Active Site Amino Acids That Participate in the Catalytic Mechanism of Nucleoside 2'-Deoxyribosyltransferase*

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The importance of eight nucleoside 2'-deoxyribosyltransferase residues for catalysis was investigated by site-directed mutagenesis. Each residue was selected because of its proximity to nucleophile Glu-98 or on its potential contribution to intrinsic protein fluorescence. Mutation of Asp-72, Asp-92, Tyr-7, Trp-12, and Met-125 resulted in over a 90% activity loss whereas mutation of Tyr-157, Trp-64, and Trp-127 produced less than a 80% activity loss. The magnitude of the perturbation on catalysis by mutation, however, was dependent on donor substrate. The $k_{cat}$ values for dino hydrolysis by these mutants were greater than 25% of that for native enzyme. Although mutant and native enzymes bound substrate analogues with comparable affinities, $K_m$ values for dino hydrolysis varied over a 1000-fold range. The pH dependence of Glu-98 esterification by dCd suggested that amino acids with $p$K values of 4.2 and 7.5 were relevant for catalysis. The intrinsic protein fluorescence was attributed primarily to Trp-127 (-80%). Pre-steady-state kinetic parameters for deoxyribosylation of mutant enzymes by dCd, dTdh, and dAdo were determined by monitoring changes in enzyme fluorescence. Collectively, results obtained with these classes of transferase mutants suggest a change in enzyme conformation attendant upon deoxyribosylation of Glu-98.

Nucleoside 2'-deoxyribosyltransferase (EC 2.4.2.6, transferase) catalyzes cleavage of the glycosidic bonds of 2'-deoxyribonucleosides (1–8) through intervention of a covalent deoxyribosyl-enzyme (EX) intermediate (9). Glu-98 is the active site nucleophile deoxyribosylated during catalysis (9). Glycoside hydrolases that catalyze analogous reactions with retention of stereochemical configuration utilize the carboxyl group from either a glutamyl (10–16) or an aspartyl residue (17–19) as the general acid catalyst and, in general, additional acidic residues that function as general acids/bases (20–24). The distance of the carboxyl group of the general acid for retaining glycosidases is 4.8 to 5.3 Å (23). In addition to these carboxylates, crystal structure data reveal that the Bacillus circulans xylanase has tyrosyl and tryptophanyl residues clustered about the active site that are postulated to contribute to catalysis (23).

In nature, nucleoside 2'-deoxyribosyltransferase is found in various Lactobacilli species and participates in nucleoside recycling in these microorganisms (3–5). The ntd gene encoding this enzyme has been cloned, and the recombinant protein expressed in Escherichia coli (8, 9, 26). Native transferase purified from E. coli and Lactobacilli, is a hexamer composed of identical ~18,000-Da subunits (9, 26). The crystal structure of this enzyme has recently been solved and refined to 2.5 Å resolution. This study identified the monomer fold as a single, doubly-wound $\alpha/\beta$-domain composed of a central 5-stranded $\beta$-sheet flanked by 4 $\alpha$-helices. Although there is one active site per subunit, each complete catalytic center defined by the position of Glu-98 requires participation of side chains from a neighboring subunit (Figs. 1 and 2). The studies described herein were initiated to examine the effect that mutagenesis of selected active site residues has on catalytic efficiency. We present results that extend our understanding of this process by comparing the pre-steady-state and steady-state kinetic properties of native transferase with nine mutant transferases.

Amino acid residues were selected for mutagenesis because of their proximity to the active site nucleophile Glu-98 or their fluorescence properties. Mutation of Glu-98, Asp-72, Asp-92, Tyr-7, and Tyr-157 defined the relative contribution of amino acid side chains to catalysis whereas the W12A, W64A, W64F, and W127F proteins defined the relative contribution of each tryptophanyl residue to the intrinsic protein fluorescence. Collectively, results obtained with these two classes of transferase mutants suggest a change in enzyme conformation attendant upon deoxyribosylation of Glu-98.

EXPERIMENTAL PROCEDURES

Materials—Purines, pyrimidines, and their corresponding 2'-deoxyribonucleosides were from Sigma, 1,2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl)thymine (dTThd) and 2,6-diarnino-9-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-9H-purine (dDAP) were gifts from J. Tuttle, and 2'-deoxy-4'-thiodenosine (dTAdo) was a gift from B. Koszalka, both at Glaxo-Wellcome.

Estimation of Transferase Active Site Concentration—Transferase mutants with the tryptophanyl content of native enzyme were assumed to have an $\epsilon_{278} = 66 M^{-1} cm^{-1}$ per active site that was determined by titrating the fluorescence of native transferase with 2,6-diaminopurine (27). Based on the assumption that equivalent absorbances were obtained with the Bradford dye binding method (28), the extinction coefficients at 278 nm for W12A, W64A, W64F, and W127F mutants were estimated to be 48, 43, 44, and 48 $M^{-1} cm^{-1}$, respectively.

Mutagenesis of the ntd Gene and Expression of Mutant Proteins—Selected transferase amino acids were replaced by site-directed mu
were employed. For example, with the gene encoding E98D, the Glu-98 was not directly attainable in a single step, two rounds of mutagenesis and a reagent kit from Amersham. In the construction of all mutant tagenesis of the cloned gene using the procedure of Taylor et al. (29) and a reagent kit from Amersham. In the construction of all mutant genes, a minimum of two nucleotides per codon was changed. When this was not directly attainable in a single step, two rounds of mutagenesis were employed. For example, with the gene encoding E98D, the Glu-98 codon was first changed to an alanine codon (GAA → GCG) followed by a second mutagenesis (GCG → GAT) on the E98A template. Following mutagenesis, the nucleotide sequence for the entire mutant gene was verified. This ensures that the mutant gene used for protein expression contained only the desired sequence changes. For expression of wild-type and mutant proteins, the coding sequences for these nucleotides were subcloned downstream of a plasmid-borne T7 promoter and transformed into BL21(DE3) (30, 31).

