Lack of Functional Significance of Cys$^{227}$ and Cys$^{234}$ in Terminal Deoxynucleotidyltransferase*

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Identification of the three functional regions (catalytic, nucleotide substrate-binding, DNA substrate-binding) of the monofunctional template independent DNA polymerase terminal deoxynucleotidyltransferase has not been completely established. The potential participation of 2 amino acid residues, Cys$^{227}$ and Cys$^{234}$, has been controversial, and conflicting data have been published. To investigate the role of Cys$^{227}$, the human terminal transferase cDNA was modified by site-directed mutagenesis to introduce a glycine codon at this position. Mutant and control wild-type human terminal transferase cDNAs had to be inserted into baculovirus genomes by homologous recombination and overexpressed in Trichoplusia ni insect larve because terminal transferase cDNAs have not been successfully expressed in bacterial systems. The Cys$^{227}$ → Gly mutant and wild-type enzymes displayed similar $k_{\text{cat}}$ values for both the nucleotide (dTTP) and DNA initiator (dA$\text{a}_0$) substrates. The $k_{\text{cat}}$ for the mutant enzyme (0.56 s$^{-1}$) was comparable to that of the native enzyme (0.58 s$^{-1}$). Additionally, catalysis by both mutant and wild-type enzymes was stimulated by Zn$^{2+}$. These results together with the observation that the amino acid residue at position 234 is not conserved across species indicated that neither Cys$^{234}$ nor Cys$^{227}$ is an essential residue in the active site of terminal transferase.

Terminal deoxynucleotidyltransferase is a DNA polymerase that is specifically expressed in immature lymphocytes and is thought to be part of a complex of proteins that mediates rearrangements of immunoglobulin and T-cell receptor genes. This enzyme is not a template-directed polymerase and may catalyze random addition of nucleotides at the junctions of the rearranged genes (Alt and Baltimore, 1982; Alt et al., 1986). Terminal transferase consists of a single polypeptide chain of 58 kDa which displays a sole catalytic activity and bears three associated functional regions, the nucleotide- and DNA substrate-binding sites and the catalytic site. As a result of its streamlined functional design, terminal transferase serves as an excellent model for mechanistic studies of DNA polymerization.

The nucleotide-binding domain of calf terminal transferase has been probed by direct cross-linking with dTTP (Pany and Modak, 1988) and with a photoaffinity analog, 8-azido-dATP (Evans and Coleman, 1989). In the presence of UV light, [α$^{32}$P]dTTP was reported to form covalent bonds with Cys$^{227}$ and Cys$^{234}$ of calf terminal transferase (Pany and Modak, 1988). The authors of this study postulated that these 2 residues form hydrogen bonds with the pyrimidine base in the nucleotide-binding domain. However, this model is inconsistent with the observation that calf terminal transferase does not require mercaptoan as a protective agent and is not very sensitive to sulfhydryl inhibitors (Kato et al., 1967). By contrast, the photoaffinity probe 8-azido-dATP covalently binds residues within the region Gly$^{343}$ to Asp$^{389}$ and does not interact with the region of the calf protein containing Cys$^{227}$ and Cys$^{234}$ (Evans et al., 1989).

The DNA-binding domain of calf terminal transferase has also been probed with photosensitive DNAs containing azido residues. Cross-linking was observed within the region of the protein which includes Cys$^{227}$ and Cys$^{234}$ (Farrar et al., 1991). However, in the model for the DNA-binding domain proposed in this study, neither of these Cys residues is in close contact with the DNA. Moreover, Gly appears at position 234 in the human form of the enzyme and Ser appears at position 234 in murine terminal transferase (Koiwai et al., 1986). It is therefore unlikely that the amino acid in this nonconserved position plays an essential role in the active site of terminal transferase. By contrast, Cys$^{227}$ is conserved in the enzyme from calf, human, and murine species. To assess the putative involvement of Cys$^{227}$ in enzyme activity by an alternate strategy, we generated recombinant human terminal transferase in which Cys$^{227}$ was substituted with Gly by site-directed mutagenesis. Although bacterial expression systems offer certain advantages for rapid screening of site-directed mutants, expression of terminal transferase in these systems has not been achieved (Chang et al., 1988). Therefore, we introduced human terminal transferase cDNA into a baculovirus and expressed the altered gene in the insect host. This procedure permitted purification of functional recombinant terminal transferase. Comparative kinetic analyses of the altered and wild-type recombinant enzymes established that Cys$^{227}$ was not critical for enzyme activity.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

