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STRUCTURAL FLUCTUATIONS IN ASPARTATE TRANSCARBAMYLASE

SUCCINIMIDE QUENCHING AND FLUORESCENCE DEPOLARIZATION OF TRYPTOPHAN AND TYROSINE RESIDUES

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The effects of binding of various effector ligands on the dynamics of aspartate transcarbamylase (ATCase, c_6r_6) and on its regulatory (r_2) and catalytic (c_3) subunits were characterized by examining succinimide quenching of the intrinsic fluorescence, and by measurement of the lifetime-resolved anisotropies. The lifetimes of the tryptophan residues in c_3 and c_6r_6 are about 1.7 ns while those of tyrosine residues in r_2 are 2.7 ns. These lifetimes are not significantly altered by the binding of various substrates, substrate analogs and nucleotides. The effects of ligand binding on the accessibility of both tyrosine and tryptophan residues to the quencher are modest in all cases, though the changes are in the same direction as seen using other physicochemical techniques such as hydrogen exchange (M. Lennick and N.M. Allewell, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 6759). The tryptophan residues in both c_3 and c_6r_6 are immobilized whereas the tyrosine residues of r_2 have some motional freedom. Ligands have no effect on the immobilized tryptophan residues in c_3 and c_6r_6, while binding of nucleotides to r_2 results in a small decrease in the motional freedom of the tyrosine residues. These results suggest that the protein matrix around the aromatic amino acids in r_2, c_3 and c_6r_6 is rather rigid and that local effects of ligands on the dynamics of these residues, and that of the surrounding protein matrix, are minor. They are in general agreement with the results of the crystal structure determination (R.B. Honzatko et al., J. Mol. Biol. 160 (1982) 219).

1. Introduction

Aspartate transcarbamylase (ATCase) catalyzes the reaction of carbamyl phosphate with L-aspartate to form *N*-carbamyl-L-aspartate in the biosynthetic pathway leading to pyrimidine biosynthesis. ATCase from *Escherichia coli* is an extensively studied regulatory enzyme which exhibits a sigmoidal dependence of activity on substrate concentration, and is subject to feedback inhibition by CTP, the end product of the pathway, and the complementary nucleotide, ATP. (for reviews, see refs. 1–3). ATCase is oligomer consisting of two catalytic trimers (c_3) and the three regulatory dimers (r_2). Evidence for large conformational changes upon ligand binding has been obtained from spectroscopic data [4,5], reactivity of sulfhydryl groups [6,7], susceptibility to proteolysis [8], dissociation by detergents [9], fluorescence spectroscopy [10,11], relaxation spectroscopy [12], changes in sedimentation behavior [6,13] and hy-

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Abbreviations: NATyA, *N*-acetyltyrosinamide; NATA, *N*-acetyltryptophanamide; c_6r_6 or ATCase, native aspartate transcarbamylase; r_2, regulatory subunit; c_3, catalytic subunit; PALA, *N*-phosphonacetyl-L-aspartate; CbmP, carbamoyl phosphate; CTP, cytidine triphosphate; ATP, adenosine triphosphate.

drogen-exchange kinetics [14]. The conformational changes resulting from effector binding probably play an important role in mediating their allosteric effects.

It is now accepted that proteins have significant internal freedom with motions occurring on time scales from picoseconds to seconds. This flexibility is believed to play an important role in such biological activities as catalysis (for reviews, see refs. 15–17). From the conformational changes which result from ligand binding and their allosteric effects, one would expect significant changes in the structural fluctuations of the enzyme. Large effects have in fact been observed by hydrogen exchange [14]. We used the intrinsic tryptophan and tyrosine fluorescence of ATCase to monitor the effects of ligands on the protein dynamics. There are two tryptophan residues in the catalytic polypeptide chain ($M_r = 33000$) while each regulatory chain ($M_r = 17000$) contains three tyrosines and no tryptophan. Tryptophan and tyrosine in both c- and r-chains appear to be involved in the allosteric mechanism. Substitution of 7-azatryptophan for tryptophan in the c-chain enhances the effects of both CTP and ATP upon the native enzyme [18], while nitration of tyrosine in the c-chain eliminates cooperativity, feedback inhibition, and enzymatic activity and reduces binding by CTP [19]. Nitrated tyrosines in the r-chain are perturbed by binding of CTP, ATP and PALA [20]. Our aim in the present investigation was to study the effects of various ligands on the dynamics of c_6r_6, c_3 and r_2 as revealed by succinimide quenching of fluorescence and by lifetime-resolved anisotropies of the aromatic amino acid residues. These fluorescence methods allow structural fluctuations and segmental motions on the nanosecond time scale to be detected. For quenching to occur, the quencher has to diffuse to the fluorophore through the protein matrix. The rate of diffusion of quencher through the matrix will thus be sensitive to the dynamics of the protein matrix [21–24] and to the extent of exposure of the residue to the aqueous phase. In the present study we used succinimide which quenches tryptophan and tyrosine fluorescence with a quenching efficiency of about 0.70 [25].