Fig. 1. The active site of nucleoside 2-deoxyriboosyltransferase. The protein model was generated using coordinates from the crystal structure refined at 2.5-Å resolution.7 The residues examined by mutagenesis are shown. The amber and blue tubes represent the peptide backbones of the two subunits that form the active site. Tyr-157 (labeled Tyr357) and Met-125 (labeled Met325) originate from the subunit adjacent to that containing Glu-98.

Fig. 2. Location of tryptophanyl residues relative to nucleophile Glu-98. Trp-12 and Trp-64 are from the same subunit as Glu-98, whereas Trp-127 (labeled Trp327) at the interface between the two subunits is from the neighboring subunit.

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Purification of Mutant Transferases—Transferase mutants were purified from E. coli cells that had been grown in a medium composed of 2 × YT (32), Vogel and Bonner minimal salts (33), and kanamycin (50 μg/ml). Cells (~5 g) were harvested, washed once with cold 50 mM sodium phosphate at pH 7.5, and suspended in an equal volume of the same buffer. The cells were broken by two passages through a French press at 14,000 p.s.i. The cell lysate was centrifuged at 27,000 × g at 4°C for 20 min in a Sorvall SS34 rotor. The supernatant was collected and diluted with an equal volume of distilled H2O. The pH of this solution was adjusted to 6.0 at 5°C with 1 N H3PO4. Transferase was absorbed onto a column (2.5 × 7 cm) of fresh Whatman DE52 resin equilibrated in 75 mM potassium phosphate at pH 6.0. The column was washed with this buffer until the absorbance at 280 nm of the effluent approached the baseline value. Transferase was eluted from the resin with a linear gradient (400 ml) from 75 mM potassium phosphate to 500 mM potassium phosphate at pH 6.0. Typically, the enzyme was eluted from the column between 80 and 160 ml. The central 40 ml of transferase fractions were pooled. The enzyme in this solution was precipitated with 60% (NH4)2SO4 (390 g/liter). The precipitate was collected by centrifugation and dissolved in 10 ml of 25 mM sodium MES at pH 6.0. This solution was dialyzed overnight at 4°C against the same buffer. Traces of denatured protein were removed by centrifugation in a SS34 rotor as above. This procedure typically yielded 14 ml of a solution that had an absorbance at 278 nm of 30 in 30% yield. Analysis of these transferase preparations by SDS-polyacrylamide gel electrophoresis indicated that over 95% of the Coomassie Blue staining material was associated with a species that had a subunit relative molecular weight of 18,000 (9).

Steady-state Kinetic Data Analysis—Hydrolysis of dino to hypoxanthine by transferase mutants was monitored as described previously (27). Equation 1, which describes the substrate dependence of the initial velocity for a ping-pong kinetic mechanism, was fitted to these data.

\[ v/W_1 = \frac{k_{cat}}{1 + \frac{K_{cat}}{[d\text{ino}]} + \frac{K_{cat}}{[Cyt]}}, \]

(Eq. 1)

The activity of transferase mutants was determined with cytosine as the acceptor substrate and three donor acceptor substrate pairs in which the concentration of each substrate was equal to 1 mM. The reaction of dino and Cyt was monitored at 292 nm as described above. The reaction of dAdo with Cyt was monitored at 285 nm (Δε285 = 2.7 mM−1 cm−1). The reaction of dUrd with Cyt was monitored at 286 nm. (Δε286 = 2.2 mM−1 cm−1).

Transient Kinetic Data Analysis—Equation 2 was fitted to the time course of the fluorescence changes during the reaction of transferase with substrates. If the time course of the reaction was biphasic, \( F(t) \) was the observed signal at time equal to \( t \), \( F_1 \) and \( F_2 \) were the respective amplitudes of the two phases, \( k_{obs,1} \) and \( k_{obs,2} \) were the respective pseudo-first-order rate constants, and \( F_0 \) was the final fluorescence intensity.

\[ F(t) = F_1 \cdot \exp(-k_{obs,1} \cdot t) + F_2 \cdot \exp(-k_{obs,2} \cdot t) + F_0 \]

(Eq. 2)

If the time course of the reaction was first-order, \( F_2 \) was set equal to 0. These pseudo-first-order rate constants (\( k_{obs,1} \)) were determined as a function of substrate concentration (S).

When the reaction of mutant enzymes with different donor substrates was examined, these results were interpreted in terms of the mechanisms of Equations 3a, 4a, and 5a. The reaction of dCyd with transferase is a monophasic process (27). Initial complex formation (\( K_c \)) occurs within the dead time of the stopped-flow spectrophotometer (1.6 ms) followed by a first-order reaction to yield deoxyribosylated enzyme (Equation 3a)

\[ K_1 \]

\[ E + d\text{Cyd} \rightleftharpoons E \cdot d\text{Cyd} \rightarrow EX \]

(Eq. 3a)

The observed first-order rate constant for this process is given by Equation 3b.

\[ k_{obs} = \frac{k_{cat} [d\text{Cyd}]}{K_c + [d\text{Cyd}]} \]

(Eq. 3b)

The reaction of dThd with transferase is a biphasic process (27). The early phase

\[ k_1 \]

\[ E + d\text{Thd} \rightleftharpoons E \cdot d\text{Thd} \rightarrow EX \]

