# Protein Structural Changes Accompanying Formation of Enzymatic Transition States: Tryptophan Environment in Ground-State and Transition-State Analogue Complexes of Adenosine Deaminase†

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ABSTRACT: The accessibility of protein tryptophan fluorescence to the quenching agent acrylamide has been studied in adenosine deaminase and in binary complexes of the enzyme with ground-state or transition-state analogues. Although the enzyme contains three tryptophan residues, Stern–Volmer plots are linear with all the fluorescence quenchable at high acrylamide concentrations. Tryptophan fluorescence is less easily quenched in the binary complexes than in the free enzyme, indicating a decrease in the accessibility of these residues. The greatest decrease in accessibility is found for the transition-state analogue complexes. Although the affinities of the transition-state analogues studied span a range of $10^6$, the Stern–Volmer constants of the complexes are the same within experimental error. Thus, as measured by this technique, changes in enzyme conformation accompanying formation of these complexes are similar for all transition-state analogues. Resonance energy transfer from tryptophan as donor to ligand as acceptor successfully explains the differing abilities of ligands to quench the enzyme's intrinsic fluorescence upon formation of complexes in the absence of acrylamide. On the basis of Forster distance calculations, it is likely that the residues partially quenched upon formation of transition-state analogue complexes are distant from the active site.

A full understanding of enzyme mechanisms requires elucidation of the structures of transition states. Studies of stable analogues of the substrate portion of the activated complex can reveal what substrate bond-making–bond-breaking steps occur during turnover (Wolfenden, 1976, 1978). It has been suggested (Wolfenden, 1978; Frieden et al., 1980) that enzyme conformation changes are also an important part of the activation process. It seems probable that the enzyme structure which is optimal for binding the transition state of the substrate (and thereby lowering its standard free energy) is not that appropriate for binding the ground state of the substrate. As a probe of the nature of these enzyme structure changes, we report here a study of the solvent accessibility of tryptophan in free enzyme and in ground-state and transition-state analogue complexes of adenosine deaminase. Such experiments with transition-state analogue inhibitors have not been previously described for any enzyme system.

Adenosine deaminase was chosen for this study because of the extensive data available for the catalytic reaction itself and for the binding of several ground-state and transition-state analogues (Wolfenden, 1976, 1978; Frieden et al., 1980; Kurz & Frieden, 1983). The chemical mechanism for this enzyme, which catalyzes the hydrolysis of (deoxy)adenosine to (deoxy)inosine, is believed to be of the addition–elimination type with direct water attack on the substrate resulting in the formation of a tetrahedral intermediate. Thus, compounds (Figure 1) which approximate the structure of the tetrahedral intermediate (or the transition state leading to it) are potent inhibitors which bind to the enzyme with apparent second-order rate constants 2–3 orders of magnitude below the encounter-controlled limit. On the other hand, compounds with a fully aromatized purine (ground-state analogues, Figure 1) are generally inhibitors of lower affinity which bind to the enzyme at the encounter-controlled rate. What appears to be characteristic of inhibition of adenosine deaminase by transition-state analogues is not only the tightness of binding (which spans a range of $10^6$ in affinity) but also the apparent slow rate constant ($k_{on}$) with which inhibitor binds to the enzyme. It has been concluded that the slow on-rates reflect an extremely weak initial binding of the inhibitor followed by a conformation change. The initial structure of the active site of the enzyme appears to be appropriate for binding the ground state of the substrate and not the transition state, and considerable readjustment of the site seems required to bind the transition state effectively (Kurz & Frieden, 1983). The nature of this critical conformation change is the subject of this study.

A strategy to probe the solution structure of proteins is to map the solvent exposure of the indole ring of tryptophan by studying the response of its fluorescence to the addition of external quenching agents such as acrylamide (Eftink & Ghiron, 1976). When the binding of the ligand itself is accompanied by a partial quenching of the enzyme's fluorescence (as is the case for transition-state analogues binding to adenosine deaminase; Kurz & Frieden, 1983), additional information can be obtained through studying the ligand quenching mechanism (Lehrer, 1976). For example, in the case of resonance energy transfer, estimates of distances between donor and acceptor are possible.

