitution in the substrate-binding pocket that is predicted to alter the substrate specificity (Fig. 1). The structures of *T. brucei* ODC in complex with several substrate and product analogs demonstrate that the δ-nitrogen of L-ornithine forms salt bridge interactions with Asp361 from the C-terminal domain, and with Asp332 from the N-terminal domain across the subunit boundary [(24,26,27); Fig. 1A]. These two residues are conserved in all functional ODCs that have been described.

To investigate the substrate preference of PBCV-1 DC we performed steady-state kinetic analysis on a range of basic amino acids. We show here that, while PBCV-1 DC contains detectable activity with L-ornithine, its activity with L-arginine is significantly higher and, therefore this enzyme should be re-classified as an ADC. The fact that it is closer in amino acid sequence to the enzymes with specificity for L-ornithine and L-lysine, than to the ADCs from bacteria and plants, suggests that PBCV-1 DC represents a new activity within the clade of the ornithine-specific enzymes.
Experimental Procedures.

Materials- Amino acids, polyamines, and the carbon dioxide kit were purchased from Sigma (St. Louis, MO). The AccQ-Fluor Reagent Kit for labeling amino acids was purchased from Waters (Milford, MA). L-[1-14C] and L-[2, 3, 3H]ornithine (47.7 mCi/ mmol) were obtained from New England Nuclear (Boston, MA) and American Radiochemicals, Inc. (St. Louis, MO), and L-[U-14C] arginine (310 mCi/mm) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Sequencing grade modified trypsin was from Promega (Madison, MI) and endopeptidase Glu-C was from Roche Applied Science (Indianapolis, IN). DFMO was obtained from ILEX Oncology (San Antonio, TX) and DFMA, originally obtained from Dr. A. Bitonti (Merrell Dow Research Institute, Cincinnati, OH), was kindly provided by Dr. Patrick M. Woster (Wayne State University, Detroit, MI).

Modeling and sequence analysis- The program InsightII (Accelrys Inc, San Diego) was used to display the active site of T. brucei ODC for the wild-type enzyme in complex with putrescine [PDB ID 1f3t; (27)]. The distance tree was built using the PHYLIP package (34) from the alignment of the displayed sequences constructed with the PCMA program (35). PSI-BLAST was used to identify eukaryotic ODC homologs in Gen Bank version 2.2.8 and to generate a local alignment. In total 53 eukaryotic sequences that had published sequence data were included (hypothetical proteins were excluded from the analysis).

Protein Expression and Purification- PBCV-1 DC was produced in E. coli from the expression plasmid for PBCV-1 DC, termed pODCTM9 which was described in (3). This vector directs the expression of an N-terminal His-tagged PBCV-1 DC from a pET-15b plasmid that contained the cDNA corresponding to an open reading frame A207R present in the PBCV-1 genome. Two
sources of PBCV-1 were used in the studies, 1) a protein with the wild-type sequence was used in the $^{14}$CO$_2$ release assay (Table 1) and for DFMO and DFMA inactivation studies (Fig. 4A and 4B), 2) Protein used for the spectrophotometric analysis, and as the background for the E296D mutant contained the substitution of Ala for Thr at position 142 (Table II, Fig. 3 and Fig. 5 data). This change was generated during cloning but has no effect on the activity or substrate specificity of the enzyme. PBCV-1 DC, PBCV-1 DC E296D, TbODC and TbODC D332E were expressed and purified as described previously for *T. brucei* ODC using Ni$^{2+}$-agarose column chromatography and Sephadex 200 (36).

*Site-directed Mutagenesis*- ODC D332E from *T. brucei* and PBCV-1 DC E296D mutants were produced using the QuickChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA). The primers listed below contain the desired mutations (in bold): D332E forward 5’-TGCATCCTGTATGAGCACGCAGTCG-3’, E296D 5’-CTAACGTTATTGTTTGAAGAATCTGTTCCA-3’.