2. Materials and methods

c_6r_6, c_3 and r_2 were prepared according to published procedures [26,27] from the derepressed diploid strain kindly provided by Dr. G. O'Donovan (Texas A. & M. University, College Station, TX). The concentrations of c_6r_6, c_3 and r_2 were determined using extinction coefficients at 280 nm of 0.59, 0.72 and 0.32 ml mg^{-1}, respectively. Values of 310000, 100000 and 34000, respectively, were assumed for molecular weights [26]. Ammonium sulfate precipitates of enzyme and the subunits were dialyzed extensively for 36 h at 4°C prior to the experiments which were conducted within 12 h of completion of dialysis.

2.1. Fluorescence lifetime and lifetime-resolved anisotropies

For determination of fluorescence lifetimes, the tryptophan residues of c_3 and c_6r_6 were excited at 295 nm, and the emission was observed through an interference filter centered at 344 nm. In the case of r_2, excitation and emission wavelengths of 290 and 313 nm, respectively, were used to measure the lifetime of tyrosine residues. The exciting beam was polarized vertically and emission observed at 54.7° from the vertical to eliminate effects of Brownian motions on the measured lifetimes [28]. No polarizers were used for the lifetime measurements of tyrosine residues. Lifetimes were measured by the phase-shift method on an SLM 4800 instrument with a modulation frequency of 30 MHz [29]. In place of the usual glycogen scatterer, 2,5-diphenyl-1,3,4-oxadiazole (PPD) in ethanol with a reference lifetime of 1.2 ns was used to correct for wavelength and geometry-dependent time response of the photomultiplier tubes [30].

For fluorescence anisotropy measurements, r_2 was excited at 290 nm and emission was measured at 313 nm by an interference filter. The emitted light was passed through a liquid filter (2 mm) containing a 0.5% solution of potassium phthalate to eliminate scatter. In the case of c_3 and c_6r_6, excitation was at 300 nm and emission was monitored at 344 nm. The excitation slits were adjusted to 8 and 2 nm and the emission slits to 8 nm.

Anisotropies were calculated from

$$r = \frac{I_{\parallel} G - I_{\perp}}{I_{\parallel} G + 2 I_{\perp}} \quad (1)$$

where G is the correction factor for monochromator efficiency in the transmission of vertically and horizontally polarized light. The total fluorescence intensity is given by

$$F = I_{\parallel} G + 2 I_{\perp} . \quad (2)$$

Quenching was achieved by adding 0.1-ml aliquots of 3 M succinimide solution to a 2.4 ml volume of protein samples. Stern-Volmer quenching constants ($K = k_q \tau_0$) were obtained from

$$\frac{F_0}{F} = 1 + K[Q] \quad (3)$$

where F_0 and F are total fluorescence intensities in the absence and presence of quencher, respectively, τ_0 the lifetime in the absence of quencher and k_q the biomolecular quenching constant. In cases where the Stern-Volmer plots showed upward curvature due to static quenching, the data were analyzed by

$$\frac{F_0}{F} = [1 + K_D(Q)][1 + K_S(Q)] \quad (4)$$

where K_S and K_D are the static and the dynamic quenching constants, respectively. In this case, the apparent quenching constant $[F_0/F - 1)/[Q])$ was plotted vs. [Q], and K_S and K_D determined from the slope ($K_S K_D$) and intercept ($K_S + K_D$).