The observation that the Cys residue at position 234 in calf terminal transferase was not conserved in either the human or mouse enzymes suggested that the residue was not crucial to the enzyme active site and prompted examination of the significance of the Cys at position 227 which is conserved in

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1. J. A. Medin and M. S. Coleman, unpublished analyses.
2. Portions of this paper (including "Materials and Methods," part of "Results," Figs. 1 and 2, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
all three forms of the enzyme. Guided by the natural substitution of Cys²²⁷ to Gly²³⁴ in human terminal transferase, Cys²²⁷ of the human enzyme was replaced with Gly by site-directed mutagenesis of cloned cDNA in a baculovirus expression system. The specific activities observed in crude extracts of SF9 cells or Trichoplusia ni larvae infected with recombinant baculovirus containing either wild-type or Gly²²⁷ mutant enzymes were comparable: in SF9 cells, 1430 - 1830 (wild-type) versus 1470 (units/mg (mutant), and in larvae, 370 (wild-type) versus 263 units/mg (mutant).

The retention of enzymatic activity, and the stability of the mutant form of the enzyme under the in vivo conditions employed permitted a more detailed functional comparison of the enzymes through kinetic analyses of partially purified preparations. The wild-type and mutant recombinant enzymes partitioned similarly during the purification procedure. In the case of both substrates, dA₉₉ and dGTP, the kₘ values for the wild-type and mutant enzymes were similar (Table I). The 1.5-fold higher values obtained for the mutant enzyme presumably reflected the Cys²²⁷ to Gly substitution. The kₘ values of both recombinant terminal transferases were comparable (0.56 s⁻¹ for wild-type enzyme and 0.58 s⁻¹ for the mutant enzyme) and the kₘ/kₖcat ratios for both dGTP and dA₉₉ were similar (Table I). These results indicated that Cys²²⁷ was apparently not directly involved in binding either substrate and that the replacement of Cys²²⁷ by Gly had only minor effects on the catalytic properties of the enzyme.

Low concentrations of Zn²⁺ have been demonstrated to increase the affinity of terminal transferase for the DNA initiator and to decrease the enzyme’s affinity for dATP resulting in an overall increase in the apparent Vₘₚ of the reaction (Chang and Bollum, 1990). The altered substrate affinities are presumably the result of a Zn²⁺-induced conformational change. This capacity to respond to Zn²⁺ has been retained in the Gly²²⁷ mutant enzyme (Table II, Miniprint).

The region of terminal transferase which contains amino acid positions 227 and 234 has been correlated with the DNA initiator-binding domain (Farrar et al., 1991) and, on the basis of three-dimensional structure predictions, includes a putative α-helix (Matsukage et al., 1987). This non-conservative amino acid substitution of Cys to Gly can theoretically destabilize an α-helix (O’Neil and DeGrado, 1990). Therefore, the Cys to Gly substitution at position 227 could have altered the conformational stability of the enzyme, particularly within the DNA initiator-binding domain. Under standard reaction conditions no alteration in the stability of the mutant enzyme was detected, and the kinetic properties of the human wild-type and mutant enzymes were similar in these assays. However, when their thermal stabilities were compared over a range of temperatures, subtle differences in the functional stabilities of the enzymes were detected (Fig. 2, Miniprint).