(Eq. 4a)
resides Participating in the Transferase Reaction

represents binding of dTthd to give E-dTthd. In contrast to dCyd binding, the binding of dTthd to transferase is observable on the stopped-flow spectrophotometer. The second phase of the reaction is the result of the mechanism are given by Equations 4b and 4c.

\[ k_{\text{obs,2}} = \frac{k_0([dTthd])}{(K_{d12})} \]  

(Eq. 4c)

The reaction of dAdo with transferase is monophasic in the presence of Ade (27). Because of its fluorescent properties, intermediates in the reaction were not much larger than the enzyme concentration, a correction for depletion solution at a ligand concentration equal to \([L]\). Because the dissociation constant \(K_d\) was fitted to the titration data to yield a dissociation constant \(K_d\) and the fractional fluorescence change upon formation of the transferase complex \(\Delta F_{\text{obs}}\).

\[ \Delta F ([L]) = 1 - \frac{\Delta F ([L])}{K_a + [L]} \]  

(Eq. 6)

Under conditions in which the concentration of Ade is sufficiently large such that EX is present mostly as EX-Ade, the observed rate constant for this mechanism is given by Equation 5b.

\[ k_{\text{obs}} = \frac{(k_0[dAdo])}{K_{a1} + [dAdo]} + k_2 \]  

(Eq. 5b)

where \(\Delta F ([L])\) is the corrected fractional fluorescence of the transferase solution at a ligand concentration equal to \([L]\). Because the dissociation constants of the substrate analogues \([L]\) used to titrate transferase were much larger than the enzyme concentration, a correction for depletion of the substrate analogue resulting from binding to transferase was not necessary.

\[ k_{\text{obs}} = \frac{k_0[dAdo]}{k_{a1} + [dAdo]} + k_2 \]  

(Eq. 5b)

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Titrations of Transferase Mutants with Substrate Analogues—The intrinsic protein fluorescence of transferase \((\lambda_{\text{ex}} = 300, \lambda_{\text{em}} = 340)\) was quenched by substrate analogues. Because the substrate analogues had relatively large dissociation constants for the enzyme and also had significant absorbance at the excitation wavelength, it was necessary to calculate relatively large inner filter corrections from the absorbance of the titrant at 300 nm. Consequently, the correction factors were determined empirically by titration of N-acetyltryptophan with the titrant. The dynamic and static quenching contributions to the fluorescence quenching of N-acetyltryptophan by the titrant are minimal at the concentration of titrant employed in these experiments (34). Equation 6 was fitted to the titration data to yield a dissociation constant \(K_d\) and the fractional fluorescence change upon formation of the transferase complex \(\Delta F_{\text{obs}}\).

\[ \frac{1}{K_{\alpha}[H^+]} = \frac{1}{K_{a1} + [H^+]} + \frac{1}{K_{a2} + [H^+]} \]  

(Eq. 9)

The reaction of dAdo with transferase is monophasic in the presence of Ade (27). Because of the fluorescent properties of intermediates in the presence of dAdo, the effect of dFDAP on the residual activity of mutant enzymes was determined after reaction of 1000 \(\mu\)M dFDAP with 200 \(\mu\)M enzyme for 50 min at 25°C. The residual transferase activity was determined with 1 \(\mu\)M dAdo and 1 \(\mu\)M Cyt as substrates. The initial velocity of product formation was monitored spectrophotometrically at 285 nm \((\Delta t_{285} = 2.7 \text{ nm}^{-1} \text{ cm}^{-1})\).

General Methods—Kinetic data, absorbance data, and fluorescence data were collected as described previously (27). The appropriate equations were fitted to the data by nonlinear least squares using SigmaPlot from Jandel Scientific (Corte Madera, CA).

RESULTS

Catalytic Activities of Mutant Transferases—Based on the demonstration that Glu-98 is deoxyribosylated during catalysis (9) and the crystal structure of native transferase, amino acids that could potentially participate in the catalytic mechanism of the 2-deoxyribosyltransferase reaction were chosen for mutagenesis. These residues were Glu-98, Asp-72, Asp-92, Tyr-7, Tyr-157, Met-125, Trp-12, Trp-64, and Trp-127. Because the substrate specificity of native transferase is very broad, the effect of these single amino acid replacements on catalytic activity was examined for several representative donor nucleoside substrates with cytosine as the acceptor substrate. Results from these studies demonstrated that: 1) mutagenesis of Glu-98, Asp-72, Asp-92, Tyr-7, Tyr-157, Met-125, Trp-12, Trp-64, and deletion of Tyr-157 caused large decreases in enzyme activity (>90%) but did not completely eliminate catalysis; 2) Trp-64, Trp-127, and the side chain of Tyr-157 did not significantly affect catalysis; and 3) the magnitude of the effects of mutagenesis on catalytic activity was dependent on the substrate (Table I).

Native enzyme catalyzes hydrolysis of 2-deoxyribosylnucleosides in addition to its transferase activity (6–9). The steady-state kinetic parameters for dIno hydrolysis by mutant transferases are summarized in Table II. The kinetic data were fitted to the databased on the demonstration that Glu-98 is deoxyribosylated during catalysis (9) and the crystal structure of native transferase, amino acids that could potentially participate in the catalytic mechanism of the 2-deoxyribosyltransferase reaction were chosen for mutagenesis. These residues were Glu-98, Asp-72, Asp-92, Tyr-7, Tyr-157, Met-125, Trp-12, Trp-64, and Trp-127. Because the substrate specificity of native transferase is very broad, the effect of these single amino acid replacements on catalytic activity was examined for several representative donor nucleoside substrates with cytosine as the acceptor substrate. Results from these studies demonstrated that: 1) mutagenesis of Glu-98, Asp-72, Asp-92, Tyr-7, Tyr-157, Met-125, Trp-12, Trp-64, and deletion of Tyr-157 caused large decreases in enzyme activity (>90%) but did not completely eliminate catalysis; 2) Trp-64, Trp-127, and the side chain of Tyr-157 did not significantly affect catalysis; and 3) the magnitude of the effects of mutagenesis on catalytic activity was dependent on the substrate (Table I).