2.2. Lifetime-resolved anisotropies

The steady-state anisotropy is dependent upon the time available for rotational displacements. If the average lifetime is decreased by quenching the anisotropy increases. For a single-exponential decay of anisotropy the dependence of r upon the lifetime is given by

$$\frac{1}{r} = \frac{1}{r_0} + \frac{\tau}{r_0 \phi} \quad (5)$$

where r_0 is the anisotropy in the absence of rotation, τ the lifetime and ϕ the rotational correlation time. Hence, a plot of $1/r$ vs. τ yields $1/r_0$ as an intercept and $1/r_0\phi$ as slope. The extrapolated limiting anisotropy ($r(0)$) will be smaller than the limiting anisotropy (r_0) and the apparent correlation time (ϕ_A) will be smaller than the correlation time expected for the protein molecule (ϕ_p) due to the presence of segmental motions. A more detailed description of lifetime-resolved anisotropies is given in earlier publications [22,23].

3. Results

The fluorescence lifetimes, Stern-Volmer quenching constants and bimolecular quenching constants for c_6r_6, c_3, and r_2 are summarized in tables 1 and 2. Some representative results are shown in figs. 1–3. The Stern-Volmer plots are either linear or curved upward as a result of static quenching. These plots suggest that all tyrosine residues of r_2 and the tryptophan residues of both c_3 and c_6r_6 are equally accessible to the quencher.

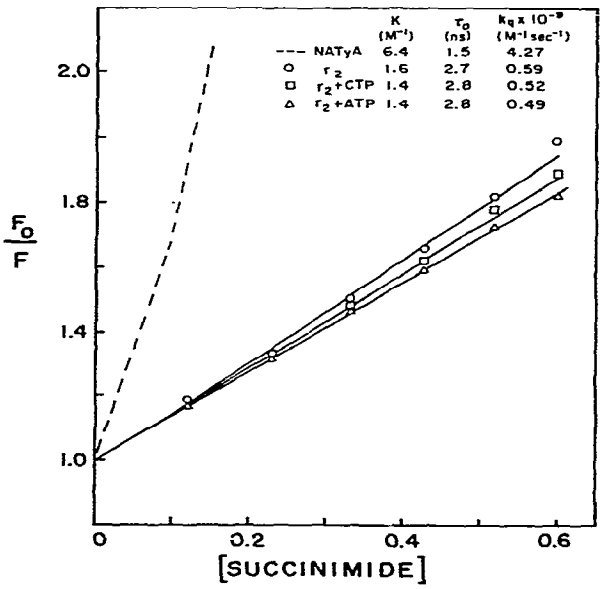

Fig. 1. Stern-Volmer plot for succinimide quenching of tyrosine fluorescence of r_2. The solutions were excited at 290 nm and the emission was measured at 313 nm. All the experiments were carried out in 0.02 M Tris-acetate buffer (pH 8.5) at 28°C.

Table 1

Fluorescence lifetimes, succinimide quenching constants, apparent limiting anisotropy and apparent correlation times for tyrosine residues in the regulatory subunit

All experiments were performed at 28°C in 0.02 M Tris-acetate (pH 8.5). The uncertainty in the lifetime measurements is about ±0.1 ns. For a protein molecule of $M_r = 34000$, the expected correlation time is about 17 ns [?2]. Under our experimental conditions r_0 for tyrosine fluorescence is 0.29 [23].

Protein/ligand	τ_0 (ns)	K_D (M^{-1})	K_S (M^{-1})	k_q ($\times 10^{-9}$) ($M^{-1}\,s^{-1}$)	Δk_q (%)	ϕ_A (ns)	$r(0)$	$r(0)/r_0$
NATyA	1.5	6.4	0.3	4.27				
r_2	2.7	1.6	< 0.05	0.59	–	10.0	0.225	0.78
r_2 + 0.3 mM CTP	2.8	1.4	–	0.52	−11.9	10.3	0.235	0.81
r_2 + 2 mM ATP	2.8	1.4	–	0.49	−17.0	10.3	0.235	0.81

Otherwise, plots of F_0/F vs. [Q] are expected to be concave towards the x-axis. The bimolecular quenching constants are about 0.6×10^9 M^{-1} s^{-1} for the tyrosine residues (r_2) and 1.2×10^9 M^{-1} s^{-1} for tryptophan residues in c_3 and c_6r_6. These k_q values are about 14 and 17% of the diffusion-controlled limits, respectively. Binding of both CTP and ATP to r_2 results in some shielding of tyrosine residues as indicated by a 12–17% decrease in k_q (table 1). In the case of c_3, the presence of PALA or succinate and CbmP results in decreased accessibility of tryptophan residues while CTP and ATP do not significantly affect k_q. Binding of PALA or CbmP and succinate results in a small increase in the accessibility of tryptophan residues in the native enzyme (table 2). This small increase in k_q for the whole enzyme contrasts with the small decrease observed for c_3. It must be emphasized, however, that for both c_3 and c_6r_6 the changes in the accessibility of the tryptophan residues are small (< 10%) but reproducible.