## Materials and Methods

*Materials.* Coformycin (COF)[1] and 2′-deoxycoformycin (DCF) were obtained from Dr. John Douros of the Developmental Therapeutic Program, Chemotherapy, National Cancer Institute. DHMPR was synthesized according to Wolfenden et al. (1977). Purine riboside (PR) was obtained from Sigma

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[1] Abbreviations: COF, coformycin; DCF, 2′-deoxycoformycin; DHMPR, 1,6-dihydro-6-(hydroxymethyl)purine riboside; PR, purine riboside; EDTA, ethylenediaminetetraacetic acid.

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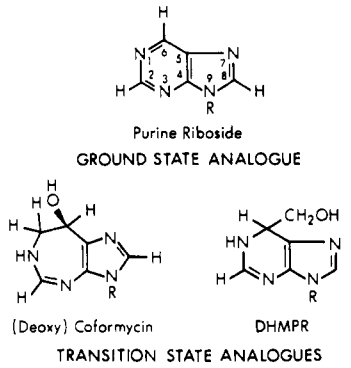


FIGURE 1: Structures of transition-state and ground-state analogues of adenosine deaminase.

Chemical Co. Acrylamide (>99.9%, electrophoresis purity grade) was the product of Bio-Rad Co.

The experiments were conducted in buffered solutions containing 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.00 at 20 °C.

Adenosine deaminase, from calf intestine, was obtained from Sigma Chemical Co. and purified essentially by the affinity chromatography method described earlier (Frieden et al., 1980). Enzyme concentrations were determined by stoichiometric fluorescence titration with the tight-binding inhibitor ($K_i = 10^{-12}$ M; Frieden et al., 1980) DCF as shown in the inset of Figure 2. The data points which depend upon DCF concentration were fitted to a straight line. The DCF concentration corresponding to the point of intersection of this (solid) line with the average end-point value (dashed line) is the enzyme concentration (0.50 $\mu$M in this example).

*Fluorometry.* Fluorescence was measured on a Spex Fluorolog fluorometer. The excitation and emission slits were set at 5 nm. Fluorescence intensity was collected digitally as the ratio of emission to reference signal. Solutions, thermostated at 20 °C, were measured in a 3-mL cuvette (1-cm path length) equipped with a Teflon stirrer to mix additions of ligands or quencher.

Titrations of protein fluorescence with acrylamide were performed at an excitation wavelength of 300 nm to avoid exciting tyrosine fluorescence (Longworth, 1971) and excessive absorption of the exciting light by high concentrations of acrylamide. While in all cases the absorbance of solutions was below 0.1 at 300 nm, small corrections for light absorption at the exciting wavelength were applied at the highest acrylamide concentrations according to Parker (1968). The extinction coefficient of acrylamide at 300 nm was determined by operation of the fluorometer in the transmission mode and was found to agree with that reported earlier (Parker, 1968). After addition of an aliquot of a 6 M acrylamide solution in buffer to the sample, at least five fluorescence values were collected, and standard deviations of each data point were calculated.

DHMPR solutions contain a fluorescent impurity which does not bind to the enzyme (Kurz & Frieden, 1983). Since the fluorescent impurity is quenched by acrylamide, blank acrylamide titrations of solutions containing DHMPR were performed and subtracted from data for acrylamide titrations of enzyme–DHMPR solutions.

