Paramecium bursaria Chlorella Virus-1 Encodes an Unusual Arginine Decarboxylase That Is a Close Homolog of Eukaryotic Ornithine Decarboxylases*

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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) of a residue that interacts with the ε-amino group of ornithine analogs in the x-ray structures of ODC. To determine whether 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 Kcat/Km for L-arginine is 550-fold higher than for either L-ornithine or L-lysine, which were decarboxylated with similar efficiency. In addition, α-difluoromethylarginine was a more potent inhibitor of the enzyme than α-difluoromethylornithine. Mass spectrometric analysis demonstrated that inactivation was consistent with the formation of a covalent adduct at Cys347. These data demonstrate that PBCV-1 DC should be reclassified as an arginine decarboxylase. The eukaryotic ODCs, as well as PBCV-1 DC, are only distantly related to the bacterial and plant arginine decarboxylases from their common β/ε-family 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 whether the Asp-to-Glu 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.

Paramecium bursaria chlorella virus-1 (PBCV-1)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 (ODCs) (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), whereas knockout of the polyamine biosynthetic enzymes causes polyamine auxotrophy (e.g. yeast (12), Trypanosoma brucei (13), and 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 anticancer and anti-parasite 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 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 eukaryotic ODCs, plant ADC, and a number of bacterial enzymes that encompass a wide range of substrate specificities (arginine, ornithine, lysine, diaminopimelate, and carboxynospermidine decarboxylases) (20).

The x-ray structures of several eukaryotic ODCs (mouse, T. brucei, and human) (23–27) and of bacterial diaminopimelate decarboxylase (BADC) (28) have been solved. These structures show that ODCs are highly conserved in comparison to ADCs and suggest that the differences in substrate specificity may result from differences in active site geometry and size. Certain points of the active site are well conserved and have been shown to be critical for substrate specificity. For example, arginine and L-ornithine, but it is not sufficient to encode the change in substrate preference.

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1 The abbreviations used are: PBCV-1, P. bursaria chlorella virus-1; ODC, ornithine decarboxylase; PLP, pyridoxal 5′-phosphate; ADC, arginine decarboxylase; DC, decarboxylase; DFMA, α-difluoromethylarginine; DFM0, α-difluoromethylornithine; TbODC, T. brucei ODC; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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late decarboxylase have been solved (28). The N-terminal domain forms a \( \beta/\alpha \)-barrel, and the C-terminal domain is folded into a \( \beta/\alpha \)-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). Although 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 diaminopimelate decarboxylase and share only \( \sim 15\% \) overall sequence identity based on pairwise comparisons. However, a bacterial enzyme from this fold type has been described that has activity on both lysine and ornithine (Lys/Orn decarboxylase (DC)) and that shares extensive sequence similarity (\( \sim 35\% \)) with eukaryotic ODCs (33).

The decarboxylase identified in chlorella virus (PBCV-1 DC) contains nearly 40\% sequence identity to the family of eukaryotic ODCs (3). Phylogenetic analysis indicates that PBCV-1 DC branches with the eukaryotic ODCs and with the bacterial Lys/OrnDCs (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 \( \alpha \)-difluoromethylarginine (DFMA) than by \( \alpha \)-difluoromethylornithine (DFMO), suggesting that it might have unusual substrate specificity.