The lifetime of tyrosine residues in r_2 is 2.7 ns and is not significantly affected by the binding of CTP or ATP. The lifetimes of tryptophan residues

Table 2

Fluorescence lifetimes and succinimide quenching constants for tryptophan residues in c_3 and c_6r_6

The uncertainty in the measurement of lifetimes is about ±0.1 ns. All the experiments were carried out in 0.05 M Tris-acetate buffer (pH 8.5) at 28°C.

Protein/ligand	τ_0 (ns)	K_D (M^{-1})	K_S (M^{-1})	k_q ($\times 10^{-9}$) ($M^{-1}\,s^{-1}$)	Δk_q (%)
NATA	2.7	19.5	1.0	7.20	
c_3	1.7	2.1	0.3	1.22	–
c_3 + PALA (mol ratio = 18)	1.8	2.0	0.3	1.11	−9
c_3 + 50 mM succinate + 10 mM CbmP	1.8	2.0	0.2	1.11	−9
c_3 + 5 mM ATP	1.7	2.1	0.3	1.21	0
c_3 + 2 mM CTP	1.7	2.1	0.2	1.25	+2
c_6r_6	1.7	2.0	0.2	1.18	–
c_6r_6 + PALA (mol ratio = 18)	1.8	2.2	0.2	1.25	+6
c_6r_6 + 50 mM succinate + 10 mM CbmP	1.8	2.2	0.2	1.23	+4
c_6r_6 + 5 mM ATP	1.6	2.0	< 0.1	1.25	+6
c_6r_6 + 2 mM CTP	1.6	2.0	< 0.1	1.25	+6
c_6r_6 + 2 mM CTP + PALA (mol ratio = 18)	1.8	2.1	0.2	1.16	−2
c_6r_6 + 2 mM CTP + 10 mM CbmP + 50 mM succinate	1.8	2.1	0.1	1.17	0

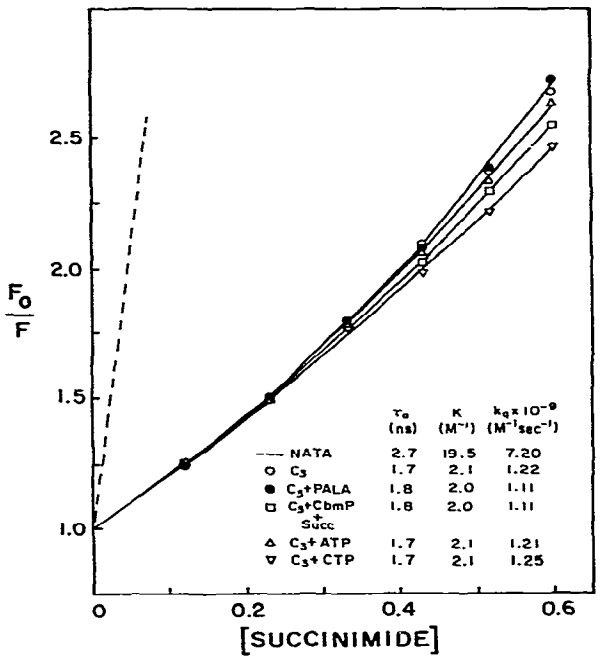

Fig. 2. Stern-Volmer plot for succinimide quenching of tryptophan fluorescence of c_3. All the experiments were carried out in 0.05 M Tris-acetate buffer (pH 8.5) at 28°C. The protein solutions were excited at 300 nm and the emission measured at 344 nm.