The quenching of the intrinsic protein fluorescence by acrylamide was analyzed by fitting the data (weighted by their standard deviations) to the modified Stern–Volmer equation for multitryptophan proteins according to eq 1 (Lehrer & Leavis, 1978) using a linear least-squares procedure. $F^0$ is the fluorescence in the absence of acrylamide, $\Delta F$ is the change in fluorescence in the presence of acrylamide, [A] is the molar concentration of acrylamide, and $f$, the fractional accessibility, is the fraction of tryptophan residues which are accessible to the quencher. The Stern–Volmer constant, $K_{SV}$, is a measure of how easily those residues are quenched. It depends upon the efficiency of the quencher (1.00 for acrylamide), the fluorescence lifetime in the absence of the quencher, and the bimolecular rate constant for collisional quenching. For a multitryptophan protein, these Stern–Volmer constants may be complex averages and may contain an unknown contribution from static quenching processes (usually small for acrylamide which is primarily a collisional quencher). In spite of these possible complexities, the validity of qualitative interpretations in the terms discussed above can be expected to be preserved.

$$\frac{F^0}{\Delta F} = \frac{1}{f} + \frac{1}{fK_{SV}[A]} \quad (1)$$

Table I: Stern–Volmer Constants for Adenosine Deaminase and Its Ground- and Transition-State Analogue Complexes

| ligand | initial quench[a] | $K_{SV}$ ($M^{-1}$) | $f$ | $N^b$ |
|---|---|---|---|---|
| none | | 4.35 ± 0.53 | 0.97 ± 0.07 | 4 |
| PR | 0.02 | 3.76 ± 0.21 | 0.92 ± 0.05 | 3 |
| DHMPR | 0.69 | 3.12 ± 0.11 | 1.21 ± 0.14 | 2 |
| COF | 0.38 | 3.13 ± 0.11 | 1.12 ± 0.05 | 1 |
| DCF | 0.41 | 3.00 ± 0.14 | 1.01 ± 0.05 | 4 |

[a] Defined in eq 2. [b] $N$, number of experiments.

When required, fluorescence emission spectra were corrected for the Raman scattering contribution of the buffer. Excitation wavelengths of 280, 290, and 300 nm were used. Digitally collected data were transmitted to a VAX 11/780 computer for processing (normalization, generation of difference spectra, etc.).

*Absorption Spectra.* Spectra of ligand solutions were collected with a Cary 118C spectrophotometer. Solutions in 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.00, were maintained at 20 °C.

## RESULTS

*Stern–Volmer Titrations.* Typical modified Stern–Volmer plots (Lehrer & Leavis, 1978) for free enzyme, the purine riboside (ground-state analogue) complex, and the coformycin (transition-state analogue) complexes are shown in Figure 2. For each complex, all the experimental points fall on a single straight line with no systematic deviations obvious at any acrylamide concentration. The apparent quenching constants, $K_{SV}$, and fractional accessibilites, $f$, derived from the slopes and intercepts of a number of such experiments are given in Table I. The value of $f$ for free enzyme and all its complexes is 1.00 within experimental error. The $K_{SV}$ values, however, are lower for the complexes compared to free enzyme, with the largest decrease for transition-state analogue complexes. The $K_{SV}$ values for all the transition-state analogue complexes are the same within experimental error.

Integrity of the enzyme and its complexes at high acrylamide concentrations is necessary for the interpretation of these data. This has been shown in several ways. The linearity of the plots and the time stability of the data suggest that no unfolding or other drastic conformation change is occurring with increasing acrylamide concentration. Normalized emission spectra for enzyme solutions whose fluorescence has been partially quenched with acrylamide superimpose exactly on the emission spectra in the absence of acrylamide (data not shown). It is clear that no irreversible changes are occurring at high acrylamide concentrations; incubation of the enzyme