*Radiolabel Release-based Enzymatic Assays*- ODC and ADC activities were determined by measuring the release of $^{14}$CO$_2$ from the enzymatic decarboxylation of L-[1-$^{14}$C] ornithine or L-[U-$^{14}$C] arginine (37). The $^{14}$CO$_2$ released was trapped in a center well containing hyamine hydroxide and counted by liquid scintillation spectrometry as described previously. Kinetic constants were measured under two conditions using either 25 mM Tris HCl buffer pH 8.2 at 37°C or 50 mM CAPSO buffer pH 9 at 42 °C. All reaction mixes contained 0.04 mM pyridoxal 5’- phosphate and 2.5 mM dithiothreitol. The concentration of L-ornithine was varied from 5 -250 mM and L-arginine from 0.05 - 5 mM. Protein was determined by the Bradford assay using bovine serum albumin as a standard.
The molar ratios of CO₂ formed from the labeled substrate were confirmed to be equivalent to the product by analyzing the products by reverse phase HPLC using a standard system for separation of polyamines (38) coupled to a Canberra Packard A140 radiomatic detector as previously described (39). L-[2, 3,3-H] ornithine was used to detect the formation of putrescine and L-[U-14C] arginine was used for the formation of agmatine. Authentic U-[14C]agmatine and [2, 3,3-H]putrescine were used as markers.

Spectroscopy–based Enzymatic Assays– Steady-state kinetics of the decarboxylation of L-ornithine (1 to 25 mM), L-arginine (0.5 to 16 mM), or L-lysine (1-50 mM) by PBCV-1 DC, TbODC, PBCV-1 DC E296E, TbODC D332E were measured spectrophotometrically at 37°C using Sigma Diagnostics carbon dioxide detection kit as previously described (36). Assays were conducted with 100 μM PLP. The Sigma kit couples decarboxylation of substrate to the oxidation of NADH (\(\lambda_{\text{max}} = 340\text{nm}\)) using phosphoenolpyruvate carboxylase and malate dehydrogenase.

HPLC Analysis of Reaction Products– The products of an enzymatic reaction with L-arginine and wtPBCV-1 DC and TbODC D332E were analyzed by HPLC using the AccQ_TAG kit (Waters, Milford, MA) in buffer (5% sodium tetraborate) and labeling reagent (6-aminoquinolyl-n-hydrozysuccinimidyl in acetonitrile) as previously described (24). PBCV-1 DC or TbODC D332E were incubated with L-arginine (20 mM for wild-type PBCV-1 DC and 170 mM for TbODC mutant) in buffer (15 mM KPO₄, 1 mM DTT, 0.15 mM PLP) at 37°C for 2.5, 5.0, and 10.0 minutes. Enzyme concentrations of 475 and 950 nM for PBCV-1 DC and 100 and 200 μM for TbODC D332E were used in the reactions. Enzymatic reactions were terminated by the addition of TCA to a final concentration of 6%. Labeled samples (5 μL) were injected onto an AccQ_TAG column using previously published buffers and gradient (24). The column was calibrated with
known amounts of the following derivatized reagents: Agmatine (RT = 27.8 min), cadaverine (RT = 40.1 min), L-ornithine (RT = 34.8 min), putrescine (RT = 44.5 min), L-arginine (RT = 21.1 min).

Inactivation of PBCV-1 DC with DFMO or DFMA- The His-tagged PBCV-1 DC protein in 50 mM NaH$_2$PO$_4$ pH 8, 300 mM NaCl, 250 mM imidazole, 0.04 mM PLP, 2.5 mM DTT, 0.5 mg/ml BSA (3) was dialyzed to remove DTT and imidazole before passing over a Talon$^R$ metal affinity column (Clontech, Palo Alto, CA) to remove BSA. After elution and further dialysis an aliquot of PBCV-1 DC protein [104 µg in 50 mM NaH$_2$PO$_4$ pH 8, 0.04 mM PLP, 0.5 mM DTT] was incubated in the absence or presence of 10 mM DFMO or 1 mM DFMA for 2 h at 37°C in a total volume of 250 µl. Aliquots (5 µl) of each reaction were removed at the times indicated and diluted accordingly to monitor inactivation of the protein.

