Carbon-13 Isotope Effect Studies of *Trypanosoma brucei* Ornithine Decarboxylase†

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ABSTRACT: Carbon isotope effect studies were undertaken with the wild-type pyridoxal 5′-phosphate (PLP)-dependent enzyme ornithine decarboxylase (ODC) from *Trypanosoma brucei* and with several active site mutants of the enzyme. For the decarboxylation of the optimal substrate, L-ornithine, by wild-type ODC, the observed carbon isotope effect (k^{12}/k^{13}) is 1.033 at pH 7.3. In comparison to the expected intrinsic isotope effect ($k^{12}/k^{13} = 1.06$) for decarboxylation, this value suggests that both the rate of decarboxylation and the rate of Schiff base interchange with L-ornithine are partially rate-limiting for the reaction steps up to decarboxylation. In contrast, with the alternate substrate L-Lys, which shows lower catalytic efficiency, the carbon isotope effect increased to 1.063, demonstrating that decarboxylation has become the rate-limiting step. For the mutant enzymes, E274A ODC and C360A ODC, with L-ornithine as substrate the carbon isotope effect also approaches the intrinsic limit. Glu-274 was previously demonstrated to play a direct role in carbanion stabilization, and thus the large carbon isotope effect ($k^{12}/k^{13} = 1.055$) is consistent with an impaired rate of decarboxylation compared to wild-type ODC. In contrast, for K69A ODC, the isotope effect is almost entirely suppressed, suggesting that Schiff-base formation (which now must occur from enzyme-bound PLP, rather than from an enzyme-bound PLP−Schiff base) has become rate-determining.

The pyridoxal 5′-phosphate (PLP)-dependent enzyme ornithine decarboxylase (ODC)[1] catalyzes the first step in the biosynthesis of polyamines. Polyamines are required for cell growth and differentiation, and, thus, the polyamine biosynthetic enzymes have generated interest as drug targets against cancer and microbial infection (*1*). Clinically, inhibitors of ODC have been most effective for the treatment of African Sleeping Sickness caused by the parasitic protozoa *Trypanosoma brucei* (*2*).

ODC is a structural homologue of alanine racemase (*3*), the structure of which has recently been reported (*4*). ODC is an obligate homodimer, and each monomer is composed of two domains. The larger N-terminal domain is a β/α barrel, and PLP binds in the center of the barrel. The active site is formed between the PLP binding site of one subunit and the C-terminal domain of the second subunit (*5*−*7*). The functional roles of several active site residues have been delineated: Lys-69 forms a Schiff base with PLP (*8*), Glu-274 interacts with the pyridine nitrogen of PLP to enhance the basicity of the nitrogen (*9*), Arg-277 stabilizes the 5′-phosphate (*10*), and Cys-360 is labeled by the mechanism-based inhibitor α-difluoromethylornithine (*8*). Additionally, the reaction mechanism has been characterized by multi-wavelength stopped-flow spectroscopy, and a rate-determining step for the overall reaction was demonstrated to be product release (*11*).

Carbon-13 kinetic isotope effects have been used to study the mechanisms of action of several PLP-dependent decarboxylases (*12*). The intrinsic isotope effect, which is observed when the decarboxylation step (k_5: Scheme 1) is entirely rate-limiting, has been reported to be 1.06 (*12*). The magnitude of the observed kinetic isotope effect depends on the rate of decarboxylation (k_5) relative to release of substrate from the enzyme−substrate Schiff base (reflected in a combination of k_4, k_3, and k_2). Steps occurring after decarboxylation have no influence on the carbon isotope effect. When decarboxylases act on their natural substrates under conditions of optimum pH (e.g., for Asp β-decarboxylase, Glu decarboxylase, Arg decarboxylase, and His decarboxylase), isotope effects of 1.01−1.03 have been observed, indicating that decarboxylation and release of substrate from the Schiff base occur at similar rates (*12*). On the other hand, isotope effects with unnatural substrates or far from optimum reaction conditions [e.g., with Arg decarboxylase (*13*)] often approach the intrinsic value, indicating that decarboxylation has become totally limiting (*12*).

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[1] Abbreviations: wtODC, wild-type *T. brucei* ornithine decarboxylase; mutant ODC's are abbreviated by single-letter codes, e.g., ODC with the Glu-274 to Ala mutation is referred to as E274A ODC.