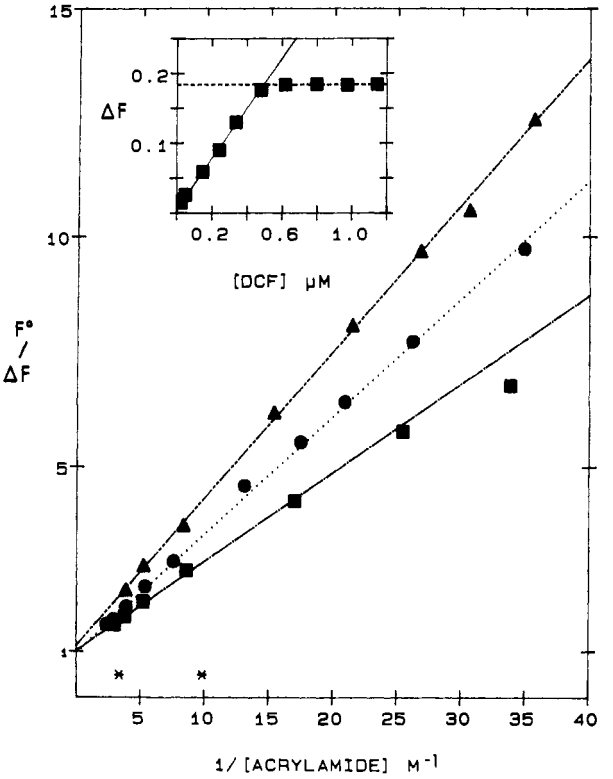


FIGURE 2: Modified Stern–Volmer plots for adenosine deaminase (■), ground-state analogue complex with PR (●), and transition-state analogue complex with COF (▲) in 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.00, 20 °C. Lines are weighted least-squares lines of best fit. Asterisks (*) near the abscissa indicate acrylamide concentrations at which stoichiometric titrations of the remaining fluorescence were performed to test for integrity of the DCF complex. Inset: Determination of enzyme concentration by stoichiometric titration of protein fluorescence with the transition-state analogue DCF. The dashed line indicates the average end point. The solid line is the least-squares line for those points which depend upon [DCF]. The value of [DCF] corresponding to the intersection of these two lines is the enzyme concentration.

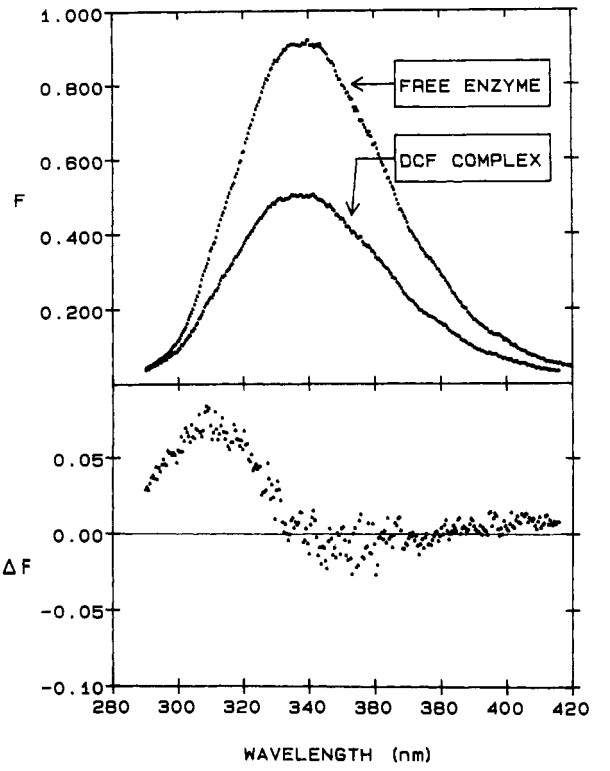


FIGURE 3: (Top panel) Emission spectra (280-nm excitation) of adenosine deaminase and its complex with the transition-state analogue DCF in 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.00, 20 °C. (Bottom panel) Normalized difference spectrum; DCF complex minus free enzyme.

at the highest acrylamide concentrations does not affect the specific activity observed upon dilution of the sample into an assay mixture (containing no acrylamide). Assay of the enzyme at the highest acrylamide level used (0.3 M) gave some decrease in activity. Assuming this decrease to be a consequence of competitive inhibition, the enzyme would remain saturated at the levels of ligands used in these experiments. Thus, the stoichiometric fluorescence titrations of the remaining enzyme fluorescence with deoxycoformycin and coformycin ($K_i$ values of $10^{-12}$ and $10^{-10}$ M, respectively; Kurz & Frieden, 1983) performed at several high acrylamide concentrations (indicated by asterisks along the abscissa of Figure 2) give results identical with those obtained for the enzyme in the absence of acrylamide (Figure 2 inset).