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activity is to treat the mutant enzyme with an inactivator of native transferase. Previously, dFDAP was shown to inhibit native transferase by covalently modifying Glu-98. When 200 μM enzyme was incubated with 1000 μM dFDAP for 50 min, the activity of D72A, D92A, and Y7A transferases with dAdo and Cyt as substrates did not markedly decrease. In contrast, native enzyme lost over 95% of its activity under these conditions. Incubation of E98A with dFDAP decreased the ratio of E98A activity to that of native enzyme from 4 × 10^{-4} to 5 × 10^{-5}.

Under certain circumstances, the activity of a mutant enzyme can be increased by providing the missing functional group in the form of a small, soluble acid or base. When catalytic rescue was attempted with a mutant β-glucosidase from Agrobacterium faecalis lacking an essential glutamyl residue, the addition of a high concentration of formate increased $k_{cat}$ (10^5-fold), although product stereochemistry was opposite that formed with the wild-type enzyme (35). When native transferase was assayed in 4 mM formate at pH 6.0 with 1 mM dAdo and 1 mM Cyt as substrates, its activity was reduced 48%. Assay of mutant transferases in buffer containing 4 mM formate reduced the activities of the D72A and E98A enzymes by 38% and 78%, respectively, but enhanced the activity of the D92A mutant 26-fold. Nonetheless, the activity of D92A transferase in 4 mM formate was less than 1% that of native transferase in this buffer.

### Intrinsic Protein Fluorescence of Mutant Transferase—Native transferase is a hexameric protein with three tryptophanyl residues (Trp-12, Trp-64, and Trp-127) per subunit. The intrinsic protein fluorescence of native transferase is substantially perturbed during deoxyribosylation of Glu-98 (27). It was of interest to determine the contribution of each tryptophanyl residue to the intrinsic protein fluorescence and to the fluorescence signal observed during catalysis.

Trp-12, Trp-64, and Trp-127 were changed individually to an alanyl or a phenylalanyl residue by site-directed mutagenesis. Substitution of an alanyl residue for Trp-12 or Trp-64 did not markedly affect the fluorescence emission intensity of the protein (Table III). Similarly, substitution of a phenylalanyl residue for Trp-64 had little effect on protein fluorescence (Table III). In contrast, substitution of a phenylalanyl residue for Trp-127 resulted in a dramatic decrease in fluorescence emission intensity. The fluorescence of W127F was 20% of that measured for native transferase (Table III and Fig. 3).

Native enzyme, W12A, and W64F had emission maximum at
The dissociation constants were estimated for each mutant from a fit of Equation 6 to these data. The data were corrected for inner filter effects as described under “Experimental Procedures.” Other conditions were as described in Fig. 3. Fluorescence values were normalized to the fluorescence of enzyme in the absence of ligand. The solid lines were calculated with Equation 6, and the values for the parameters are tabulated in Table IV.

Titrations of the fluorescence of native, W12A, and W64A transferases by N⁶-methyladenine are presented in Fig. 4. For native enzyme, dissociation constants (Kₐ) with dTAdo and dThd were 30 ± 1 μM and 263 ± 9 μM, respectively (Table IV). These values were similar to the Kₐ values of 46 ± 3 μM and 490 ± 20 μM, respectively, determined for these substrate analogues in a transfer reaction between dCyd and Ade. Thus, the dissociation constants represent substrate analogue binding to the active site. Except for a few instances, binding of nucleobase or nucleoside analogue by native and mutant proteins was similar; the ratio of Kₐ values for mutant to native enzymes was, in general, less than 10 (Table IV).

Half-reactions of Selected Donor Substrates with Mutant Transferases—The relative activities of native and mutant transferases with selected substrates are given in Table I. The kcat values for the dIno/Cyt reaction (Table II) are a function of the rate constants of the donor and acceptor half-reactions. For native enzyme, these half-reactions have been monitored by changes in enzyme fluorescence (27). The kinetic constants are estimated from a fit of Equation 6 to these data. When the fluorescence of mutant transferases was titrated with acceptor substrates N⁶-methyladenine, adenine, and thymine, these substrates bound to the enzyme without further reaction so the fit of Equation 6 to these data yielded the dissociation constant. In contrast, donor substrates deoxyribosylate the enzyme with nucleobase release (9). At low enzyme concentrations, this reaction will appear irreversible and cannot be treated as a simple binding isotherm. Consequently, the affinity of mutant proteins was determined for nucleoside analogues that very inefficiently deoxyribosylated the enzyme. The sugar moiety of 200 μM dTAdo or dThd was transferred to cytosine (200 μM) at a rate less than 0.05% of that of dAdo or dThd (determined spectrophotometrically).

### Table III

| Enzyme       | Relative fluorescence<sup>a</sup> |
|--------------|----------------------------------|
|              | 0 mM Guanidine HCl | 6 mM Guanidine HCl |
| Native       | 1.00              | 1.00              |
| D72A         | 1.00              | 0.99              |
| D72N         | 0.98              | 0.99              |
| D92A         | 1.03              | 1.01              |
| D92N         | 0.93              | 0.98              |
| E98A         | 0.93              | 0.87              |
| E98D         | 1.09              | 0.95              |
| M125A        | 1.00              | 0.95              |
| Y7A          | 1.08              | 1.00              |
| Y157F        | 1.10              | 1.03              |
| Y157         | 1.24              | 0.97              |
| W12A         | 0.95              | 0.63              |
| W64A         | 1.13              | 0.52              |
| W64F         | 1.18              | 0.63              |
| W127F        | 0.20              | 0.63              |

<sup>a</sup> For mutant enzymes in 0.1 M MES at pH 6.0, the ratio of the fluorescence emission intensity at 322 nm of the mutant enzyme to that of native enzyme was determined with an excitation wavelength of 280 nm. For mutant enzymes in 0.1 M MES with 6 M guanidinium chloride at pH 6.0, the ratio reported represents the fluorescence emission intensity at 360 nm of the mutant enzyme to that of native enzyme determined with an excitation wavelength of 280 nm.