**Table I**

**Kinetic properties of terminal transferases**

| Enzyme   | kₘ (dATP) | kₘ (dGTP) | kₘ (dA₉₉) | kₘ/kₖcat (dATP) | kₘ/kₖcat (dGTP) | kₘ/kₖcat (dA₉₉) |
|----------|------------|------------|------------|-----------------|-----------------|-----------------|
| Wild-type| 361 ± 37   | 3.7 ± 0.4  | 0.56       | 1.55 × 10⁴      | 152 × 10⁴       | 109 × 10⁴       |
| Gly²²⁷   | 591 ± 30   | 5.3 ± 0.4  | 0.58       | 0.97 × 10⁴      | 92.7 × 10⁴      | 69.8 × 10⁴      |

While the inflection point in the temperature inactivation curve occurred at about 45 °C for both enzymes (Fig. 2A, Miniprint), analyses of the time course of inactivation at 45 °C revealed differential thermostability which peaked at 10 min (Fig. 2B, Miniprint). This phenotype did not affect the specific activity of the mutant enzyme in crude extracts since these cells and insects were cultured at 27 °C. Indeed, the low optimal temperature employed in the baculovirus expression system facilitates the preparation of mutationally altered proteins with potential thermostabilities.

The similarity between the kinetic properties of the wild-type and Gly²³⁴ mutant forms of human terminal transferase indicated that the presence of Cys in position 227 was not essential to enzyme activity. In order to assess the functional significance of the Cys which occurs at position 234 of the calf enzyme, the kₘ/kₖcat ratios for the 58-kDa form of the calf enzyme (Robbins and Coleman, 1988) were calculated from unpublished analyses. The values (214 × 10⁴ s⁻¹ M⁻¹ for dA₉₉, and 3.7 × 10³ s⁻¹ M⁻¹ for dGTP) were in agreement with corresponding values for the human wild-type and Gly²²⁷ mutant enzymes (Table I). Thus, the replacements of Cys with Gly at position 234 (human terminal transferase), and at both positions 234 and 227 (human Gly²²⁷ mutant) were not accompanied by appreciable alterations in catalytic efficiency. The origin of the observed cross-linking of calf terminal transferase Cys²²⁷ and Cys²³⁴ to dTTP in the presence of UV light reported in an earlier study (Pandey and Modak, 1988) is unknown. However, there is evidence that irradiation of sulfur-containing amino acids leads to the formation of radicals where an unpaired electron localizes on sulfur atoms (Vladimirov et al., 1970). It is conceivable that the 2 Cys residues in this region of the protein were activated and cross-linked to dTTP since it is expected that the nucleotide would be in close proximity with the DNA-binding domain. The data presented in this article, however, provided direct evidence that neither Cys²²⁷ nor Cys²³⁴ is essential for enzyme activity of terminal transferase.

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SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Materials- Restriction endonucleases Avr II and Nco I were obtained from New England Biolabs. T4 DNA ligase and the Klcent fragment of DNA polymerase I were purchased from BRL. [3H]dATP and [3H]dGTP were purchased from DuPont. [3H]dGTPs were synthesized on an ABI 380B synthesize (Applied Biosystems, Inc.) at the University of Kentucky Macromolecular Structure Facility. UltraspHERE MsO4 and MsO2 were purchased from Amersham Chemicals. All other chemicals were reagent grade from various commercial sources.

The transfer vector pAc-C4 was kindly provided by E. Kawasaki (Carni Corp.).

Table II. Effect of Divalent Cation on the Relative Rates of Terminal Transferase Activity at 35°C (wt) and Terminal Transferase (Gly227) (wt)

| Enzyme | Divalent Cation Added | Relative Rates (wt) |
|--------|-----------------------|---------------------|
| Terminal transferase | none | 1.00 |
| Transferase | 62.5 μM ZnSO4 | 2.20 |
| (Gly227) | (wt) | |
| Terminal transferase | none | 1.00 |
| Transferase | 62.5 μM ZnSO4 | 2.12 |

The experiments in which divalent cation was added to the standard reactions were carried out as described in Materials and Methods. The reactions were carried out in triplicate and the enzyme activity values were the average values obtained.

Figure 3. Effect of ZnSO4 on the Relative Rates of Terminal Transferase Activity at 35°C (wt) and Terminal Transferase (Gly227) (wt)

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| Terminal transferase | none | 1.00 |
| Transferase | 62.5 μM ZnSO4 | 2.12 |

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