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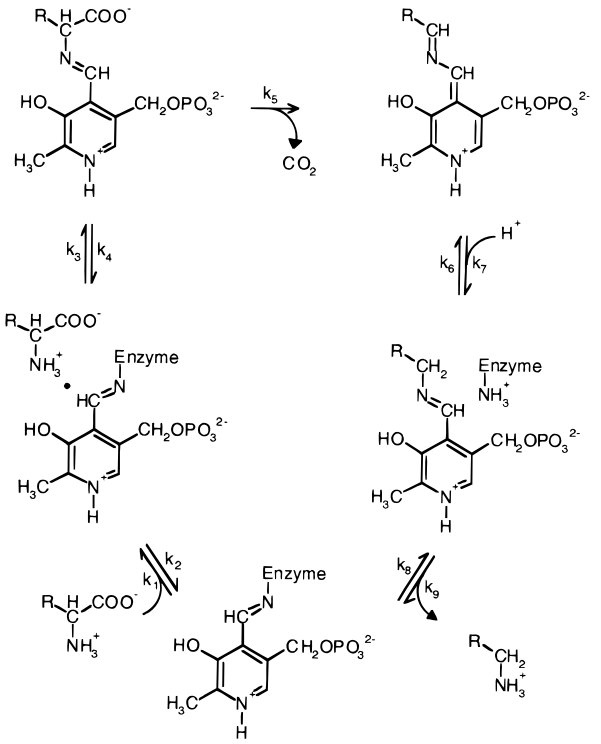
Scheme 1



In this paper, we describe carbon-13 isotope effect studies on wild-type *T. brucei* ODC and on several active site mutants of the enzyme. The wild-type enzyme was also studied with two different substrates and under several different pH, temperature, and solvent conditions. The full range of carbon isotope effects were observed, providing insight into the reaction mechanism and the roles of Lys-69, Cys-360, and Glu-274 in catalysis. Like previous studies (*14*−*16*), these isotope effect experiments demonstrate that the combination of site-directed mutagenesis and heavy-atom isotope effects provides a powerful approach to the identification of the contributions of individual residues to catalysis.

EXPERIMENTAL PROCEDURES

Materials

All chemicals used were reagent grade or better. Dabsyl chloride (4-dimethylaminoazobenzene-4′-sulfonyl chloride) was purchased from Fluka. HPLC-grade acetonitrile, trifluoroacetic acid, PLP, cadaverine, putrescine, L-Lys, L-ornithine, 1,3-diaminopropane hydrochloride salts, phosphoric acid-d_3 (99+ % D) 85% solution in D_2O, and sodium deuterioxide (99+ % D) 40% solution in D_2O were purchased from Sigma (St. Louis, MO). Deuterium oxide (99% D) was purchased from Cambridge Isotope Labs (Andover, MA).

Methods

ODC Expression and Purification. ODC was expressed from the cloned gene as a His-tag fusion protein in BL21/DE3 cells from the T7 promoter as described (*11*). The enzyme was purified by Ni^{2+}−agarose column chromatography and gel filtration as previously described (*7*, *9*). The construction of clones encoding the mutant enzymes C360A, K69A, and E274A ODC has been previously described (*7*, *9*).

Carbon Isotope Effects. Carbon isotope effects were measured by the competitive method using enzyme substrates of natural abundance as described by O′Leary (*17*). Briefly, this method involves isolation of the product CO_2 from a low conversion sample (approximately 10% reacted) and from a complete reaction (100% reacted). The isotopic ratio of the CO_2 is then determined using an isotope ratio mass spectrometer. The reaction extent was monitored by HPLC analysis of substrates and products (see below for method). HPLC analysis of both L-ornithine and putrescine in the sample confirmed that the complete reaction was 100% reacted (100% L-ornithine was converted to putrescine). Further, the carbon isotope ratios from CO_2 purified from the complete reactions were constant and did not change with the addition of more enzyme and additional incubation time.