Alignment of the primary amino acid sequence of this enzyme with the x-ray structures for mammalian and trypanosomal 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 (TbODC) in complex with several substrate and product analogs demonstrate that the \( \delta \)-nitrogen of L-ornithine forms salt bridge interactions with Asp\(^{361} \) from the C-terminal domain and with Asp\(^{332} \) from the N-terminal domain across the subunit boundary (Fig. 1A) (24, 26, 27). 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, although PBCV-1 DC contains detectable activity with L-ornithine, its activity with L-arginine is significantly higher; therefore, this enzyme should be reclassified 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 a carbon dioxide kit were purchased from Sigma. The AccQ-Fluor reagent kit for labeling amino acids was purchased from Waters (Milford, MA). \( L-[1,14^C] \) Ornithine and \( L-[2,3,3^H] \) ornithine (47.7 mCi/mmol) were obtained from PerkinElmer Life Sciences and American Radiolabeled Chemicals (St. Louis, MO).
enzymatic decarboxylation of L-[1-14C]ornithine or L-[U-14C]arginine and 2.5 mM dithiothreitol. The concentration of L-ornithine was varied used in these studies. 1) A protein with the wild-type sequence was used expression of N-terminally His-tagged PBCV-1 DC from a pET-15b pODCTM9, which was described previously (3). This vector directs the sequence data were included. (Hypothetical proteins were excluded ties were determined by measuring the release of 14CO2 from the (37). The 14CO2 released was trapped in a center well containing Hya —mine hydroxide and counted by liquid scintillation spectrometry as 37 °C using a Sigma diagnostics carbon dioxide detection kit as described previously (36). Assays were conducted with 100 μL PLP. The Sigma kit couples decarboxylation of substrate to the oxidation of NADH (λmax = 340 nm) using phosphonopyruvate carboxylase and malate dehydrogenase. HPLC Analysis of Reaction Products—The products of an enzymatic reaction with t-arginine and wild-type PBCV-1 DC and TbODC D332E were analyzed by HPLC using an AccQ-Tag kit (Waters) in 5% sodium tetraborate and labeling reagent (6-diminoquinolyl-n-hydroxysuccinimidyl in acetonitrile) as described previously (24). PBCV-1 DC or TbODC D332E was incubated with t-arginine (20 mM for wild-type PBCV-1 DC and 170 mM for the TbODC mutant) in 15 mM KPO4, 1 mM dithiothreitol, and 0.15 mM PLP at 37 °C for 2.5, 5.0, and 10.0 min. 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 trichloroacetic acid to a final concentration of 6%. Labeled samples (5 μL) were injected onto an AccQ-Tag column using the buffers and gradient described previously (24). The column was calibrated with known amounts of the following derivatized reagents: agmatine (retention time ~ 27.8 min), cadaverine (40.1 min), t-ornithine (34.8 min), putrescine (44.5 min), and t-arginine (21.1 min). Inactivation of PBCV-1 DC with DFMO or DFMA—The His-tagged PBCV-1 DC protein in 50 mM NaH2PO4 (pH 8), 300 mM NaCl, 250 mM imidazole, 0.04 mM PLP, 2.5 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin (3) was dialyzed to remove dithiothreitol and imidazole before passing over a Talon metal affinity column (Clontech, Palo Alto, CA) to remove bovine serum albumin. After elution and further dialysis, an aliquot of PBCV-1 DC protein (104 μg in 50 mM NaH2PO4 (pH 8), 0.04 mM PLP, and 0.5 mM dithiothreitol) 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 Spectrometry/Proteomics Core Facility of the Pennsylvania State College of Medicine. Reactions containing 4.2 μg (~100 nmol) of untreated or DFMO- or DFMA-inactivated PBCV-1 DC protein in a total volume of 100 μL were subjected to tryptic or endoproteinase Glu-C digestion using a protease/protein ratio over the range of 1:50 to 1:250 (w/w) as indicated. Samples were digested with modified trypsin in 50 mM NH4HCO3 (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 analysis by MALDI-TOF. Samples were digested with Glu-C in 25 mM NH4HCO3 (pH 7.8) containing 10% (v/v) acetonitrile for 2–16 h at 25 °C, stopped by the addition of glacial acetic acid, and stored frozen until analysis by MALDI-TOF using an Applied Biosystems 4700 Proteomics Analyzer. Digested samples were evaporated and resuspended three times in 200 μL of deionized water to remove volatile digestion buffers (NH4HCO3), which can interfere with subsequent binding to strong cation exchange
TABLE I
Steady-state kinetic analysis of PBCV-1 DC on L-ornithine and L-arginine

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

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 (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, although the $k_{cat}$ was only slightly lower, the $K_m$ was -400 times higher ($K_m = 180$ 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 (pH 9) at 42 °C. The assays were therefore repeated using these conditions, and the ability to decarboxylate L-ornithine was slightly improved ($K_m = 46$ mM) (Table I). However, even under these conditions, the protein still preferred L-arginine as a substrate, with a $K_m$ 2 orders of magnitude lower than for L-ornithine.