Preparation of samples of PBCV-1 DC protein for MALDI-TOF analysis-MALDI analyses were performed at the Mass Spec/Proteomics Core Facility at the Penn State College of Medicine. Reactions containing 4.2 µg (~100 nmol) of untreated, DFMO or DFMA inactivated PBCV-1 DC protein in a total volume of 100 µl were subjected to tryptic or endoprotease Glu-C digestion using a protease:protein ratio over the range 1:84 to 1:21 (w/w) as indicated. Samples were digested with modified trypsin in 50 mM NH$_4$HCO$_3$ buffer pH 8, containing 10 % v/v acetonitrile for 16 h, stopped by the addition of 4 µl of glacial acetic acid and stored frozen until analyzed by MALDI-TOF. Samples were digested with Glu-C in 25 mM NH$_4$HCO$_3$ buffer pH 7.8 containing 10 % v/v acetonitrile for 2-16 h at 25°C, stopped with glacial acetic acid and stored frozen until analysis by MALDI-TOF using an Applied Biosystems 4700 Proteomics analyzer.
Digested samples were evaporated and resuspended in 200 µl of deionized water three times to remove volatile digestion buffers (NH₄HCO₃) which can interfere with subsequent binding to strong cation exchange resins. The final resuspension was evaporated to ~10 µl, then 1/9th volume of 1.0% TFA was added to bring the final TFA concentration to 0.1%. Using a BioHit Multipipettor, ZipTip SCX tips were equilibrated with three times 10 µl of 0.1 % TFA, then the sample aliquots were pipetted up and down across the resin 15 times to bind and concentrate peptides. The bound peptides were washed with 5 X 10 µl of 0.1% TFA to remove salts, followed by elution of the bound peptides by carefully pipeting 2 µl of freshly prepared 5% NH₄OH/30% MeOH up and down 4-5 times. The droplet containing the eluted peptides was deposited onto a polished stainless steel MALDI target plate (Applied Biosystems) and allowed to dry. After drying, each spot was overlaid with 0.6 µl of freshly prepared CHCA matrix (5 mg per ml recrystallized α-cyano-hydroxycinnamic acid/ 2 mg/ml NH₄H₂PO₄/50% acetonitrile/0.1% TFA).
Results.

Analysis of PBCV-1 DC Substrate Preference and Activity – Decarboxylation of L-ornithine or L-arginine by PBCV-1 DC was measured by following $^{14}$CO$_2$ release from L-[1-$^{14}$C]ornithine or L-[U-$^{14}$C]arginine (Table I). In studies carried out in Tris-HCl buffer, pH 8.2 at 37°C, the protein was highly active on L-arginine with a $K_m$ of 0.45 mM and a $k_{cat}$ of 15 s$^{-1}$. It also had detectable activity on L-ornithine; however, while the $k_{cat}$ was only slightly lower, the $K_m$ was about 400 times higher ($K_m$ of 180 mM; Table I), giving an overall difference in substrate preference of 550-fold for $k_{cat}/K_m$. These results contrast with a previous report of a $K_m$ of 0.78 mM for L-ornithine (3). The previously reported assays were conducted in CAPSO buffer pH 9 at 42°C. The assays were therefore repeated using these conditions and the ability to decarboxylate L-ornithine was slightly improved ($K_m$ of 46 mM; Table I). However, even under these conditions, the protein still preferred L-arginine as a substrate with a $K_m$ two orders of magnitude lower than that for L-ornithine. The products of the reaction were identified by HPLC (38,39) after the protein had acted upon either L-[U-$^{14}$C]arginine or L-[2, 3-$^3$H]ornithine. As expected, $[^{14}$C] agmatine was found in stoichiometric amounts with $^{14}$CO$_2$ when L-[U-$^{14}$C]L-arginine was decarboxylated, and $[^3$H]putrescine was formed in equivalent amounts when L-[2, 3-$^3$H]ornithine was the substrate.

The substrate preference of PBCV-1 DC was also characterized using a NADH coupled spectrophotometric assay. Steady-state decarboxylation was measured for L-arginine, L-ornithine and L-lysine over a wide range of concentrations (Fig. 3). The relative substrate preferences for L-arginine over L-ornithine were similar to those observed by the $^{14}$CO$_2$-release assay, however the $k_{cat}/K_m$ on both substrates was 8-fold lower under the conditions of the spectrophotometric assay (Table II). L-Lysine was also a substrate for the reaction and was decarboxylated with similar
efficiency to L-ornithine (Fig. 3). This result is in contrast to the ODCs from both mouse and T. brucei for which the $k_{\text{cat}}/K_m$ for L-lysine is 300-fold lower than for L-ornithine (31,37). The similar catalytic efficiency of PBCV-1 DC on L-ornithine and L-lysine, however, is reminiscent of the bacterial Lys/OrnDC from Selenomonas ruminantium (33).

Inhibition of PBCV-1 DC with DFMO and DFMA – A previous report indicated that DFMA was a more potent inhibitor of PBCV-1 DC than was DFMO (3). To confirm this finding, we undertook similar inhibition experiments with DFMA and DFMO (Fig. 4A). PBCV-1 DC was irreversibly inactivated by incubation with either DFMO or DFMA. Consistent with previous reports, DFMA inactivated the enzyme more rapidly than DFMO; after a 30 min incubation with 1 mM DFMA only 6% of the activity remained, while 10 mM DFMO reduced the activity to only 26% during the same incubation time. By 1 hr only 0.8% of the activity remained with DFMA, yet 17% of the activity was still present with DFMO.