Both complete and low conversion reactions were typically scaled to yield approximately 50 μmol of CO_2. To use convenient volumes of solution, the 100% reaction solution contained 25 mM buffer and 10 mM substrate, and the low conversion reaction solution contained 25 mM buffer and 25 mM substrate. The following buffers were used: pH 7.3, sodium phosphate; pH 7.8, sodium *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES); pH 9.0, sodium 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES). For wild-type ODC, buffers also included 1 mM dithiothreitol (DTT) and 0.02 mM PLP; for E274A and K69A ODC, they contained 2 mM DTT and 0.4 mM PLP; and for C360A, they contained 10 mM DTT and 1.5 mM PLP. Enzyme concentrations in the reactions were 0.2−5 μM for wild-type ODC, 7−10 μM for E274A and C360A ODC, and 30 μM for K69A ODC. Reaction times for complete and low conversion samples were determined empirically. For the low percent conversion sample, the incubation times were 10 min for wild-type ODC, 20−30 min for E274A ODC and C360A ODC, and 65 h for K69A ODC. The isotope ratios for CO_2 for the 100% reacted samples of L-ornithine and L-Lys were determined multiple times using wild-type ODC under different reaction conditions (e.g., pH, temperature, and solvent) using reaction times of 10−20 h.

Because the isotopic ratio of the CO_2 is critical to this analysis, various precautions were taken to avoid contamination by extraneous CO_2 from air or bicarbonate from solutions. All solutions used in isotope reactions were bubbled with CO_2-free nitrogen gas to sweep dissolved CO_2 from the solutions. More extensive bubbling was required for solutions with alkaline pH. Isotope effect experiments were performed in airtight flasks fitted with stopcocks and rubber septa to allow addition of materials. Control reactions were performed to show that additions of volumes of ODC solution that were less than 1% of the total solution volume produced no detectable CO_2. When larger volumes of enzyme were used, they were degassed as described by O'Leary (*17*). For samples containing D_2O, additional precautions were taken because deuterium oxide is enriched in the isotope ^{17}O. Because the oxygen atoms in the bicarbonate ion are in equilibrium with the oxygen atoms in water, the CO_2 sample is also enriched in ^{17}O. This enrichment causes inaccurate measurements of carbon isotope ratios. To prevent this, purified CO_2 samples were equilibrated in 10 mM H_3PO_4 before isotopic analysis.

Enzyme reactions were stopped by addition of 1 M H_2SO_4 containing 1,3-diaminopropane (0.5 M), the internal

standard for analysis of fraction reaction (see below), to a final concentration of 10 mM 1,3-diaminopropane and a pH <2. The CO_2 produced by the ODC reaction was then purified by cryodistillation on a high-vacuum line to remove traces of water. The isotope ratio of the purified CO_2 was determined by isotope ratio mass spectrometry on a Finnigan Delta S isotope ratio mass spectrometer.

HPLC Measurement of the Fraction of Reaction. The substrate and product (amino acid and polyamine) of the ODC reaction were labeled with dabsyl chloride, separated on reverse-phase HPLC, and quantitated by comparing areas under the peaks to those of known standards. Internal standard, 1,3-diaminopropane, was included in each standard and sample to correct for variations in the completeness of labeling reaction or the injection amount. The concentration of internal standard was determined by measuring the volume of solution remaining after isolation of CO_2 and using the known amount of 1,3-diaminopropane added in the acid quench solution.

Standard mixtures were prepared for amino acid (10 mM) and polyamine (10 mM) pairs, e.g., L-ornithine and putrescine and L-Lys and cadaverine. These solutions were serially diluted to yield a set of standard solutions ranging in concentration from 0.25 to 10 mM. These solutions were diluted 10-fold into a solution containing 0.1 M H_2SO_4 plus internal standard. The concentration of the internal standard was varied from 0.2 to 1 mM.

Both samples and standards were labeled with dabsyl chloride using the same procedure. An aliquot (5 μL) of reaction or standard mixture was added to 0.2 M $NaHCO_3$, pH 9.0 (25 μL). Dabsyl chloride (0.05 mL of 1.5 mg/mL in acetone) was added to each sample, and the reactions were incubated at 70 °C for 10 min. Completion of the labeling reaction was indicated by a color change from red to orange. The reactions were cooled to room temperature and diluted with 70% ethanol (120 μL). The samples were centrifuged to remove any precipitated material, and aliquots were analyzed on HPLC. Labeling reactions were performed in triplicate.

Samples were analyzed on a Waters 625 HPLC system using a 4.6 mm × 250 mm Vydac 201HS54 reverse-phase C18 column and detection at 436 nm. Reaction mixtures were separated using a 15 min gradient from 50% to 100% solvent B (0.1% trifluoroacetic acid, 90% acetonitrile, 10% water), where solvent A was 0.1% trifluoroacetic acid in water. The column was washed with 100% solvent B and reequilibrated to 50% solvent B between runs. Data collection and analysis were done using Waters Maxima 825 software.