The products of the reaction were identified by HPLC (38, 39) after the protein had acted upon either L-[1-$^{14}$C]arginine or L-[2,3-3H]ornithine. As expected, [1$^{14}$C]agmatine was found in stoichiometric amounts with $^{14}$CO$_2$ when L-[U-$^{14}$C]arginine was decarboxylated, and [3H]putrescine was formed in equivalent amounts when L-[2,3-3H]ornithine was the substrate.

The substrate preference of PBCV-1 DC was also characterized using a coupled NADH 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 $k_{cat}/K_m$ for 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 compared with L-ornithine (Fig. 3). This result is in contrast to the ODCs from both mouse and T. brucei, for which the $k_{cat}/K_m$ for L-lysine is 300-fold lower than for L-ornithine (31, 37). However, the similar catalytic efficiency of PBCV-1 DC for L-ornithine and L-lysine 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 is a more potent inhibitor of PBCV-1 DC compared with 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, whereas 10 mM DFMO reduced the activity to only 26% during the same incubation time. By 1 h, 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))methylcysteine adduct at Cys$^{366}$.
Chlorella Virus Arginine Decarboxylase

Steady-state kinetic analysis of mutant and wild-type PBCV-1 DC and TbODC

Kinetic assays were performed using the spectrophotometric method that measures the oxidation of NADH in a coupled assay.

| Substrate and enzyme | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|----------------------|-----------|-------|---------------|
|                      | s$^{-1}$  | mM    | s$^{-1}$ mM   |
| L-Arg                | 1.5 ± 0.3 | 3.5 ± 0.2 | 4.2 × 10^3 |
| PBCV-1 DC$^a$        | 0.14 ± 0.004 | 42 ± 2.7 | 3.3 |
| TbODC$^b$            | 0.025 | 14 | 1.8 |
| TbODC D332E$^a$      | 0.04 ± 0.005 | 34 ± 10 | 1.2 |
| 1-Orn                | 0.9 ± 0.2 | 136 ± 44 | 7.4 |
| PBCV-1 DC            | 0.02 ± 0.003 | 145 ± 30 | 0.14 |
| TbODC$^c$            | 12 | 0.4 | 3.0 × 10^4 |
| TbODC D332E          | 10 ± 0.6 | 61 ± 1.2 | 1.6 × 10^3 |

$^a$ Data for these enzymes were also collected by HPLC analysis of agmatine formation using AcetQ-Tag labeling as described under "Experimental Procedures," and similar results were obtained.

$^b$ Data were taken from Refs. 30 and 32.

(major product, 90%) and a minor product (~10%) at Lys$^{60}$ (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 native PBCV-1 DC are Cys$^{347}$ and Lys$^{71}$, which correspond to Cys$^{340}$ and Lys$^{71}$ in mouse ODC.

Endopeptidase Glu-C digestions of untreated PBCV-1 DC or of PBCV-1 DC inactivated by DFMO or DFMA were analyzed by linear MALDI-TOF mass spectrometry in positive ion mode using PBCV-1 DC and Glu-C self-digested peptides for internal calibration of the spectra. The spectra of the inactivated enzyme had new peptide fragments with masses corresponding to the theoretical 81.1-Da difference previously observed for the Glu$^{361}$ peptide, which, within experimental error, agrees with Glu$^{361}$ in mouse ODC. The Glu$^{361}$ peptide is the key determinant in substrate binding and catalysis (Fig. 1) (44). The only residues within 4.5 Å of the putrescine side chain that are variable between mouse and T. brucei also contains the equivalent position in PBCV-1 DC is residue 296, 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 shown in Fig. 4B, these masses (m/z 4681.0 and 4740.8) were not observed in the control digest, whereas all three digests contained a peak slightly smaller than the theoretical value (4592.2 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-digested peptides to the observed peak cannot be separated definitively.)