Mouse ODC is inactivated by DFMO with the formation of a covalent S-[(2-(1-pyrroline)]methyl adduct at Cys360 (major product 90%) and a minor product (c. 10%) at Lys69 (40,41). Although previous studies have shown that DFMA is an irreversible inhibitor of bacterial and plant ADCs (42), the adduct formation site has not been identified. However, based on the inactivation of mouse ODC, the likely sites of interaction in the native PBCV-1 DC are residues Cys347 and Lys71, which correspond to Cys360 and Lys69 in mouse ODC.

Endoproteinase Glu-C digestions of untreated PBCV-1 DC or of PBCV-1 DC inactivated by DFMO or DFMA were analyzed by linear MALDI-TOF MS in positive ion mode, using PBCV-1 DC and Glu-C self-digested peaks for internal calibration of the spectra. The spectra of the inactivated enzyme had new peptide fragments with masses corresponding to adducts on the
Lys$^{321}$-Glu$^{361}$ peptide (KSVPTPQLRDVPDDEEYVPSVLYGCTCDGVDVINHNVALPE) containing Cys347. The presence of this peptide in the Glu-C digest and the absence of shorter fragments cleaved at the internal DDEEY sequence is probably due to the digestions being carried out in NH$_4$CO$_3$ buffer which increases the specificity of Glu-C but restricts activity and the known inactivity of Glu-C to cut at clusters of acidic residues (43). As seen in Fig. 4B, these masses (m/z 4681.0 and 4740.8) were not observed in the control digests, while all three digests contained a peak slightly smaller that the theoretical value (4599.2 average mass) of the equivalent unmodified fragment. (It should be noted that within the limited resolution of these linear spectra, the potential contributions of the unmodified peptide and either of two similar mass Glu-C self-digest peptides to the observed peak cannot be separated definitively).

The new peptide that appears after DFMO inactivation (m/z 4681.0) is 81.8 Da heavier than the unmodified peptide Lys$^{321}$-Glu$^{361}$, which within experimental error agrees with the theoretical 81.1 Da difference previously observed for the S-[(2-(1-pyrroline)]methyl-cysteine adduct formed by DFMO at Cys360 of mouse ODC (41). In the digest of the protein inactivated by DFMA, the new peptide observed at m/z 4740.8 is 141.6 Da larger than the unmodified peptide Lys$^{321}$-Glu$^{361}$. This value is within experimental error of the theoretical adduct mass of 140.2 Da which would occur with the analogous linear DFMA adduct to a Cys in the peptide. This result is consistent with DFMA inactivation occurring similarly to DFMO inactivation of ODC, with decarboxylation of the DFMA-PLP Schiff base leading to the loss of a fluoride ion to generate a reactive electrophilic conjugated imine that binds covalently to Cys 347. Subsequent elimination of the second fluoride anion followed by an internal transaldimination reaction with Lys71 would generate the adduct. This adduct cannot cyclize in the same way as the adduct derived from DFMO.
because of the replacement of the terminal amino group with a guanidino group. The Lys\textsuperscript{321}-Glu\textsuperscript{361} peptide does contain another Cys residue at position 345. Since we were unable to produce interpretable ms/ms spectra of the modified peptides, addition to this site cannot be ruled out but, based on the existing data on the structure and inactivation of mammalian and trypanosomal ODC by DFMO (24-26,41), Cys347 is the likely site of attachment.

Assuming that the peaks in the DFMO- and DFMA-inactivated protein spectra at m/z 4597.9 and 4598.6 respectively do represent unmodified peptide, then not all of the PBCV-1 DC was modified at this site. Although MALDI analysis is not quantitative, the relative sizes of the modified and parent peaks are similar, which suggests that only about half the protein was altered at Cys347, in contrast to the almost total loss of enzyme activity observed, with >99% inhibition (with DFMA) and >90% (with DFMO). One explanation for this discrepancy is that interaction with another site such as Lys71 occurs to a larger extent than with the inactivation of mouse ODC with DFMO. However, we were unable by MS to identify a putative DFMO or DFMA adduct to peptides containing Lys71 after digestion by Glu-C or by trypsin (where an adduct at Lys71 would prevent tryptic cleavage at that site). Alternative explanations could be that: (a) only one of the two PBCV-1 DC subunits making up the homodimer needs to be modified to cause loss of catalytic activity; (b) that analytical workup resulted in the preferential loss of some of the modified peptide; (c) that, although we did not find any other peptides that were significantly different between control and DFMO- or DFMA-inactivated PBCV-1 DC in the analysis of either Glu-C or trypsin digests, modification of an additional residue at the active site occurs. The sequence coverage was about 50% in the Glu-C digests, so modifications of the unrepresented proteolytic fragments would not have been observed; (d) that some proportion of the recombinant protein
extract may be enzymatically inactive prior to the start of the reaction, and thus unable to react with DFMO or DFMA. It is also possible that a significant portion of the “unmodified” peak represents Glu-C self-digestion fragments.