3 We previously reported that the half-reaction of dIno with native transferase was described by an equation analogous to Equation 3b with Kₐ = 420 μM and kₐ = 3.7 s⁻¹ (27). Because the amplitude of the fluorescence signal decreased as the dIno concentration increased, data were not collected for dIno concentrations above 700 μM in these experiments. When the dIno concentration was varied between 2.5 and 20...
The dissociation constant \( K_d \) and the fractional fluorescence quenching upon formation of a complex with \( \Delta F \), with the indicated ligands were calculated from fluorescence emission data \( \lambda_{\text{excitation}} = 300 \text{ nm} \) and \( \lambda_{\text{emission}} = 340 \text{ nm} \) and Equation 6 as described under "Experimental Procedures."

### Table IV

Parameters for binding of transferase to substrate analogues

| Enzyme          | Ka, \( \mu \text{M} \) (\( \Delta F_0 \)) |
|-----------------|------------------------------------------|
| Native          | 78 ± 4 (0.292 ± 0.004)                  |
| D72A            | 170 ± 10 (0.076 ± 0.006)                |
| D72N            | 41 ± 4 (0.276 ± 0.005)                  |
| D92A            | 360 ± 30 (0.29 ± 0.03)                  |
| Y157F           | 128 ± 2 (0.349 ± 0.002)                 |
| E98A            | 70 ± 3 (0.314 ± 0.004)                  |
| E98D            | 54 ± 3 (0.233 ± 0.003)                  |
| M125A           | 72 ± 1 (0.309 ± 0.001)                  |
| Y7A             | 88 ± 2 (0.385 ± 0.002)                  |
| Y157            | 300 ± 10 (0.208 ± 0.007)                |
| W124A           | 250 ± 60 (0.12 ± 0.01)                  |
| W64A            | 260 ± 20 (0.233 ± 0.008)                |
| W64F            | 146 ± 6 (0.02 ± 0.003)                  |
| W127F           | 53 ± 8 (0.159 ± 0.005)                  |

*The change in fluorescence signal upon addition of 1 mM ligand to the enzyme was less than 5%.*

derived from the half-reaction data with dAdo and Cyt as substrates are consistent with the steady-state kinetic parameters determined for the complete transfer reaction (27). This technique was extended to the mutant transferases (Table IV).

Reactions of native transferase with dThd and dAdo, analyzed by the mechanism of Equations 4a and 5a, have been presented previously (27). The time course for reaction of native transferase with dCyd (Fig. 5, A and B) was analyzed by the mechanism of Equation 3a. Binding of dCyd to transferase is a rapid equilibrium process that yields an initial complex \( \text{E-dCyd} \) from which deoxyribosylated enzyme \( \text{EX} \) is formed. For dCyd concentrations much greater that the \( K_d \) for initial complex formation, a maximal fluorescence change is observed and represents the transformation of E-dCyd to EX and Cyt (Fig. 5A). The fluorescence change for EX formation from E and dCyd decreases as the dCyd concentration is decreased (Fig. 5B). Because the concentration of E-dCyd accumulating transiently at low dCyd concentrations and the net fluorescence change approaches 0 under these conditions, E and EX have similar fluorescence but that for E-dCyd is quenched. Equivalent results were observed with W127F transferase at high dCyd concentrations (Fig. 5A). At low dCyd, however, the sign of the fluorescence change reversed (Fig. 5B), which suggested that, for this mutant enzyme, fluorescence of E was greater than that of \( \text{EX} \). The concentration dependences of the pseudo-first-order rate constant describing the half-reaction for native and W127F transferases with dCyd are described by Equation 3b (Fig. 6). The kinetic parameters for native enzyme were \( k_{cat} = 280 ± 10 \text{ s}^{-1} \) and \( K_{cat} = 240 ± 30 \text{ \mu M} \). The analogous values for W127F were \( 260 ± 10 \text{ s}^{-1} \) and \( 250 ± 40 \text{ \mu M} \).

Kinetic parameters for the half-reaction of each mutant transferase with dCyd, dThd, and dAdo are summarized in Tables V and VI. Substitution of Glu-98 with either an alanyl or aspartyl residue profoundly affected the rate of EX formation from both dCyd and dThd. This effect on catalysis was similar to that observed for these mutant enzymes in their transfer reaction rates (Table I) and in their rate of dIno hydrolysis (Table II). It should be noted that, although the fluorescence of W127F was reduced by 80%, the pre-steady-state parameters determined for this mutant enzyme were equivalent to those of native transferase (Tables V and VI).