The intrinsic protein fluorescence of the binary complexes is less than that of the free enzyme in several cases. Data for the various complexes (Table I) are expressed as

$$\text{initial quench} = 1 - F_C/F_E \qquad (2)$$

where $F_E$ is the fluorescence of free enzyme and $F_C$ is the fluorescence of the complex. The transition-state analogues differ in their ability to quench the enzyme's intrinsic fluorescence by a factor of ~2. The ligand having the lowest affinity (DHMPR $K_i = 10^{-6}$ M; Frieden et al., 1980) shows the greatest initial quench while COF and DCF [affinities differing by $10^2$ but having the same UV chromophore ($K_i$'s = $10^{-10}$ and $10^{-12}$ M, respectively; Frieden et al., 1980)] show the same initial quench. The formation of the ground-state analogue complex with PR ($K_i = 3 \times 10^{-6}$ M; Frieden et al., 1980) results in only a very small initial quench.

*Emission Spectra.* In the absence of acrylamide, the free enzyme (no ligand), and complexes (saturating ligand), emission spectra (Figure 3, top panel) have maxima at 340 nm which are independent of the exciting wavelength between 280 and 300 nm. The half-widths of the free enzyme spectra are ~54 nm. Differences between the spectra of the DCF complex and those of the free enzyme are revealed when the former are normalized to the latter (by making the maximum value equal 1 in each case). A significantly greater portion of the total fluorescence of the transition-state analogue complex is found on the blue side of the maximum. This difference, which is most evident at an exciting wavelength of 280 nm and is present to a lesser degree at an exciting wavelength of 290 nm, has disappeared at an exciting wavelength of 300 nm (data not shown). The maximum of the normalized difference spectrum (280-nm excitation) is found at 305–310 nm (Figure 3, bottom panel).

## Discussion

The results of this paper concern the nature of the structural change in the enzyme which accompanies formation of the catalytic transition state. Very little information of this type, obtained from experiments in solution, is available for any enzyme system. The solvent accessibility study reported here appears to be the first example of an attempt to characterize these differences using the Stern–Volmer method.

*Tryptophan Environment.* The quenching patterns for a multitryptophan protein such as adenosine deaminase (containing three tryptophans) frequently cannot be analyzed precisely (Lehrer & Leavis, 1978). Nevertheless, useful (though qualitative) information as to the nature of ligand-

induced protein conformation changes can usually be extracted.

The intercept of the modified Stern–Volmer plot, $f$, represents the fraction of the fluorescence which is accessible to the quencher. Since the value of $f$ is 1.0 in the free enzyme and all the complexes, none of the tryptophans are buried in a protected hydrophobic protein core as has been found in some cases [e.g., azurin (Eftink & Ghiron, 1976) and 7S nerve growth factor (Rao & Neet, 1982)]. This is consistent with the type of emission spectra of the free enzyme and its complexes (vide infra).

While the precise significance of the linearity of such plots for a multitryptophan protein is difficult to assess (Eftink & Ghiron, 1976; Lehrer & Leavis, 1978), it is likely that the emitting residues do not differ greatly in their accessibility. This conclusion is strengthened by the observation that the plots remain linear even for transition-state analogue complexes where up to 69% of the original fluorescence of the free enzyme has already been quenched upon formation of the complex.

The values of the quenching constants (Table I) for the free enzyme and its complexes indicate that tryptophan is moderately shielded from the quencher (Eftink & Ghiron, 1976). Accessibility is somewhat reduced for the ground-state complex with purine riboside and markedly reduced in the transition-state analogue complexes. This is consistent with a globular protein which becomes more compact as complexes are formed. While the role of this conformation change in the catalytic process for adenosine deaminase is at present unknown, conformation changes in the active site which limit solvent access and/or orient catalytic groups around a substrate can make an important contribution to enzymatic catalytic strategies (Anderson et al., 1978; Huber & Bennet, 1983; Janin & Wodak, 1983). Frequently, these changes in the active-site region result in remote structural changes as well (Chothia et al., 1983; Lesk & Chothia, 1983). We believe this to be the case with adenosine deaminase (vide infra).