Concentrations of the substrate and product were determined by calculating detector response curves for each compound at five concentrations of standard. Detector response was the ratio of the compound peak area to the internal standard peak area multiplied by the concentration of internal standard. Standard curves of detector response versus standard concentration were analyzed by linear regression. All response curves for all standards were linear with $r^2 \geq 0.98$. Concentration of unknown was determined by interpolating the concentration from the detector response of the unknown. This procedure was validated by assaying CO_2 production by the enzyme in parallel with disappearance of substrate and appearance of product (data not shown).

Table 1: Carbon Isotope Effects Measured for *T. brucei* Wild-Type and Mutant Ornithine Decarboxylases[a]

enzyme	substrate	temp	pH or pD	solvent	k^{12}/k^{13}(obsd)	*n*
wtODC	ornithine	37	7.3	H_2O	1.0325 ± 0.0002	4
wtODC	ornithine	37	7.8	H_2O	1.0313 ± 0.0003	4
wtODC	ornithine	4	7.8	H_2O	1.0297 ± 0.0010	4
wtODC	ornithine	37	7.3	D_2O	1.0232 ± 0.0004	4
wtODC	ornithine	37	8.0	D_2O	1.0193 ± 0.0011	3
wtODC	ornithine	37	9.0	H_2O	1.0384 ± 0.0002	4
wtODC	lysine	37	7.3	H_2O	1.0633 ± 0.0003	5
E274A ODC	ornithine	37	7.3	H_2O	1.0551 ± 0.0005	5
C360A ODC	ornithine	37	7.3	H_2O	1.0525 ± 0.0002	4
K69A ODC	ornithine	37	7.3	H_2O	1.0033 ± 0.0009	5

[a] Temperature is given in degrees centigrade. k^{12}/k^{13}(obsd), observed kinetic isotope effect comparing *V*/*K* for decarboxylation of $^{12}CO_2$-ornithine to $^{13}CO_2$-ornithine, where the error is the standard deviation of the mean for *n* determinations.

Calculation of Kinetic Isotope Effects. The observed carbon isotope effects (k^{12}/k^{13}), which equal the ratio of *V*/*K* for the decarboxylation of $^{12}CO_2$ vs $^{13}CO_2$, were calculated using eq 1 (*18*), where R_f is the isotope ratio ($^{13}C/^{12}C$) of the product after the reaction proceeded to fraction *f* and R_o is the isotope ratio of the product at 100% completion of the reaction. The isotope ratio of the product at 100% complete reaction is equal to the isotope ratio of the carboxyl carbon atom in the substrate.

$$k^{12}/k^{13}(\text{obsd}) = \frac{\log (1 - f)}{\log [1 - f(R_f/R_o)]} \qquad (1)$$

RESULTS AND DISCUSSION

Carbon isotope effects on the decarboxylation of L-ornithine and L-Lys by wild-type and mutant *T. brucei* ODCs are summarized in Table 1. The isotope effects were calculated using eq 1. For wild-type ODC using L-ornithine as a substrate, the observed k^{12}/k^{13} is 1.0325 at pH 7.3 and is not significantly affected by changes in temperature (37 vs 4 °C). The carbon isotope effect is increased at pH 9.0 and slightly suppressed if D_2O is substituted for H_2O at both pH 7.3 and pH 8.0. In contrast, when L-Lys is used as a substrate, the observed k^{12}/k^{13} increases to 1.0633, a value which is equal to the reported intrinsic ^{13}C isotope effect for amino acid decarboxylation (*12*). These results for wild-type ODC are very similar to what has been reported for a number of other PLP-dependent decarboxylases (*12*). For the decarboxlyation of L-ornithine by the three mutant enzymes, E274A ODC, K69A ODC, and C360A ODC, we observe the full range of isotope effects from 1.0033 for K69A ODC to 1.0551 for E274A ODC (Table 1).

The observed isotope effect can be related to the isotope effect on the decarboxylation step (called the "intrinsic isotope effect" = k_5^{12}/k_5^{13}) by eq 2 (*17*−*20*), where k_2−k_5 are rate constants for the individual steps in the mechanism of ODC-catalyzed decarboxylation (Scheme 1). This equation assumes that decarboxylation is the only isotopically sensitive step in the reaction and that decarboxylation is irreversible.

$$\frac{k^{12}}{k^{13}}(\text{obsd}) = \frac{k_5^{12}/k_5^{13} + k_5/k_4\,(1 + k_3/k_2)}{1 + k_5/k_4\,(1 + k_3/k_2)} \qquad (2)$$

The ^{13}C isotope effect data provide no insight into steps which occur after decarboxylation. Thus, the contribution of subsequent steps, e.g., product release, demonstrated by stopped-flow analysis to be a rate-limiting step in the steady-state reaction of ODC with L-ornithine (*11*), cannot be assessed by this method. According to eq 2, the observed isotope effect equals the intrinsic isotope effect only when the term $(k_5/k_4)(1 + k_3/k_2)$ is small. Chemically, this means that the intrinsic isotope effect is observed only when substrate release from the enzyme−substrate Schiff base (upper left structure in Scheme 1) is rapid compared to decarboxylation of this same intermediate.