**Sequence analysis of PBCV-1 DC active site changes.** The amino acid sequence for PBCV-1 DC was aligned with ODC family members, including those for which structural information is available. This allowed amino acid changes to be mapped onto the three-dimensional structure of the active site complexed with the putrescine product (Fig. 1). The only residues within 4.5 Å of the putrescine side-chain that are variable between mouse and *T. brucei* ODCs and PBCV-1 DC are Tyr331 (replaced by Phe) and Asp332 (replaced by Glu). When a wider range of ODCs from various species are considered, the residue at position 331 may be either Tyr or Phe, thus the difference in this position for PBCV-1 DC is a previously observed variation.

Asp-332 is highly conserved in the ODC family and plays an important role in substrate binding and catalysis [Fig. 1; (44)]. The equivalent position in PBCV-1 DC is residue 296, which contains a Glu, suggesting that this substitution is a key determinant in the change in substrate specificity observed for PBCV-1 DC (Fig. 1B). To determine if this sequence substitution occurs in any other ODC, database analysis of Genbank 2.2.8 was conducted. Asp332 is invariant in the eukaryotic ODCs examined in the database; the analysis included 53 sequences that were linked to publications (hypothetical proteins were not included in the analysis). The prokaryotic Lys/OrnDC identified in *S. ruminantium* also contains an Asp at this position. Several sequences for antizyme inhibitor (human: NP_680479, mouse: NP_061215, rat: NP_072107), an inactive ODC homolog that regulates ODC activity (45), do contain the D332E substitution. However, the analysis suggests that D332E substitution in PBCV-1 DC is unique among the currently known
eukaryotic ODC-like sequences that encode active enzymes. Thus, since PBCV-1 DC is the only member of the eukaryotic ODC branch that has a preference for L-arginine, and the only member containing the Asp to Glu substitution, it appeared likely that the D296E alteration is a structural determinant responsible for the change in substrate specificity.

Kinetic Analysis of TbODC and PBCV-1 DC mutants – To investigate the impact of the active site difference at position 332 on substrate specificity differences between the ODCs and PBCV-1 DC, mutational analysis was undertaken. The Asp at position 332 was mutated to Glu in *T. brucei* ODC (TbODC D332E) and the equivalent swap was conducted for PBCV-1 DC (PBCV-1 DC E296D). For the wild-type enzymes the substrate selectivity of *T. brucei* ODC for L-ornithine is more stringent than observed for PBCV-1 DC on L-arginine, and thus the conversion would potentially be more difficult. Spectrophotometric and HPLC analyses of the substrate specificity of both wild-type and mutant enzymes demonstrated that these mutations decreased the activity on both L-arginine and L-ornithine (Table II). Significant increases in the $K_m$ on the preferred substrate are observed for both TbODC D332E and PBCV-1 DC E296D compared to wild-type enzymes. In addition for PBCV-1 DC, $k_{cat}$ decreased for both L-ornithine and L-arginine. The $k_{cat}$ effects are not observed in the background of the *T. brucei* sequence. While the mutant enzymes are both much less active than the wild-type enzymes, a small (20-fold) change in the ratio of $k_{cat}/K_m$ when comparing L-arginine and L-ornithine has occurred in both backgrounds, which relaxes the substrate preference towards the alternate amino acid (Fig. 5). The data demonstrate that the residue at position 332 is an important determinant for substrate binding in both *T. brucei* ODC and PBCV-1 DC. However this substitution alone is insufficient to produce the observed substrate specificity change, and clearly additional amino acid changes are required.
Discussion.

