

Synthesis and Characterization of Stacked and Quenched Uridine Nucleotide Fluorophores*

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Intramolecular aromatic interactions in aqueous solution often lead to stacked conformation for model organic molecules. This designing principle was used to develop stacked and folded uridine nucleotide analogs that showed highly quenched fluorescence in aqueous solution by attaching the fluorophore 1-aminonaphthalene-5-sulfonate (AmNS) to the terminal phosphate via a phosphoramidate bond. Several fold enhancement of fluorescence could be observed by destacking the molecules in organic solvents, such as isopropanol and dimethylsulfoxide or by enzymatic cleavage of the pyrophosphate bond. Stacking and destacking were confirmed by ¹H NMR spectroscopy. The extent of quenching of the uridine derivatives correlated very well with the extent of stacking. Taking 5-H as the monitor, temperature-variable NMR studies demonstrated the presence of a rapid interconversionary equilibrium between the stacked and open forms for uridine-5'-diphospho-β-1-(5-sulfonic acid) naphthylamidate (UDPAmNS) in aqueous solution. ΔH was calculated to be -2.3 Kcal/mol, with 43–50% of the population in stacked conformation. Fluorescence lifetime for UDPAmNS in water was determined to be 2.5 ns as against 11 ns in dimethyl sulfoxide or 15 ns for the pyrophosphate adduct of AmNS in water. Such a greatly reduced lifetime for UDPAmNS in water suggests collisional interaction between the pyrimidine and the fluorophore moieties to be responsible for quenching. The potential usefulness of such stacked and quenched nucleotide fluorophores as probes for protein-ligand interaction studies has been briefly discussed.

Since the seminal work of Weber and Laurence (1) with 1-anilidonaphthalene-8-sulfonic acid, extrinsic fluorescent probes have been extensively used to monitor various aspects of protein-ligand or enzyme-substrate interactions. Among others, these probes have been used (i) to establish the degree of polarity or hydrophobicity of a particular region of a protein, (ii) to measure the distance between groups on protein surface, (iii) to measure the extent of flexibility of protein in solution, (iv) to measure the rate of very rapid conformational transitions, and (v) to measure kinetic constants of interaction between protein and ligand (2). To facilitate these studies, a variety of derivat-

ized fluorescent ligands or substrate analogs have been synthesized over the years without any serious attention being given to their solution conformations or their transitions to new conformations on interaction with the target proteins. Such conformational transitions are often crucial steps in biological interactions as typically exemplified by NAD, the common cofactor for a very large number of dehydrogenases. The molecule exhibits reversible stacking between the adenine and pyridine moiety with both the open and the closed forms in rapid interconversionary equilibrium in aqueous solutions (3, 4). During catalysis, NAD takes a totally extended conformation on the enzyme surface; the conserved tertiary structure of the pyridine nucleotide binding site being a very characteristic feature of all these oxidoreductases (5).

Etheno-ATP was originally synthesized as a fluorescent analog of ATP. It had a high quantum yield and a long fluorescence lifetime and could be used to follow ATP interactions primarily by polarization studies (6–8). In contrast, as in case of NAD, significant population of etheno-NAD was in a folded conformation in aqueous solution as a result of aromatic interactions between the pyridine and the modified adenine moiety, leading to dynamic collisional quenching of fluorescence and short fluorescence life-time (9). This stacked and quenched fluorophore was brilliantly used to establish negative co-operativity for glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle for the binding of the tetrameric apoenzyme to the coenzyme. The conformational transition of etheno-NAD from folded to stretched conformation as reflected by its enhanced fluorescence on interaction with the target protein was the monitoring parameter for this purpose (10). Although stacked fluorophore with quenched fluorescence can be of immense use in protein-ligand binding studies, as is exemplified by etheno-NAD, it is surprising to note that no deliberate effort has so far been made to design such compounds taking advantage of the potential aromatic interaction between the attached fluorophore and a suitable moiety of the desired biomolecule.