**pH Dependence**—The kinetic parameters describing the half-reaction of dCyd with native transferase were determined for selected pH values between 3.95 and 8.26. These results show that \( k_2 \) values were independent of pH between pH 3.95 and 8.0 (Fig. 7A). \( K_f \) for this reaction was sufficiently large at pH values greater than 8.0 that a reliable estimate for \( k_2 \) could not be obtained. The \( k_2/K_f \) values had a bell-shaped pH dependence. The slope of the basic limb of the titration curve was consistent with a single ionization, whereas the slope of the acidic limb suggested that the value of \( k_2/K_f \) was dependent on two ionizations. Because only ionizations of free enzyme and free substrate are reflected in the pH dependence of \( k_2/K_f \) and the \( pK \) of dCyd under these experimental conditions was 4.26 ± 0.02, one of the ionizations of the acidic limb was assigned to dCyd. Equation 8, which was derived for the mechanism of Equation 7, described the pH dependence of \( k_2/K_f \) with \( K_f = (6 \pm 1) \times 10^{-5} \text{ M} \) (pKeA = 4.22 ± 0.03), \( K_B = (3.2 ± 0.5) \times 10^{-8} \text{ M} \) (pKeB = 7.49 ± 0.02) and a pH independent value for \( k_2/K_f = 0.94 ± 0.03 \text{ \mu M}^{-1} \text{ s}^{-1} \).
ionizations associated with this binding process should be related only to pK values for free enzyme as dFThd does not have a pK value between pH 5 and 8.5. The dependence of 1/Kd of native enzyme for dFThd (Fig. 7B) was fitted to Equation 9 to give a pH independent Kd = 290 ± 20 μM, Ka = (2.0 ± 0.6) × 10⁻² M (pKa = 4.7 ± 0.1), and Kb = (1 ± 0.3) × 10⁻⁸ M (pKb = 8.0 ± 0.1). When the ionizable carboxymethyl group of Glu-98 of native enzyme was replaced by a methyl group in mutant E98A, the pH dependence of dFThd binding to this mutant enzyme was eliminated (Fig. 7B).

DISCUSSION

The intrinsic protein fluorescence of transferase is markedly perturbed by substrates and substrate analogues. Recently, these fluorescence changes were used to monitor transfer of the deoxyribosyl sugar of a donor substrate to the enzyme (27). Native transferase is a hexamer composed of identical subunits, each of which contains three tryptophanyl residues (9). Crystal structure data places Trp-12 and Trp-64 in the vicinity of the active site defined by the position of Glu-98, whereas Trp-127 is located at the subunit interface distal from the active site nucleophile (Fig. 2). The effect of substitution on each of these tryptophanyl residues with an alanyl or phenylalanyl residue individually established that 80% of the intrinsic protein fluorescence is due to Trp-127. Substitution of an alanyl residue for Trp-12 or Trp-64 caused a 5% decrease or a 13% increase in the intrinsic protein fluorescence, respectively (Fig. 2 and Table III). The wavelength for maximal fluorescence emission of native transferase was 322 nm and is characteristic for a tryptophanyl residue buried in an hydrophobic environment (36). Furthermore, the relatively larger quantum yield of Trp-127 relative to either Trp-12 or Trp-64 is consistent with their location in hydrophilic environments.

The magnitude of the fluorescence changes (~20%) during catalysis and the relative contributions of the fluorescence of Trp-12, Trp-64, and Trp-127 to the intrinsic fluorescence of the enzyme suggested that most of the fluorescence change was due to perturbation of the Trp-127 environment. This was confirmed by comparison of the fluorescence changes associated with reaction of dCyd with native and W127F transferases (Fig. 5A). The reaction of 1 mM dCyd with native enzyme (Kd = 240 μM) and W127F (Kd = 250 μM) resulted in fractional fluorescence changes of 0.28 and 0.09, respectively. For comparison of the absolute fluorescence changes associated with each protein, the fractional fluorescence change associated with W127F was multiplied by the ratio of the relative fluorescence of W127F to that of native enzyme. Thus, the normalized fractional change in fluorescence of native enzyme during the reaction with dCyd was 0.28 and that with W127F was 0.018. Clearly, over 90% of the fluorescence change associated with the half-reaction of dCyd with native transferase is due to perturbation in the fluorescence of Trp-127. Because Trp-127 is located 16 Å from the active site nucleophile, these fluorescence changes result from a conformation change at the subunit-subunit interface and not from a direct interaction of dCyd with Trp-127 (Fig. 2).

The nucleoside 2'-deoxyribosyltransferase-catalyzed reaction results in retention of product configuration (6, 7). This result plus the fact that initial velocity data for the transfer reaction is described by a ping-pong kinetic mechanism suggests formation of a covalently deoxyribosylated enzyme intermediate (8). Recently, this intermediate was trapped by reaction of transferase with 2,6-diamino-9-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-9H-purine, and the residue modified was 4 The average of the distances between Trp-127 (N) and Glu-98 (Oz) plus Glu-98 (Oz2).
The assignment of Glu-98 as the active site nucleophile (9) and its position within the crystal structure of native transferase2 define those amino acids that form the active site pocket and which could participate in catalysis. Identification of the transferase general acid/base is of particular interest. The combination of structural data and the mechanism-based requirement of a second carboxylate residue guided our selection of amino acids to be replaced by site-directed mutagenesis. For retaining glycosidases, the average distance between the pair of catalytic carboxylates is 4.8 to 5.3 Å (23). Only Asp-72 and Asp-92 of catalytic carboxylates is 4.8 to 5.3 Å (23). Only Asp-72 and Glu-98 are always replaced by site-directed mutagenesis. For the definition of a second carboxylate residue guided our selection of amino acids to be replaced by site-directed mutagenesis. Identification of the active site nucleophile and its position within the crystal structure of native transferase2 define those amino acids that form the active site pocket and which could participate in catalysis. Identification of the general acid/base is of particular interest. The combination of structural data and the mechanism-based requirement of a second carboxylate residue guided our selection of amino acids to be replaced by site-directed mutagenesis. For retaining glycosidases, the average distance between the pair of catalytic carboxylates is 4.8 to 5.3 Å (23). Only Asp-72 and Glu-98 are always replaced by site-directed mutagenesis.