For wild-type ODC with L-ornithine as substrate, the observed isotope effect is significant but smaller than the intrinsic isotope effect, suggesting that both decarboxylation and Schiff-base interchange are partially rate-limiting for the reaction up to the decarboxylation step. Further, as it is likely that loss of substrate from the Michaelis complex (k_2) is fast (although compelling evidence for this assumption is not available), it is reasonable to assume that $k_3/k_2 \ll 1$. The same assumption has been made during the analysis of a number of other decarboxylases by carbon isotope effects (*12*). Under this condition, eq 2 simplifies to eq 3, which can then be used to calculate the ratio of k_5/k_4.

$$\frac{k^{12}}{k^{13}}(\text{obsd}) = \frac{{k_5}^{12}/{k_5}^{13} + k_5/k_4}{1 + k_5/k_4} \qquad (3)$$

For an observed isotope effect of 1.0325, the calculated k_5/k_4 is 1, suggesting that for wild-type ODC with L-ornithine as a substrate the rate of decarboxylation (k_5) is similar to the rate of Schiff-base decay (k_4) (or, in the more general case, substrate release from the E−S Schiff base). The rate of decarboxylation was previously reported to be 20 s^{-1} at 4 °C (*11*), and the ^{13}C isotope data suggest k_4 will be of similar magnitude. The smaller isotope effect observed in D_2O compared to H_2O parallels previous results with Glu decarboxylase (*21*) and suggests that the Schiff-base interchange step has a large solvent isotope effect, as would be expected for a reaction involving a number of proton transfers.

In contrast, for wild-type ODC with L-Lys as substrate, the observed isotope effect equals the intrinsic value ($k^{12}/k^{13} = 1.06$), indicating that decarboxylation is fully rate-limiting. It has previously been demonstrated that L-Lys is a less efficient substrate than L-ornithine; the K_m is 100-fold higher, although the k_{cat} remains similar to that observed for L-ornithine (*9*). By analogy with previous studies of Arg decarboxylase (*13*), this probably reflects the importance of the exact size of the substrate amino acid side chain in orienting the Schiff base for decarboxylation in the active site; Schiff-base formation is relatively tolerant of side-chain variations, but decarboxylation (which leads to a structurally rigid quinonoid intermediate) is very sensitive to the configuration of the C(α)−N bond, which in turn is very sensitive to the length of the side chain.

In previous studies, we demonstrated that Glu-274 stabilizes the positive charge on the pyridine nitrogen of PLP, enhancing the electron-withdrawing ability of the ring (*9*). Mutation of Glu-274 to Ala decreased k_{cat} by 50-fold, while substitution of PLP by *N*-methyl-PLP restored activity to wild-type levels (*9*). Based on these data, substitution of Glu by Ala would be expected to decrease the rate of the decarboxylation step. In support of this hypothesis, the ^{13}C isotope effect increases from 1.0325 in the wild-type enzyme to 1.0551 for E274A ODC using L-ornithine as the substrate (Table 1). For E274A ODC, the isotope effect is near the intrinsic limit, suggesting that decarboxylation is nearly fully rate-limiting.

The increase in the ^{13}C isotope effect upon increasing the pH from 7.3 to 9.0 may also be reflective of the acid−base chemistry of the pyridine nitrogen. We previously reported spectral analysis of PLP bound to both wild-type and R277A ODC over a range of pH (*10*). The spectral data are consistent with the interpretation that deprotonation of the pyridine nitrogen occurs with a pK_a of 8.5. The steady-state turnover number (k_{cat}) of the reaction of ODC with L-ornithine also decreases at high pH, with a dependency that is consistent with the loss of a catalytically important proton dissociating with a pK_a of 8.5. Thus, similar to the effect of mutating Glu-274 to Ala, high pH would be expected to slow the rate of the decarboxylation step without necessarily changing the rates of prior steps. At a sufficiently high pH, the observed ^{13}C isotope would be expected to approach the intrinsic value.