The new peak that appeared after DFMO inactivation (m/z 4681.0) is 81.8 Da heavier than the unmodified Lys$^{321}$–Glu$^{361}$ peptide, which, within experimental error, agrees with the theoretical 81.1-Da difference previously observed for the S-(5-(1-pyrroline)-methyl) cysteine adduct formed by DFMO at Cys$^{340}$ of mouse ODC (41). In the digest of the protein inactivated by DFMA, the new peak observed at m/z 4740.8 is 141.6 Da larger than the unmodified Lys$^{321}$–Glu$^{361}$ peptide. 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 residue 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 trans-aldimination reaction with Lys$^{71}$ would generate the adduct. This adduct cannot cyclize in the same way as the adduct derived from DFMA because of the replacement of the terminal amino group with a guanidino group. The Lys$^{347}$–Glu$^{361}$ peptide does contain another Cys residue at position 345. Because we were unable to produce interpretable tandem mass spectrometric 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 trypanosome ODC by DFMO (24–26, 41), Cys$^{347}$ 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 Cys$^{347}$, in contrast to the almost total loss of enzyme activity observed with DFMA (>99% inhibition) and with DFMO (>90% inhibition). One explanation for this discrepancy is that interaction with another site such as Lys$^{71}$ occurs to a larger extent compared with inactivation of mouse ODC with DFMO. However, we were unable to identify by mass spectrometry a putative DFMO or DFMA adduct to peptides containing Lys$^{71}$ after digestion by Glu-C or trypsin (where an adduct at Lys$^{71}$ would prevent tryptic cleavage at that site). Alternative explanations could be (a) that 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 work-up 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 occurred (the sequence coverage was ~50% in the Glu-C digests, so modifications of the unrepresented proteolytic fragments would not have been observed); and (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-digested fragments.

Sequence Analysis of PBCV-1 DC Active-site Changes—The amino acid sequence of PBCV-1 DC was aligned with the sequences of 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 Tyr$^{331}$ (replaced by Phe) and Asp$^{332}$ (replaced by Glu). When a wider range of ODCs from various species are considered, the residues 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$^{322}$ 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 is Glu, suggesting that this substitution is a key determinant in the change in substrate specificity observed for PBCV-1 DC (Fig. 1B). To determine whether this sequence substitution occurs in any other ODC, analysis of the GenBank™ Data Bank (Version 2.2.8) was conducted. Asp$^{322}$ is invariant in the eukaryotic ODCs examined in the Data Bank; 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 residue at this position. Several sequences for antizyme
inhibitor (human, NP_680479; mouse, NP_061215; and rat, NP_072107), an inactive ODC homolog that regulates ODC activity (45), 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, because 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 appears likely that the D296E alteration is one of the structural determinants 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. Asp332 was mutated to Glu in TbODC (TbODC D332E), and the equivalent substitution was conducted for PBCV-1 DC (PBCV-1 DC E296D). For the wild-type enzymes, the substrate selectivity of TbODC for L-ornithine was more stringent than that of PBCV-1 DC for 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 $K_m$ for the preferred substrate were observed with both TbODC D332E and PBCV-1 DC E296D compared with the wild-type enzymes. In addition, with PBCV-1 DC, the $k_{cat}$ values decreased for both L-ornithine and L-arginine. The $k_{cat}$ effects were not observed in the background of the T. brucei sequence. Although the mutant enzymes were both much less active than the wild-type enzymes, a small change (20-fold) in the $k_{cat}/K_m$ ratio upon comparison of L-arginine and L-ornithine occurred in both backgrounds, which relaxed the substrate preference for the alternative amino acid (Fig. 5). The data demonstrate that the residue at position 332 is an important determinant for substrate binding in both TbODC 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 $beta/alpha$-barrel fold are from both bacterial and eukaryotic origins, 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 for L-ornithine and L-lysine (e.g. S. ruminantium Lys/OrnDC) (33) share high sequence similarity, whereas 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, whereas 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 $k_{\text{cat}}/K_{\text{m}}$ observed for L-arginine at all pH values and temperatures studied. DFMA is a more potent inhibitor of 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 Glu in PBCV-1 DC. This residue interacts directly with substrate, and site-directed mutagenesis established that it is an important residue for substrate binding in both ODCs and PBCV-1 DC. However, the residue at position 332 is not sufficient on its own to determine substrate preference. Swapping the residue at position 332 between Asp and Glu did not significantly increase the relative activity of either TsODC 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 that do not contact ligand directly are often important in the change of function between homologous proteins (e.g. Refs. 46–49). Indeed, we have previously demonstrated that 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, whereas 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 PBCV-1 DC is more closely related to enzymes with ornithine specificity than to bacterial ADcs in the $\beta$-a-fold class, despite their common substrate preference. A potentially important link in this evolution is the observation that a group of bacterial enzymes that 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.

to be more closely related to bacterial enzymes than to plant enzymes (50), suggesting that 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 because the host cell also makes both polyamines. However, the virus is known to inhibit protein translation in altered host cells (52), and altered levels of polyamines could play a role in this inhibition. Eukaryotic initiation factor 5A plays an essential role in translation of selective messengers 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 eukaryotic initiation factor 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 eukaryotic initiation factor 5A, thereby affecting host cell translation.