### Table V

Pre-steady state parameters for reaction of transferase mutants with dCyd and dThd

| Enzyme | dCyd | dThd |
|--------|------|------|
|        | $k_1$ | $k_2$ | $k_{-1}$ | $k_{-2}$ |
| Native | 240 ± 30 | 280 ± 10 | 4.43 ± 0.003 | 42.2 ± 0.9 |
| D72A   | 40 ± 20  | 0.29 ± 0.02 | 0.9 ± 0.1 | 310 ± 20 |
| D72N   | 160 ± 20 | 16.2 ± 0.9  | 1.23 ± 0.04 | 112 ± 6 |
| D92A   | $k_2/K_1 = (3.5 ± 0.3) \times 10^5 \mu M^{-1} s^{-1}$ | NSa | NSa | NSa |
| D92N   | NSa | NSa | NSa |
| E98A   | NSa | NSa | NSa |
| E98D   | NSa | NSa |
| M125A  | 1100 ± 200 | 18 ± 1 | 0.08 ± 0.01 | 130 ± 10 |
| Y7A    | 210 ± 30  | 2.00 ± 0.09 | 1.6 ± 0.1 | 160 ± 10 |
| Y157F  | 340 ± 50  | 124 ± 6 | 1.04 ± 0.008 | 50 ± 3 |
| ΔY157F | $k_2/K_1 = 7 ± 0.3 \times 10^3 \mu M^{-1} s^{-1}$ | 1.0 ± 0.1 | 160 ± 10 | 3.06 ± 0.01 |
| W12A   | 1700 ± 500 | 0.36 ± 0.07 | 1.6 ± 0.1 | 160 ± 10 |
| W64A   | NSa | NSa |
| W64F   | NSa | NSa |
| W127F  | 250 ± 40  | 260 ± 10 | 0.33 ± 0.02 | 50 ± 9 |

a The observed change in fluorescence signal upon addition of 1 mM substrate to the protein with the stopped-flow spectrophotometer was less than 3% of the initial protein fluorescence.

### Table VI

Pre-steady state parameters for reaction of selected transferase mutants with dAdo

| Enzyme | dCyd | dThd |
|--------|------|------|
|        | $k_1$ | $k_2$ | $k_{-1}$ | $k_{-2}$ |
| Native | 200 ± 20 | 173 ± 3 | 52 ± 3 | 0.53 ± 0.03 |
| W64A   | 700 ± 300 | 140 ± 20 | 36 ± 4 |
| W64F   | 560 ± 80  | 250 ± 10 | 90 ± 3 |
| Y157F  | 400 ± 90  | 127 ± 7 | 74 ± 4 |
| W127F  | 230 ± 40  | 216 ± 7 | 30 ± 7 |

The fluorescence changes observed with the stopped-flow spectrophotometer after mixing 1 mM substrate with the following transferase mutants were less than 3% of the initial protein fluorescence: E98A, E98D, D72A, D72N, D92A, W12A, Y7A, Y157F, and M125A. The reaction of D72N enzyme with dAdo and 100 μM Ade was described by Equation 4c where $k_2 = 0.24 ± 0.02 \mu M^{-1} s^{-1}$, $k_1 = 179 ± 5 s^{-1}$, and $k_2 = 2.9 ± 0.1 s^{-1}$.

### Figure 7

**A** The pH dependence of $k_2/K_1$ and $k_2$ for the reaction of dCyd with native transferase. Experimental values for these parameters were calculated from data similar to those presented in Fig. 4. The solid line was calculated by Equation 8, and the values for the parameters are given under “Results.” **B** The pH dependence for the binding of dThd to native and E98A transferases. $K_d$ values were calculated from fluorescence quenching data as described in Fig. 4. The solid line for the binding data with native transferase was calculated with an equation analogous to Equation 8, and the parameters are given under “Results.”

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Asp-92 are candidates for the role of the general acid/base in transferase. The distance between Glu-98 (O\text{e}2) and Asp-72 (O\text{e}2) is 6.0 Å and that between Glu-98 (O\text{e}2) and Asp-92 (O\text{e}2) is 5.6 Å (Fig. 1). Thus, based on geometry, either Asp-72 or Asp-92, or possibly both, could function as the general acid/base. The role of each in catalysis was assessed by the effects that replacement with alanyl or asparaginyl residues had on substrate binding and catalysis. The ratio of catalytic activity of native enzyme to D92A was approximately 10\textsuperscript{4} for all substrates examined whereas this ratio for D72A was as small as 30 for d\textsubscript{11}no and Cyt as substrates (Table I). In accord with these findings, only the activity of D92A was rescued by 4 \text{m} formate. These results suggest that Asp-92 functions as the general acid/base. However, the ratio of catalytic activity of native transferase to that of D92N was less than 10 with d\textsubscript{11}ur and Cyt as substrates (Table I). Since asparagine would not be expected to function efficiently as a general acid/base, this result brings into question the exclusivity of Asp-92 as the acid catalyst. Alternatively, this observation may reflect the relationship between acid/base-assisted catalysis and the nature of the leaving group. For example, the nucleophile and general acid/base of the exoglucanase/xylanase from Cellulomonas fimi (Cex), which has a mechanism similar to that of transferase, are Glu-233 and Glu-127, respectively (20). When the ratio of catalytic efficiency (k\textsubscript{cat}/K\text{m}) of native Cex to E127A enzyme was systematically studied, this value varied between 6000 and 1 depending on the leaving group ability of the substrate (20). If a similar circumstance exists for transferase, these studies implicate Asp-92 as the general acid/base. The role of Asp-72 is less clear; perhaps this residue participates in stabilizing the oxycarbonium ion-like transition state.