The isotope effect for C360A ODC is also near the intrinsic limit (Table 1), again suggesting that decarboxylation has become fully rate-limiting in the mutant enzyme. Cys-360 is the site of covalent attachment of the mechanism-based inhibitor α-difluoromethylornithine (*8*). These results place the residue in the active site of the enzyme near the Cα carbon. Furthermore, mutation of Cys-360 decreases k_{cat} by 50-fold (*7*, *22*), demonstrating that it is a catalytically important residue. The large observed ^{13}C isotope effect suggests a role for this residue in decarboxylation. Cys-360 may play a role in dictating the configuration of the C(α)−N bond, which in turn would dictate the position of the carboxyl group relative to the plane of the PLP ring. Improper positioning of the carboxyl group would result in a decreased rate of decarboxylation.

Lys-69 forms a Schiff base with PLP in the native enzyme (*8*). Mutation of Lys-69 to Ala decreases the reaction rate (k_{cat}) by 10^4-fold, presumably because enzyme−substrate Schiff-base formation and decay are slower from enzyme-bound PLP aldehyde (mutant enzyme) than from enzyme-bound PLP Schiff base (wild-type enzyme). The lack of a significant carbon isotope effect ($k^{12}/k^{13} = 1.0033$) for the reaction of K69A ODC with L-ornithine (Table 1) is consistent with this interpretation.

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REFERENCES

1. Marton, L. J., and Pegg, A. E. (1995) *Annu. Rev. Pharmacol. Toxicol. 35*, 55−91.
2. Wang, C. C. (1995) *Annu. Rev. Pharmacol. Toxicol. 35*, 93−127.

3. Grishin, N. V., Phillips, M. A., and Goldsmith, E. J. (1995) *Protein Sci. 4*, 1291−1304.
4. Shaw, J. P., Petsko, G. A., and Ringe, D. (1997) *Biochemistry 36*, 1329−1342.
5. Coleman, C. S., Stanley, B. A., Viswanath, R., and Pegg, A. E. (1994) *J. Biol. Chem. 269*, 3155−3158.
6. Tobias, K. E., and Kahana, C. (1993) *Biochemistry 32*, 5842−5847.
7. Osterman, A. L., Grishin, N. V., Kinch, L. N., and Phillips, M. A. (1994) *Biochemistry 33*, 13662−13667.
8. Poulin, R., Lu, L., Ackerman, B., Bey, P., and Pegg, A. E. (1992) *J. Biol. Chem. 267*, 150−158.
9. Osterman, A., Kinch, L. N., Grishin, N. V., and Phillips, M. A. (1995) *J. Biol. Chem. 270*, 11797−11802.
10. Osterman, A. L., Brooks, H. B., Rizo, J., and Phillips, M. A. (1997) *Biochemistry 36*, 4558−4567.
11. Brooks, H. B., and Phillips, M. A. (1997) *Biochemistry 36*, 15147−15155.
12. O'Leary, M. H. (1988) *Acc. Chem. Res. 21*, 450−455.
13. O'Leary, M. H., and Piazza, G. J. (1981) *Biochemistry 20*, 2743−2748.
14. Waldrop, G. L., Turnbull, J. L., Parmentier, L. E., Lee, S., O'Leary, M. H., Cleland, W. W., and Schachman, H. K. (1992) *Biochemistry 31*, 6592−6597.
15. Wedler, F. C., Ley, B. W., Lee, B. H., O'Leary, M. H., and Kantrowitz, E. R. (1995) *J. Biol. Chem. 270*, 9725−9733.
16. Lee, B. H., Ley, B. W., Kantrowitz, E. R., O'Leary, M. H., and Wedler, F. C. (1995) *J. Biol. Chem. 270*, 15620−15627.
17. O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83−104.
18. Northrop, D. B. (1975) *Biochemistry 14*, 2644−2651.
19. Cleland, W. W. (1975) *Biochemistry 14*, 3220−3224.
20. Cleland, W. W. (1982) *Crit. Rev. Biochem. 13*, 385−428.
21. O'Leary, M. H., Yamada, H., and Yapp, C. J. (1981) *Biochemistry 20*, 1476−1481.
22. Coleman, C. S., Stanley, B. A., and Pegg, A. E. (1993) *J. Biol. Chem. 268*, 24572−24579.

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