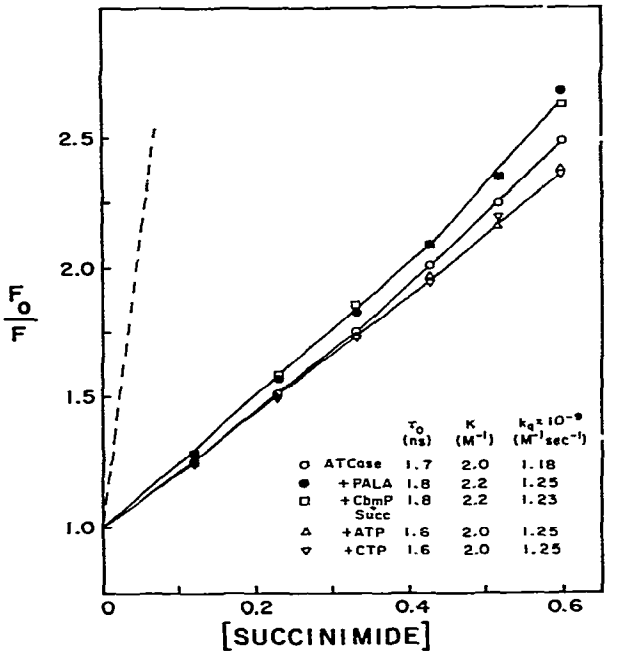

Fig. 3. Stern-Volmer plot for succinimide quenching of tryptophan fluorescence from c_6r_6. Experimental conditions are the same as those given in fig. 2.

in c_6r_6 and c_3 are about 1.7 ns. The tryptophan lifetimes increase slightly (about 0.1 ns) upon binding of CbmP and succinate or PALA while binding of nucleotides results in a small decrease (about 0.1 ns).

The steady-state anisotropy of tryptophan residues in both c_3 and c_6r_6 is about 0.265–0.270, the same as that observed for NATA in propylene glycol at −60°C, where rotational diffusion is completely absent [22]. The steady-state anisotropies of c_3 and c_6r_6 are not influenced by the binding of effectors. This similarity in the steady-state anisotropy and limiting anisotropy suggests that tryptophan residues in c_3 and c_6r_6 are immobile, perhaps due to hydrogen bonding with polar residues in the protein matrix. As c_3 ($M_r = 100000$; $\phi_p = 50$ ns) and c_6r_6 ($M_r = 310000$, $\phi_p = 150$ ns) are large molecules ($\tau \ll \phi$), the Brownian motion of the whole molecule will not significantly de-

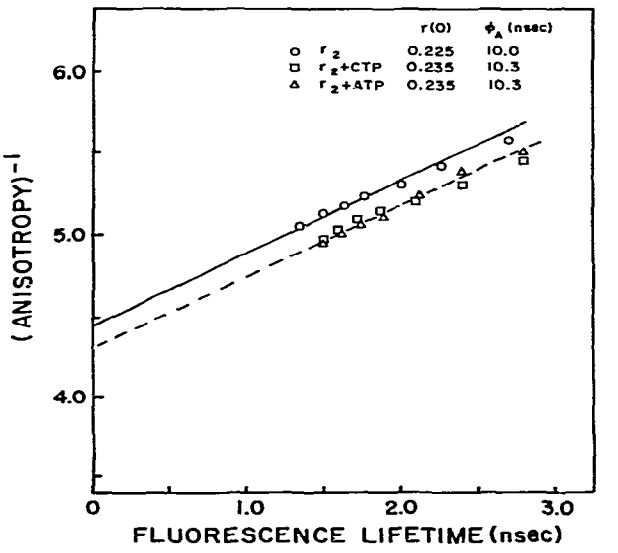

Fig. 4. Lifetime-resolved anisotropies of r_2 in the presence of ATP and CTP.

crease the steady-state anisotropies.

We observed a value of 0.18 for the steady-state anisotropy of tyrosine residues in r_2, which is smaller than the fundamental anisotropy of the tyrosine (0.29 [23]). In table I, the apparent correlation time (ϕ_A), apparent limiting anisotropy ($r(0)$) and the corresponding correlation times (ϕ_ρ) expected for protein molecules of the molecular weight of r_2 are given. Both $r(0)$ and ϕ_A for r_2 are smaller than r_0 and ϕ_ρ, suggesting that nanosecond segmental motions of tyrosine residues occur. Binding of CTP and ATP results in small increases in both $r(0)$ and ϕ_A, indicating a modest decrease in the motional freedom of the tyrosine residues. This increase suggests that binding of ATP and CTP increases the rigidity of the protein matrix of r_2.

4. Discussion

The emission maximum near 340 nm for the tryptophan fluorescence of c_3 and c_6r_6 is somewhat lower than that expected for a tryptophan residue which is completely exposed to water (355 nm). This could be due either to the surrounding environment being less polar than water or to the rigidity of its microenvironment. The latter appears more probable given the immobility of the fluorophores. Our polarization results and lifetimes for tryptophan residues are in agreement with the results of Kempe and Stark [10] and Matsumoto and Hammes [11]. The relatively high accessibility to succinimide, which is a large polar molecule, suggests that the tryptophan residues are on or near the surface.