It is remarkable that (within 5%) the quenching constants for all the transition-state analogue complexes are identical although the binding constants for these inhibitors span a range of $10^6$. Thus, by what is admittedly only a rather low-resolution probe, all transition-state analogues are able to induce the same enzyme conformation change regardless of the overall thermodynamic stability of the complex.

What then is responsible for the ~8-kcal differences in the stability of these complexes? The answer may lie not in the ability of the different analogues to trigger the conformation change which results in an enzyme form appropriate for stabilizing the catalytic transition state but in the differing ability of the analogues to interact favorably with that enzyme form. These inhibitors are after all imperfect analogues of the catalytic transition state, and it is not difficult to imagine steric or other factors interfering with an optimal local interaction at the active site while the overall enzyme conformation remains essentially the same for all the complexes.

*Emission Spectra.* The wavelength maximum and half-width of these emission spectra place the tryptophans of adenosine deaminase and its complexes into the classification of Burstein (1973) characterized by limited contact with water at the protein surface. The enzyme contains 12 tyrosines as well as 3 tryptophans (Phelan et al., 1970), and the small tyrosine contribution to the emission spectrum with 280-nm excitation is apparent when tryptophan fluorescence has been partially quenched by binding the transition-state analogues. The normalized difference spectra between the transition-state analogue complex (DCF) and free enzyme (Figure 3, bottom panel) have a maximum at 305–310 nm which is characteristic of tyrosine emission (Longworth, 1971; Kronman & Holmes, 1971).

*Quenching Mechanism of Transition-State Analogues.* We have noted that a correlation exists between the amount of overlap of the absorption spectrum of a ligand with the protein emission spectrum and the amount of quench observed upon formation of the enzyme complex with that ligand. We have tested the hypothesis that resonance energy transfer is responsible for this ligand initial quench (Stryer, 1978). The size of the overlap integral, $J$ (eq 3), calculated for the two types of ligand chromophores (DCF and DHMPR) correlates closely with the ability of these ligands to quench the protein fluorescence.

The overlap integral, $J$, is given (Stryer, 1978) by

$$J = \frac{\int_0^\infty F(\lambda)\epsilon(\lambda)\lambda^4\, d\lambda}{\int_0^\infty F(\lambda)\, d\lambda} \quad (3)$$

where $\lambda$ is the wavelength (in centimeters), $\epsilon(\lambda)$ is the molar extinction coefficient of the ligand (acceptor) as a function of wavelength, and $F(\lambda)$ is the fluorescence (in arbitrary units) as a function of wavelength. The values of the overlap integrals for the two types of chromophores (DCF, COF, and DHMPR) were calculated by numerical integration every 2 nm between 290 and 428 nm. The fluorescence spectra were first corrected for the tyrosine contribution. Forster theory predicts that if resonance transfer is responsible for the ligand quench of the protein fluorescence then the ratio of the values of the overlap integrals should be related to the efficiencies of transfer, $E$, by

$$\frac{J(\text{DHMPR})}{J(\text{DCF})} = \frac{E(\text{DHMPR})/[1 - E(\text{DHMPR})]}{E(\text{DCF})/[1 - E(\text{DCF})]} \quad (4)$$

If resonance energy transfer is occurring, the efficiency of transfer, $E$, may be identified with the initial quench for the ligand (Table I). The calculated value of the ratio of overlap integrals (left side of eq 4) is 3.0; the right side of eq 4 has a value, 3.2, in substantial agreement.