The interaction between aromatic rings is of wide chemical and biological interest, because it plays important roles in vital biological processes, such as stabilization of protein and nucleic acid structure and recognition of mRNA cap-binding proteins, and in the biological reduction by NADH (11–14). Studies with model systems such as benzene, naphthalene, and their fluorinated derivatives have shown formation of both T-type and parallel stacking in the gas and solution phases. The energetics of such interactions have been calculated (15–19). In general, the interaction between two nonpolar aromatic ring systems is so weak that it is easily compensated by the entropy factor. This is expected to be predominant only in concentrated solutions or when the interacting groups are brought together by some other interactions such as coulombic interactions or hydrogen bonds as in the designing of devices for molecular recognition, catalysis, and development of self-replicating molecules and molecular clips (20–24). Such interactions are

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facilitated when the aromatic moieties are brought close to each other, making them substituents of the same molecule (25–28). In this report, we demonstrate that following this designing principle, stacked nucleotide fluorophores that are in rapid equilibrium with the stretched forms can be synthesized quite easily. More importantly, rapid collision between the fluorophore and the heterocyclic base often leads to dramatic quenching of fluorescence that can be used to monitor conformational changes of the ligand on interaction with its target protein.

In search of a suitable fluorescent nucleotide substrate for the DNA-dependent RNA polymerase, Yarbrough and colleagues synthesized a new class of fluorescent nucleotide triphosphate analogs that contained the fluorophore 1-aminonaphthalene-5-sulfonate (AmNS)¹ attached via a γ -phosphoramidate bond (29, 30). A linear increase of fluorescence was observed during RNA synthesis with UTPAmNS as one of the substrates. This was assumed to be attributable to the pyrophosphorolysis of UTPAmNS during catalysis, because independent enzymatic cleavage of the analog with phosphodiesterase also led to severalfold enhancement of fluorescence. The possibility of stacking was implied but was not systematically explored. UTPAmNS has since been regularly used for studies on the topology of the RNA polymerase active site without actually realizing the significance of the solution conformation of the synthesized fluorophore (31). We have now reinvestigated the problem to understand the reason for this intense quenching of fluorescence. For this purpose we synthesized all the three AmNS derivatives of uridine nucleotides with appropriate controls. The extent of quenching of fluorescence of the uridine phosphates could be clearly correlated with their degree of stacking interactions. The molecules showed reversible intramolecular stacking between uracil and naphthalene moieties with both the open and the closed forms in rapid interconversionary equilibrium in aqueous medium. Phosphodiester bond cleavage is not a prerequisite for fluorescence quenching. Such enhancement can also be brought about by destacking the molecules in nonaqueous solvents or on binding to a protein in a stretched conformation. In the following paper we shall demonstrate the usefulness of one of these stacked and quenched fluorophores to follow its conformational transition from stacked to the stretched form as it lands on the substrate binding site of UDP-galactose-4-epimerase as a substrate analog (32). It is anticipated that designing of such stacked fluorophores for other nucleotides will greatly facilitate analysis of many aspects of nucleotide-protein interactions of biological importance and also for development of monitors for high throughput screening systems.

MATERIALS AND METHODS

All common chemicals such as buffering salts and solvents were purchased from SD Fine Chemicals, SRL, Qualigen, or Merck. The spectrograde solvents were purchased from Spectrochem. AmNS was purchased from Fluka. The nucleotides 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, DEAE-cellulose, and all other chemicals unless otherwise mentioned were purchased from Sigma.

Spectrophotometric analyses were carried out in a Hitachi U3200 spectrophotometer. Fluorimetric measurements were done in a Hitachi F4010 spectrofluorimeter. ¹H NMR experiments were done in either a 100-MHz Jeol or a 200-MHz Bruker NMR spectrometer. Temperature-variable NMR was done in a 100-MHz Jeol NMR spectrometer.