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REFERENCES

1. Van Etten, J. L. (2003) Annu. Rev. Genet. 37, 153–195
2. Van Etten, J. L., and Meints, R. H. (1999) Annu. Rev. Microbiol. 53, 447–494
3. Morehead, T. A., Guron, J. R., Adams, B., Nicerson, K. W., Fitzgerald, L. A., and Van Etten, J. L. (2002) Virology 301, 165–175
4. Pegg, A. E., Shantz, L. M., and Coleman, C. S. (1994) Biochem. Soc. Trans. 22, 846–852
5. Taher, C. W., and Taher, H. (1984) Annu. Rev. Biochem. 53, 749–790
6. McCann, P. P., and Pegg, A. E. (1992) Pharmacol. Ther. 54, 195–215
7. Morgan, D. M. L. (1999) Mol. Biotechnol. 11, 229–250
8. Thomas, T., and Thomas, T. J. (2001) CMSL Cell. Mol. Life Sci. 58, 244–258
9. Igarashi, K., and Kashigawa, K. (2000) Biochem. Biophys. Res. Commun. 271, 559–564
10. O’Byrne, T. G., Megsh, L. C., Gilliard, G., and Soler, A. P. (1997) Cancer Res. 57, 2630–2637
11. Hanfrey, C., Franceschetti, M., Mayer, M. J., Bliwise, C., and Michael, A. J. (2002) J. Biol. Chem. 277, 5413–5419
12. White, W. H., Gunyuzlu, P. L., and Toyn, J. H. (2001) J. Biol. Chem. 276, 10784–10800
13. Li, F., Hsu, S. B., Wang, C. C., and Gottschedien, K. M. (1998) Exp. Parasitol. 87, 255–267
14. Jiang, Y., Roberts, S. C., Jardin, A., Carter, N. S., Shih, S., Aripinayagam, M., Fairlamb, A. H., and Ullman, B. (1999) J. Biol. Chem. 274, 3781–3788
15. Roberts, S. C., Scott, J., Gasteiger, E. J., Jiang, Y., Brooks, B., Jardin, A., Carter, N. S., Heby, O., and Ullman, B. (2002) J. Biol. Chem. 277, 5902–5909
16. Nishimura, K., Nakatsu, F., Kashigawa, K., Ohno, H., Saite, H., Saito, T., and Igarashi, K. (2002) Genes Cells 7, 41–47
17. Pendeville, H., Carpino, N., Marine, J. C., Takahashi, Y., Muller, M., Martial, J. A., and Cleveland, J. L. (2001) Mol. Cell. Biol. 21, 6549–6558
18. Pegg, A. E., Shantz, L. M., and Coleman, C. S. (1995) J. Biol. Chem. 270, 132–138
19. Wang, C. C. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 95–127
20. Grishin, N. V., Phillips, M. A., and Goldsmith, E. J. (1995) Protein Sci. 4, 1293–1304
21. Sandmeier, E., Hale, T. I., and Christen, P. (1994) Eur. J. Biochem. 221, 997–1002
22. Momany, C., Ghosh, R., and Hackert, M. L. (1995) Protein Sci. 4, 849–854
23. Kern, A. D., Oliveira, M. A., Coffino, P., and Hackert, M. (1999) Structure 7, 567–578
24. Grishin, N. V., Osterman, A. L., Brooks, H. B., Phillips, M. A., and Goldsmith, E. J. (1999) Biochemistry 38, 15174–15184
25. Almudr, J. J., Oliveira, M. A., Grishin, N. V., Phillips, M. A., and Hackert, M. L. (2000) J. Mol. Biol. 295, 7–16
26. Jackson, K. L., Goldsmith, E. J., and Phillips, M. A. (2003) J. Biol. Chem. 278, 20375–20385
27. Jackson, K. L., Brooks, H. B., Osterman, A. L., Goldsmith, E. J., and Phillips, M. A. (2000) Biochemistry 39, 11247–11257