If we assume a quantum yield ($Q_0$) of 0.3 [according to the correlation between emitter class and quantum yield found by Burstein (1973)], a random orientation factor for the dipole–dipole interaction ($K^2$), and a refractive index for the solution ($n$) of 1.33, we can calculate the distance ($R$) between the emitter and the ligand according to

$$R = R_0\left(\frac{1}{E} - 1\right)^{1/6} \quad (5)$$

where $E$ is the efficiency of transfer; $R_0$ is given by

$$R_0 = (JK^2Q_0n^{-4})^{1/6}(9.7 \times 10^3\ \text{Å}) \quad (6)$$

and $J$ is the overlap integral. Values of 15.9 and 16.1 Å were obtained for the DCF and DHMPR data, respectively. Essentially the same values were obtained whether the overlap integral was calculated by using the emission obtained with excitation at 300 or 280 nm (with correction to the latter for the contribution of tyrosine).

Thus, resonance energy transfer from tryptophan as donor to the ligand as acceptor is responsible for the quenching of protein fluorescence observed when the complexes are formed. Although the value of the calculated distance is reasonable for a globular protein with an estimated radius of 22 Å, several factors preclude a quantitative interpretation. These factors include the unknown orientation factor, the likely presence of heterogeneous emission from three separate residues, the lack

of precise measurements of quantum yield, and slight differences between the absorption spectrum of bound vs. free ligand. Nevertheless, the approximate distance obtained does strongly suggest that changes in protein conformation upon binding transition-state analogues occur in areas of the protein fairly remote from the actual binding site.

That only negligible quench is obtained with purine riboside is not surprising since the absorption spectrum (maximum at 263 nm) does not overlap the protein emission spectrum to any significant degree.

*Conclusion.* Consistent with kinetic and equilibrium data, the conformation of adenosine deaminase changes to accomodate binding of analogues of the substrate transition state. This conformation change, as reflected in the change in solvent accessibility of residues likely to be remote from the actual binding site, is the same for transition-state analogues which differ greatly in their affinity. Resonance energy transfer between emitter and ligand successfully explains the differing abilities of the ligands to quench the enzyme's intrinsic fluorescence upon formation of complexes.

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# Interaction of Pyrophosphate Moieties with $\alpha$-Helixes in Dinucleotide Binding Proteins

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ABSTRACT: A detailed analysis has been carried out of the binding of dinucleotide pyrophosphate moieties to those proteins where helixes play an important role in the binding. For this analysis, the three-dimensional structures and amino acid sequences of six proteins interacting with three different dinucleotides were available. As glutathione reductase binds two dinucleotides, seven enzyme–dinucleotide complexes have been studied. In all these complexes the pyrophosphate moiety is located near the N-terminus of at least one $\alpha$-helix: the "dinucleotide binding helix". In dihydrofolate reductase two helixes interact with the pyrophosphate of NADP. Only two common characteristics of all complexes are observed: (i) the occurrence of a glycine at the N-terminus of the helix and (ii) the favorable interaction of the $\alpha$-helix dipole with the negatively charged pyrophosphate moiety. In virtually every other respect, the dinucleotide binding by dihydrofolate reductase differs from the mode of binding by the five other proteins. The helixes of these five proteins have been grouped together as "category I" dinucleotide binding helixes. The six category I helixes form part of a compact $\beta\alpha\beta$ unit of highly similar structure and very dissimilar sequences. Nevertheless, a characteristic fingerprint for the sequence of this unit can be deduced. Only the ADP moieties of the dinucleotides occupy very similar positions with respect to these compact $\beta\alpha\beta$ units. Therefore, an appropriate name for these units would be "ADP binding $\beta\alpha\beta$ folds".

During the last decade, intriguing observations on the similarity in structure and in dinucleotide binding properties of a number of enzymes that are very different in amino acid sequence have been reported (Rao & Rossmann, 1973; Rossmann et al., 1974, 1975; Ohlsson et al., 1974; Matthews et al., 1979; Wierenga et al., 1983; Birktoft & Banaszak, 1985). These structural investigations have been one of the cornerstones of the generalization by Hol et al. (1978) that

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