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Short Communication

New Insights into the Roles of Critical Residues in the Active Site of Thymidylate Synthase: Results from Site-Directed Mutagenesis Interpreted in Light of a “High-Resolution” Kinetic Mechanism for Wild-Type Enzyme¹⁾

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Introduction

TS catalyzes the conversion of dUMP and CH₂H₄folate to dTMP and H₂folate by reductive methylation. In this reaction CH₂H₄folate serves both as methyl donor and reducing agent. Such complex chemistry [see (1) for details] has made TS of interest as a model for enzymatic catalysis. The reaction is very important *in vivo* where it provides the only *de novo* source of dTMP required for DNA synthesis. Thus maintaining adequate levels of TS activity is usually critical in rapidly growing cells where DNA synthesis is correspondingly rapid. This enzyme is, therefore, an important target for chemotherapeutic agents such as the anti-tumor agent 5-fluorouridine which is metabolized to the active agent 5-fluoro-2'-deoxyuridylate. Consequently TS has received a great deal of attention for its clinical relevance.

We have defined a detailed (“high-resolution”) kinetic mechanism for catalysis by TS from rate and equilibrium constants for individual steps in the catalytic cycle (Appleman, Spencer, and Villafranca, unpub-

lished results). Rate constants governing these steps were measured by stopped-flow spectroscopy. This kinetic scheme has provided the framework for examining the roles of individual amino acids in TS by comparison of altered enzymes generated by oligonucleotide-directed mutagenesis with wild-type (wt) enzyme. Mutations at key residues Cys¹⁴⁶, Arg¹⁶⁶, and Asp¹⁶⁹ lead to enzymes that are crippled in their ability to carry out catalysis.

Material and Methods

Materials and methods are as described previously (1).

Results and Discussion

The active-site amino acid residues Cys¹⁴⁶, Arg¹⁶⁶, and Asp¹⁶⁹ were selected for investigation based upon the three-dimensional structure of TS (2, 3) and conservation in known primary sequences of thymidylate synthases. Altered enzymes (C146A, R166Q, and D169N) with single amino acid substitutions designed to eliminate the proposed functionality of these residues were prepared. We demonstrated by x-ray crystallography that the three-dimensional structure of each of the three mutant enzymes was like that of the wild type. The affinities of these enzymes for dUMP and the folate analog MTX-glu₅ were determined by titration with fluorescence detection, and the meas-

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Abbreviations: TS, thymidylate synthase (methylene-tetrahydrofolate: dUMP C-methyltransferase, ec 2.1.1.b) from *Escherichia coli*; CH₂H₄folate, [6R]-5,10-CH₂-H₄folate; MTX-glu₅, 4-amino-4-deoxy-10-methylpteroyl-glu₅

Table 1. Comparison of dissociation constants (K_d) for ligand binding and of the steady-state kinetic parameters k_{cat} and K_m s for wild type and mutant thymidylate synthases

	dUMP K_d (μ M)	mut/ wt ¹⁾	MTX-glu ₅ ²⁾ K_d (μ M)	mut/ wt	k_{cat} (s ⁻¹)	mut/ wt	dUMP K_m (μ M)	mut/ wt	CH ₂ H ₄ folate K_m (μ M)	mut/ wt
wt	4.		1.4		1.6		3.		13.	
C146A	10.	2.5			<<0.0001	<<0.0006				
R166Q	720.	180.	7.9	5.6	0.015	0.009	9700.	3200.	140.	11.
D169N	28.	7.	6.9	4.9	<<0.0001	<<0.0006				

¹⁾ Ratio of value for mutant enzyme to that of wild type²⁾ For binding to enzyme.dUMP complex

ured values of K_d are reported in Table 1. The catalytic activities of the mutant enzymes have also been compared with that of wt (Table 1).

Role of Cys¹⁴⁶

During the course of catalysis, a covalent complex between dUMP and TS is formed by the nucleophilic attack of Cys¹⁴⁶ on C6 of dUMP (1). Dev *et al* have demonstrated that replacement of Cys¹⁴⁶ by several other residues including Ala produces an inactive enzyme (4) and our results with C146A are consistent with their findings (Table 1). Elimination of the active-site sulfhydryl prevents attack upon dUMP, and thus blocks subsequent steps in the catalytic cycle. The mutant C146A is competent with respect to binding of substrates and products as reflected in binding of dUMP and MTX-glu₅ (Table 1). We have found C146A to be extremely helpful in identifying spectral changes observed in our stopped-flow spectroscopic studies that are associated solely with ligand binding rather than with chemical reactions (data not shown).