Replacement of the active site nucleophile Glu-98 by an alanyl or an aspararyl residue had a more profound effect on catalysis than did substitution of other amino acid residues (Tables I, II, and IV). However, the ratio of the catalytic activity of E98A to that of native enzyme was greater than expected from previous work with a comparable mutant of the A. faecalis \(\beta\)-glycosidase (42). The source of this difference is unknown but is too large to be accounted for by translational misincorporation. It is noteworthy that the carboxylates of Asp-92 and Asp-72 are separated by 9 to 11 Å which is similar to the distance between the catalytic carboxylate pair of the inverting glycosidases (23, 35). However, when Glu-98 was replaced by an aspararyl residue, the ratio of catalytic activity of the mutant transferase relative to that of the native enzyme was roughly comparable to the analogous ratios for the A. faecalis \(\beta\)-glycosidase and the B. circulans xylanase (35, 42). Because substrates bind to mutant transferases with similar affinity (Table IV), the effects of substitution at Glu-98 reside in the chemistry of the reaction.

The amino acids in the native enzyme were chosen for mutagenesis based on their proximity to Glu-98. Three of these, Asp-72, Asp-92, and Tyr-7 originate from the subunit containing Glu-98 while Met-125, Trp-127, and Tyr-157 belong to the neighboring subunit (see Figs. 1 and 2). Mutant enzymes with a phenylalanyl residue in place of Trp-64, Trp-127, or Tyr-157 suffered less than an 85% decrease in catalytic activity and substrate affinity (Tables I and IV). In contrast, replacement of Tyr-7, Trp-12, or Met-125 with an alanyl residue and deletion of Tyr-157, the C-terminal residue, caused a significantly larger decrease in catalytic activity; but, as noted above, the magnitude of these effects was substrate-dependent. Comparison of the of Y157F and ΔY157 suggest that the C-terminal carboxylate and not the phenolic oxygen is important for catalysis. For Y7A and M125A, the reduction in enzymatic activity is consistent with these mutations affecting reaction chemistry since the effects of substrate binding were minor (Table IV). The effects of these substitutions on catalysis may be due to subtle changes in orientation of either the substrates within the active site or the groups involved in catalysis. The phenolic oxygen of Tyr-7 is 3.0 Å from the catalytic carboxylate of Glu-98 and could serve to orient this group for catalysis or hydrogen bond to the 3'-OH of the deoxynucleoside substrate. A similar function for a tyrosyl residue has been suggested following mutagenesis of the B. circulans xylanase (25). The role of Met-125 remains to be defined, but the consequence of its replacement and proximity to Trp-127, the source of 90% of the fluorescent change observed during catalysis, is most interesting. Further insight into the roles of these residues in catalysis may come from characterization of revertant enzymes derived from genetic selections that require expression of a functional transferase.

Except for mutation of Glu-98, the mutations examined here had little effect on k\textsubscript{cat} for transferase hydrolyase activity, but had a large effect on K\text{m}. This result is consistent with hydrolysis of deoxyribosylated enzyme being the rate-limiting step that is not subject to significant catalysis by any of the amino acid side chains mutated for this study.

The pH dependence of k\textsubscript{cat}/K\text{m} for the deoxyribosylation of native transferase by dCyd indicated two groups with pK\textsubscript{a} values of 4.2 and 7.5 (Fig. 7A). Because substrate ionization was corrected for, these represent pK\textsubscript{a} values for free enzyme. When the pH dependence for binding of dFThd to native enzyme was measured, the pK\textsubscript{a} values obtained were 4.7 and 8.0 (Fig. 7B). The pH dependence for binding of dFThd to E98A suggests that the lower pK\textsubscript{a} value measured with native enzyme is the property of active site nucleophile Glu-98, although a definitive assignment is not possible due to inaccuracy inherent in signal strength as the assay pH became more alkaline (Fig. 7B). The bell-shaped profile noted for the pH dependence of k\textsubscript{cat}/K\text{m} of the native enzyme suggests that the monoprotonated form of the free enzyme binds substrate as described by Equation 7. For native enzyme, k\textsubscript{cat}/K\text{m} was independent of pH. This suggests that, upon ES formation, other ionizations are unimportant for catalysis or that substrate binding results in their pK\textsubscript{a} values being shifted out of the pH range of these experiments. The pH dependence of the transferase reaction is similar to that for the hydrolysis of 2',4'-dinitrophenyl-\(\beta\)-cellobiose by the xylanase from C. fimi (13). For this enzyme, the active site nucleophile was identified as Glu-233 and the general acid as Glu-127. The pH dependence data for transferase and C. fimi xylanase (13) indicate that two ionizable groups contribute to catalysis and that only one of these groups is protonated in the active form of the enzyme. These data do not, however, indicate the protonation state of the specific transferase residues in the active form of the enzyme; but, it seems likely that one of these two ionizable residues is Glu-98.

In summary, we have established that Asp-72, Asp-92, Trp-12, Met-125, Tyr-7, and the C-terminal carboxylate of Tyr-157 contribute significantly to the catalytic efficiency of nucleoside 2-deoxyribosyltransferase. The detailed role of each residue in catalysis will have to await the crystal structures of the mutant enzymes complexed with appropriate substrates in their active sites. We had hoped that these mutagenesis studies would identify the general acid for the transferase reaction, but Asp-92 and Asp-72 both remain as possible candidates. Perhaps the ambiguity in assigning the role of general acid exclusively to one of these residues is founded in the relaxed substrate specificity of the transferase that may allow both residues to function as the general acid in a substrate-dependent manner.
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Active Site Amino Acids That Participate in the Catalytic Mechanism of Nucleoside 2′-Deoxyribosyltransferase

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