The PLP-dependent decarboxylases that belong to the β/α-barrel fold are from both bacterial and eukaryotic origin, and they include enzymes capable of decarboxylating a range of basic amino acid substrates (20). Within this fold class, the eukaryotic ODCs, and a group of bacterial enzymes with dual specificity on L-ornithine and L-lysine [e.g. *S. ruminantium* Lys/OrnDC (33)], share high sequence similarity, while the enzymes with specificity for L-arginine and other basic amino acids share very low sequence similarity with the ornithine specific enzymes (Fig. 2). Thus, PBCV-1 DC is an anomaly because it shares greater sequence identity with the ODCs, while it strongly prefers L-arginine as a substrate. The preference for L-arginine over L-ornithine and L-lysine is reflected in the 550-fold higher \( k_{cat}/K_m \) observed for L-arginine at all pHs and temperatures studied. DFMA is a more potent inhibitor of the PBCV-1 DC than DFMO, although both compounds form adducts at the same site. Thus, our data clearly indicate that PBCV-1 DC should be reclassified as an ADC.

The X-ray structures of ODC provide insight into amino acid variations that are likely to be important for changing substrate specificity. The only residue in direct contact with the substrate that differs between ODCs and PBCV-1 DC is Asp332, which is a Glu in PBCV-1 DC. This residue interacts directly with substrate, and site-directed mutagenesis establishes that it is an important residue for substrate binding in both ODCs and PBCV-1 DC. However, the residue at position 332 is not on its own sufficient to determine substrate preference. Swapping of the residue at position 332 between an Asp and a Glu did not significantly increase the relative activity of either *T. brucei* ODC, or PBCV-1 DC, on the less preferred substrate. These results demonstrate that amino acid changes at residues distant from the active site are also necessary to switch the
substrate preference. A number of studies have shown that amino acid residues that are distant from the active site, and do not contact ligand directly, are often important in the change of function between homologous proteins [e.g. (46-49)]. Indeed we have previously demonstrated that for ODC amino acid residues in the dimer interface that are distant from the active site are important for enzyme activity (44).

PBCV-1 is the first virus known to encode polyamine biosynthetic enzymes (1). In addition to encoding PBCV-1 DC, the virus contains the enzymes necessary to produce putrescine from agmatine (agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase), and it also contains homospermidine synthase, but not spermidine synthase, suggesting that the end product of the pathway in the virus is homospermidine (50). It is interesting to speculate on the evolutionary driving force for the substrate specificity of PBCV-1 DC for L-arginine. In algae, including chlorella isolates, amino acid analyses demonstrated that L-arginine is one of the most abundant amino acids, while L-ornithine is one of the least abundant (51). Thus the lack of L-ornithine in these cells suggests that the switch in substrate specificity of PBCV-1 DC to L-arginine was required for efficient production of putrescine.

The high amino acid sequence identity (40%) between PBCV-1 DC and the ODCs suggests that the PBCV-1 DC is more closely related to enzymes with ornithine specificity than to bacterial ADCs in the β/α fold class, despite their common substrate preference. A potentially important link in this evolution is the observation that a group of bacterial enzymes which also share strong sequence similarity to the eukaryotic ODCs, have dual specificity on L-lysine and L-ornithine (33). PBCV-1 DC groups most closely to these bacterial enzymes in phylogenetic analysis, and these sequences form a bridge between the eukaryotic ODCs and the more distantly related ADCs from
bacteria and plants (Fig. 2). Interestingly, PBCV-1 DC, like the bacterial enzymes has equal activity on both L-lysine and L-ornithine, though it is distinct in preferring L-arginine. These observations suggest that the PBCV-1 DC may have been acquired from a bacterial source, or from a common ancestor, that contained a dual specificity Lys/OrnDC, and that it has evolved a minimum set of amino acid substitutions to switch specificity without adversely affecting activity. Interestingly, the PBCV-1 homospermidine synthase was also proposed to be more closely related to bacterial enzymes than plant enzymes (50), suggesting the entire polyamine biosynthetic pathway in the virus may have been acquired from bacteria.

The role of putrescine and homospermidine in viral pathogenesis remains unclear, since the host cell also makes both polyamines. However, the virus is known to inhibit protein translation in the host (1,2), and altered levels of polyamines could play a role in this inhibition. Eukaryotic translation initiation factor 5A (eIF-5A) plays an essential role in translation of selective messages required for cell division and proliferation (52). This factor is synthesized as an inactive precursor that is modified to its active form by the covalent attachment of hypusine via two enzymatic steps. The first step is catalyzed by deoxyhypusine synthase and utilizes spermidine as the donor substrate. Recently it was reported that this same enzyme can catalyze the reverse reaction using putrescine as an acceptor producing homospermidine and unmodified eIF-5A (53). Putrescine levels increase significantly in chlorella cells after infection with PBCV-1 (50), and this increase could potentially alter the levels of hypusine modified eIF-5A thereby affecting host cell translation.
Acknowledgements. We would like to thank Tariq Ibrahim for technical assistance.
Figure Legends.