Role of Arg¹⁶⁶

Arg¹⁶⁶ is one of four residues that interact with phosphate of bound nucleotide and thus is expected to play an important role in the binding of dUMP (2, 3). Further, it has been proposed that by charge-charge interactions the residue might stabilize Cys¹⁴⁶ in the thiolate form, which is essential for its attack on dUMP (4). Replacement of Arg¹⁶⁶ by Gln should eliminate hydrogen bonds to dUMP and electrostatic interaction with Cys¹⁴⁶. As anticipated, the affinity of the enzyme for dUMP is greatly decreased when interaction between the phosphate group on the nucleotide and arginine is eliminated as demonstrated by the increase in K_d for dUMP binding (Table 1) and K_m for dUMP (Table 1) compared to those for

wt. Further, the slow catalytic rate [110-fold decrease in k_{cat} (Table 1)] is also consistent with unfavourable formation of the thiolate of Cys¹⁴⁶. Binding of ligands at the folate site is weaker to R166Q than to wt as shown by the increased K_d for MTX-glu₅ and K_m for CH₂H₄folate (Table 1), even though no direct interactions occur between Arg¹⁶⁶ and the folate (2, 3). This is probably explained by a slight shift in the position of the dUMP in the mutant relative to wt, leading to an altered orientation of the folate.

Role of Asp¹⁶⁹

After formation of the covalent complex between dUMP and TS, the resulting enolate form of dUMP attacks the N5-iminium ion CH₂⁺ = H₄folate, the CH₂H₄folate species that is presumed to be bound to enzyme. This produces a covalent ternary complex of substrates with enzyme as a catalytic intermediate (1, 5). A role originally proposed for Asp¹⁶⁹ was that of a proton donor that would assist in the breakdown of this covalent ternary complex (3). As anticipated, replacement of Asp¹⁶⁹ with Asn produces a completely inactive mutant (Table 1) with only slightly altered affinities for dUMP and MTX-glu₅ (Table 1). However, D169N is no longer capable of forming the ternary covalent complex with substrates. Further the enzyme does not seem to be capable of opening the bridge between N5 and N10 of CH₂H₄folate, even though binding of folates and folate analogs lacking such a bridge is perfectly normal. This points to a new and completely unanticipated role for Asp¹⁶⁹. Since D169N cannot carry out those steps in the catalytic cycle necessary to form the covalent ternary complex between enzyme and substrates, we cannot yet unambiguously determine whether or not Asp¹⁶⁹ has a role in the decomposition of this complex as originally postulated.

Conclusion

We have shown that Cys¹⁴⁶, Arg¹⁶⁶, and Asp¹⁶⁹ are critical residues in the function of TS, and have successfully defined roles for these residues through the study of mutant enzymes. Our investigations have demonstrated the necessity for the use of diverse approaches to characterize the structural and catalytic properties of such enzymes in order to elucidate the alterations in enzyme function produced by substitution of one amino acid residue by another.

References

1. Appleman, J. R. & Villafranca, J. E. (1992) in: *Techniques in Protein Chemistry III* (Angelletti, R. H., ed.) pp. 407–416, Academic Press, Inc. Orlando, Florida.
2. Matthews, D. A., Appelt, K., Oatley, S. J. & Xuong, Ng. H. (1990) *J. Mol. Biol.* 214, 923–936.
3. Matthews, D. A., Villafranca, J. E., Janson, C. A., Smith, W. W., Welsh, K. & Freer, S. (1990) *J. Mol. Biol.* 214, 937–948.
4. Dev, I. K., Yates, B. B., Leong, J. & Dallas, W. S. (1988) *Proc. Natl. Acad. Sci.* 84, 1472–1476.
5. Moore, M. A., Ahmed, F. & Dunlap, R. B. (1986) *Biochemistry* 25, 3311–3317.