There is ample evidence for both gross conformational changes and localized perturbations in ATCase due to ligand binding (see references in section 1). Evidence for dynamic changes has been obtained by hydrogen-exchange measurements [14]. The results of our fluorescence studies also indicate some perturbation by ligand binding of the dynamics of the protein matrix, although the changes are small. r_2 becomes slightly more rigid as a result of the binding of CTP and ATP, as indicated by a decrease in k_q and the rotational freedom of tyrosine residues. Similarly, small decreases in k_q for quenching of tryptophan residues in c_3 and an increase in k_q for the native enzyme upon substrate and substrate analog binding indicate that these ligands affect c_3 and c_6r_6 in opposite directions. These changes are in the same direction as those seen by differential ultracentrifugation and hydrogen-exchange measurements [13,14]. The lack of motional freedom for tryptophan residues in both c_3 and c_6r_6 suggests that both tryptophan residues are involved in hydrogen bonding. Model studies using fluorophores in polar solvents have indicated that hydrogen bonding of fluorophores can decrease their rates of rotation by a factor of 10 [31].

The high steady-state anisotropy of both tyrosine and tryptophan residues suggests that the protein matrix around these fluorophores is fairly rigid. There are wide variations seen in the motional freedom for both tyrosine and tryptophan residues in proteins. Immobilized tryptophan residues were observed in the case of aldolase and liver alcohol dehydrogenase. Similarly, limited motional freedom for tyrosine residues were also found in bovine pancreatic trypsin inhibitor and ribonuclease [22,23]. The small magnitude of changes in bimolecular quenching constants, lack of change in the steady-state anisotropy of the tryptophan residues in both c_3 and c_6r_6 and small changes in $r(0)$ and ϕ_A for r_2 due to the binding of various effectors all indicate that the ligand-promoted effects on the dynamics of the microenvironment of these fluorophores are minor. These small effects on the protein dynamics are in sharp contrast to the substantial alterations observed in the kinetics of hydrogen exchange [14] and X-ray structures of ATCase [2,3,32]. These differences presumably reflect the differences in the types of motions detected by these techniques. Whereas fluorescence measurements are sensitive to the dynamics of the protein only in the region of the fluorophore, hydrogen exchange and X-ray diffraction monitor the entire polypeptide chain. Furthermore, while only motions on the nanosecond time scale can be detected by fluorescence anisotropy measurements, hydrogen exchange is sensitive to motions with rate constants in the range of min^{-1} to $year^{-1}$. These slower motions which are the determining factor in hydrogen exchange will

not affect the steady-state anisotropy. It is of interest, however, that the ^{13}C results of Moore and Browne [33] also indicate that ligands have only minor effects on r_2, and that the changes seen were attributed to direct interactions between specific residues and effector molecules.

In ATCase, both the tyrosine and tryptophan residues appear to be located near or on the surface. Hence, these residues are already easily accessible to the quencher and any further increase in their accessibility due to the binding of the ligands will be small. We chose succinimide as the quencher because, being large and polar, it is likely to be more sensitive to steric hindrance in its approach to the fluorophore. For single-tryptophan proteins, the range of k_q is about 80-fold for succinimide when compared to about 20-fold for acrylamide which is also a polar but slightly smaller quencher (M. Eftink, personal communication; B.P. Maliwal and J.R. Lakowicz, unpublished observations) and about 5-fold for oxygen [22]. Nevertheless, the effects seen from succinimide quenching are small, and probably rule out any direct involvement of tyrosine residues in the binding sites of r_2 and tryptophan residues in the binding sites of c_3. In the case of lysozyme and trypsin, where tryptophan residues are present at the binding site, interactions with ligands resulted in a significant decrease in their accessibility to the quencher [24].

The major conclusions of this study, that the tyrosine residues of r_2 and the tryptophan residues of c_3 are relatively immobilized, exposed to solvent, and not part of the ligand-binding sites, are consistent with the results of the crystal structure determination [32]. The planes of the tyrosine and tryptophan rings are generally well defined and in contact with solvent. Trp-209 and -284 of c_3 are both more than 15 Å from the active site. One tyrosine of r_2, Tyr-89, is close to the allosteric domain of r_2, but not necessarily in contact with bound nucleoside (K. Volz and W. Lipscomb, personal communication).

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