**Fig. 1. Active-site of *T. brucei* ODC.** A. Structure of the active-site of *T. brucei* ODC complexed with putrescine [PDB ID 1f3t; (27)]. Residues that are within 4.5 Å of putrescine are displayed. Those contributed from the N-terminal domain of monomer A are displayed in purple, and residues contributed from the C-terminal domain of monomer B are displayed in turquoise. Atoms other than carbon are colored as follows: Nitrogen atoms are blue, oxygen atoms are red, sulfur atoms are yellow, and phosphate atoms are orange.

B. Alignment of a sequence segment for a representative set of eukaryotic ODCs and PBCV-1 DC. Sequences accession numbers are as follows: PBCV-1 DC, NP_048554; *Chlamydomonas reinhardtii* ODC, CAE46409; TbODC, *Trypanosoma brucei* ODC, INJJ_A; Mouse ODC, P00860; *Nicotiana tabacum* ODC, AAQ14852; *Saccharomyces cerevisiae*, NP_012737.

**Fig. 2. Sequence analysis of the β/α-barrel PLP decarboxylase family.** Phylogenetic analysis of a representative set of ODC and ADC sequences. The scale bar corresponds to evolutionary distance of 0.1 amino acid substitutions per site. The distance tree was built using PHYLIP package (34) from the alignment of the eight displayed sequences constructed with PCMA program (35). The branching order of PBCV-1 DC and the bacterial Lys/OrnDC’s relative to the eukaryotic ODCs cannot be conclusively determined by this analysis. Accession numbers for the eukaryotic ODCs are listed in Fig. 1B; for the remaining sequences they are as follows: *Selenomomas ruminantium* Lys/OrnDC; BAA24923; *Pirellula* sp. putative Lys/OrnDC, NP_868377; *Nicotiana tabacum* ADC, BAD06581; *E. coli* ADC, BAB37237.
Fig. 3. Steady-state kinetic analysis of PBCV-1 DC on L-arginine (A) over L-ornithine (B) or L-lysine (C). Decarboxylation was measured using the coupled NADH oxidation assay (see Experimental Procedures). Data points (filled squares) are the average of at least two experiments and error bars represent standard error of the mean. Data were fitted by non-linear regression to the Michaelis-Menten equation and the fitted parameters are: L-arginine ($K_m = 3.5 \pm 0.2$ mM, $k_{cat} = 15 \pm 0.3$ s$^{-1}$), L-ornithine ($K_m = 136 \pm 44$ mM, $k_{cat} = 1.0 \pm 0.2$ s$^{-1}$) and L-lysine ($K_m = 115 \pm 27$ mM, $k_{cat} = 0.45 \pm 0.08$ s$^{-1}$). Data were analyzed with GraphPad Prism version 4.0.

Fig. 4. Inactivation of PBCV-1 DC by DMFA and DFMO. Panel A shows the loss of decarboxylase activity when PBCV-1 DC was incubated with 1 mM DMFA or 10 mM DFMO as indicated. Panel B shows a portion of the linear MALDI-TOF MS spectra (positive ion mode) of Glu-C digested PBCV-1 DC (upper plot), PBCV-1 DC inactivated with 10 mM DFMO (middle plot) and PBCV-1 DC inactivated with 1 mM DFMA (lower plot).

Fig. 5. Comparison of substrate specificity for L-arginine and L-ornithine for wild-type and mutant TbODC and PBCV-1 DC. The ratio of the catalytic efficiency ($k_{cat}/K_m$) on L-arginine vs that for L-ornithine is plotted for wtTbODC (black box), TbODC D332E (horizontal hatch), wtPBCV-1 DC (white box), and PBCV-1 DC E296D (diagonal hatch). Data are displayed in log-scale. An equal catalytic efficiency on both substrates would give a value of zero.
Table I. Steady-state kinetic analysis of PBCV-1 DC on L-ornithine and L-arginine.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$M$^{-1}$)</th>
<th>Buffer</th>
<th>pH/°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arg</td>
<td>0.45</td>
<td>15</td>
<td>$3.3 \times 10^4$</td>
<td>Tris</td>
<td>8.2/37</td>
</tr>
<tr>
<td>L-Arg</td>
<td>0.48</td>
<td>24</td>
<td>$5.0 \times 10^4$</td>
<td>CAPSO</td>
<td>9.0/42</td>
</tr>
<tr>
<td>L-Orn</td>
<td>180</td>
<td>10</td>
<td>60</td>
<td>Tris</td>
<td>8.2/37</td>
</tr>
<tr>
<td>L-Orn</td>
<td>46</td>
<td>14</td>
<td>$3.0 \times 10^2$</td>
<td>CAPSO</td>
<td>9.0/42</td>
</tr>
</tbody>
</table>