28. Ray, S. S., Bonanno, J. R., Fajahankark, R. B., Pinho, M. G., He, G., De Lencaste, H., Tomasa, A., and Burley, S. K. (2002) Structure 10, 1499–1508
29. Cookman, C. S., Stanley, B. A., and Pegg, A. E. (1993) J. Biol. Chem. 268, 24572–24579
30. Osterman, A. L., Brooks, H. B., Rizo, J., and Phillips, M. A. (1997) Biochemistry 36, 4556–4567
31. Osterman, A., Kinch, L. N., Grishin, N. V., and Phillips, M. A. (1995) J. Biol. Chem. 270, 11797–11802
32. Osterman, A. L., Brooks, H. B., Jackson, L., Abbott, J. J., and Phillips, M. A. (1999) Biochemistry 38, 11814–11826
33. Takatsuka, Y., Yamaguchi, Y., Ono, M., and Kamio, Y. (2000) J. Bacteriol. 182, 6732–6741
34. Felsenstein, J. (1989) Cladistics 5, 164–166
35. Pei, J., Sadreyev, R., and Grishin, N. V. (2003) Bioinformatics 19, 427–428
36. Osterman, A. L., Grishin, N. V., Kinch, L. N., and Phillips, M. A. (1994) Biochemistry 33, 13662–13667
37. Pegg, A. E., and McGill, S. (1979) Biochim. Biophys. Acta 568, 416–427
38. Pegg, A. E., Wechter, R., Poulin, R., Woster, P. M., and Coward, J. K. (1989) Biochemistry 28, 8446–8453
39. Coleman, C. S., Hu, G., and Pegg, A. E. (2004) Biochem. J. 370, 849–855
40. Metzger, B., Bey, P., Danzin, C., Jung, M. J., Casara, P., and Vevert, J. P. (1978) J. Am. Chem. Soc. 100, 2551–2553
41. Poulin, B., Lu, L., Ackermann, B., Bey, P., and Pegg, A. E. (1992) J. Biol. Chem. 267, 150–158
42. Bitonti, A. J., Casara, P. J., McCann, P. P., and Bey, P. (1987) Biochem. J. 242, 69–74
43. Coligan, J. E., Dunn, B. M., Ploeg, H. L., Speicher, D. W., and Wingfield, P. J. (1996) in Current Protocols in Protein Science (Wingfield, P. J., ed) Vol. 2, p. 11.11.12, John Wiley & Sons, Inc., New York
44. Myers, D. P., Jackson, L. K., Ipe, V. G., Murphy, G. E., and Phillips, M. A. (2001) Biochemistry 40, 13230–13236
45. Murakami, Y., Ichiba, T., Matsufuji, S., and Hayashi, S. (1996) J. Biol. Chem. 271, 3340–3342
46. Hedstrom, L., Sylagi, L., and Rutter, W. J. (1992) Science 255, 1249–1253
47. Oue, S., Okamoto, A., Yano, T., and Kagamihara, H. (1999) J. Biol. Chem. 274, 2344–2349
48. Rajagopalan, P. T. R., Lutz, S., and Benkovic, S. J. (2002) Biochemistry 41, 12618–12626
49. Jeffrey, C. J., Gloss, L. M., Petako, G. A., and Ringe, D. (2000) Protein Eng. 13, 105–112
50. Kaiser, A., Vollmert, M., Tholl, D., Graves, M. V., Gurnon, J. R., Xing, W., Liser, A. D., Nicerson, K. W., and Van Etten, J. L. (1999) Virology 263, 254–262
51. Brown, M. R., and Jeffrey, S. W. (1992) J. Exp. Mar. Biol. Ecol. 161, 91–113
52. Tome, M. E., and Gerner, E. W. (1997) Biol. Signals 6, 150–156
53. Park, J.-H., Wolff, E. C., Folk, J. E., and Park, M. H. (2003) J. Biol. Chem. 278, 32683–32691