Assays were performed using the $^{14}$CO$_2$-trapping method as described in the experimental procedures.
Table II. Steady-state kinetic analysis of mutant and wild-type DC’s from PBCV-1 and *T. brucei*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBCV-1 DC</td>
<td>15 ± 0.3</td>
<td>3.5 ± 0.2</td>
<td>4.2 x 10$^3$</td>
<td></td>
</tr>
<tr>
<td>PBCV-1 DC E296D</td>
<td>0.14 ± 0.004</td>
<td>42 ± 2.7</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>TbODC</td>
<td>0.025</td>
<td>14</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>TbODC D332E$^1$</td>
<td>0.04 ± 0.005</td>
<td>34 ± 10</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>PBCV-1 DC</td>
<td>0.9 ± 0.2</td>
<td>136 ± 44</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>PBCV-1 DC E296D</td>
<td>0.02 ± 0.003</td>
<td>145 ± 30</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>TbODC</td>
<td>12</td>
<td>0.4</td>
<td>3.0 x 10$^4$</td>
<td></td>
</tr>
<tr>
<td>TbODC D332E</td>
<td>10 ± 0.6</td>
<td>6.1 ± 1.2</td>
<td>1.6 x 10$^3$</td>
<td></td>
</tr>
</tbody>
</table>

Kinetic assays were performed using the spectrophotometric method that measures the oxidation of NADH in a coupled assay. $^1$Data for these enzymes were also collected by HPLC analysis of agmatine formation using AccQ_tag labeling as described in the Experimental Procedures, and similar results were obtained. $^2$Data were taken from (30,32).
Figure 1A.

![Diagram showing amino acid interactions and residues](image)

Figure 1B.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino Acid Sequence</th>
<th>Residue Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>MouseODC</td>
<td>GSFNCILYDAHVKALQKRPKDE------KYYSSISWGPTCGL</td>
<td>332-377</td>
</tr>
<tr>
<td>TbODC</td>
<td>GSFNCILYDAHVRPLQREPPIPNE-----KLYPSSWGPTCGL</td>
<td>332-375</td>
</tr>
<tr>
<td>ChlamyODC</td>
<td>GSFNCILYDQNPGYKVRSPMLADSTDSE-RTLSTLWGPTCGLSA</td>
<td>332-375</td>
</tr>
<tr>
<td>PBCV-1 DC</td>
<td>GSFSNIFKEKVPYPTQLLRQVFDDE-----ETYPSVLIGCQVDVHNCVAP-ELHI</td>
<td>343-381</td>
</tr>
<tr>
<td>NicotaODC</td>
<td>GSNMCVLKYDAVNAQPLAVLSDAKTSGS-(GS)KFPTTVFQPTCGL</td>
<td>343-395</td>
</tr>
<tr>
<td>ScerODC</td>
<td>GNMCILFDHQEPHPTRTLYHNLEFHYDFDESTTAVLSINKTRSEYPYKVSISWGPTCGLDCAKEYNKHDVIV</td>
<td>343-429</td>
</tr>
</tbody>
</table>
Figure 2.
Figure 3

A

\[ v (s^{-1}) \]

\[ \text{L-Arg (mM)} \]

B

\[ v (s^{-1}) \]

\[ [\text{L-Orn}] \text{ mM} \]

C

\[ v (s^{-1}) \]

\[ [\text{L-Lys}] \text{ mM} \]
Figure 4A

![Graph showing the effect of 10 mM DFMO and 1 mM DFMA on enzyme activity over time. The graph plots enzyme activity remaining (in %) against time in minutes. The x-axis represents time in minutes ranging from 0 to 120, and the y-axis represents enzyme activity remaining ranging from 0.1 to 100. Two lines are depicted: one for 10 mM DFMO and another for 1 mM DFMA. The enzyme activity decreases over time for both treatments, with 10 mM DFMO showing a steeper decline.]
Figure 4B.
References:


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Chlorella virus PBCV-1 encodes an unusual arginine decarboxylase that is a close homolog of eukaryotic ornithine decarboxylases

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