Synthesis and Purification of Uridine Nucleotide-1-(5-sulfonic acid) Naphthylamides—The nucleotide-AmNS derivatives were synthesized according to the procedure of Yarbrough *et al.* (29) with some

modifications. 223.5 mg (1 mmol) of AmNS was added to 10 ml of water, and the pH was adjusted to 5.8 with 0.1 N NaOH. Any insoluble material was removed by centrifugation. 4 ml of 12.5 mM nucleotide and 2 ml of 1 M EDC at pH 5.8 were added to a reaction vessel maintained at 20 °C. The reaction was initiated by adding 10 ml of AmNS solution and allowed to continue for 2.5–3 h. The pH was kept between 5.65 and 5.75 by periodic addition of 0.1 N HCl. After the completion of the reaction, the mixture was neutralized with 0.1 N NaOH and made 0.05 M in ammonium bicarbonate. This was then centrifuged to remove any insoluble material. The clear supernatant was then loaded onto a 40-ml DEAE-cellulose column equilibrated with 0.05 M ammonium bicarbonate and washed with 60 ml of 0.05 M ammonium bicarbonate, followed by a 600-ml gradient of ammonium bicarbonate (0.05–0.5 M) with a flow rate of 25 ml/h. The fluorescent analog eluted out after the unreacted AmNS and showed a brilliant blue fluorescence. 6-ml fractions were collected, and the absorbance was monitored at 260 and 320 nm. The fractions for which the ratio of A_{260} and A_{320} fell within 1.75–1.85 were pooled. The value for unreacted AmNS was ~0.8. The pooled fractions were then subjected to repeated evaporation with water under reduced pressure at 35 °C to drive out ammonium bicarbonate. The purified material was dissolved in 0.5–2 ml of water. Purity was assessed by TLC on cellulose plates developed with absolute alcohol and 0.5 M ammonium acetate, pH 7.5, in the ratio 7:3. If traces of free AmNS were detected, the purified fluorescent nucleotide was rechromatographed on a 15-ml DEAE-cellulose column using a 300-ml gradient of ammonium bicarbonate (0.05–0.25 M). All other nucleotide derivatives were synthesized essentially following the same protocol. The yield was between 30 and 40% of the starting nucleotide.

The pyrophosphate adduct of AmNS (PPAmNS) was prepared by reacting sodium pyrophosphate with AmNS and the water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide under the same conditions described above for the synthesis of uridine nucleotide analogs. It was purified by chromatography on a DEAE-cellulose column and was shown to be homogeneous on TLC as described earlier.

The uridine nucleotide derivatives had a absorbance peak at 320 nm compared with PPAmNS and AmNS, both of which had peaks at 330 nm. To take advantage of this peak shift and also to compare our results with that of earlier published values by Yarbrough *et al.* (29), all fluorimetric experiments were carried out by exciting the fluorophores at 360 nm.

Acid hydrolysis and phosphodiesterase digestion of the phosphoramidates were performed according to the procedure of Yarbrough *et al.* (29) and Yarbrough (33).

¹H NMR Spectroscopy—The ¹H NMR spectra of the samples were usually taken in the 200-MHz Bruker NMR spectrometer or the 100-MHz Jeol NMR spectrometer. In all cases, ~5–10-mg samples were taken in 250–300 μ l D₂O or d₆-Me₂SO. The acetone peak at 2.20 ppm was taken as the internal standard for experiments in D₂O, and for experiments in d₆-Me₂SO, the signal attributable to the residual protons of the solvent at 2.49 ppm was taken as the internal standard. The temperature-variable ¹H NMR in D₂O was done in the 100-MHz Jeol NMR spectrometer using an acetone signal at 2.20 ppm as standard throughout the experiment. The NMR tube was tightly capped to avoid escape of acetone at high temperature (maximum 80 °C). An external thermocouple sensor fitted with the instrument was used as a temperature probe.

RESULTS

Enhancement of Fluorescence in Absence of Phosphodiester Bond Cleavage—Fig. 1A clearly shows that phosphodiester bond cleavage is not a prerequisite for fluorescence enhancement of uridine-5'-diphospho- β -1-(5-sulfonic acid) naphthylamidate (UTPAmNS), because decrease of medium polarity alone can lead to complete dequenching of fluorescence. A 8-fold enhancement of fluorescence could be observed in 100% isopropanol. Similar results were also obtained with Me₂SO. For comparison, the relative fluorescence values of free AmNS, PPAmNS, and UTPAmNS, are presented in Fig. 1B. Formation of a phosphoramidate bond leads to a 5-fold increase in fluorescence compared with AmNS, but introduction of uridine moiety dramatically quenches the fluorescence (Fig. 1B, trace c), which becomes much lower than that of the free fluorophore. In a reverse experiment, the quenched fluorescence of UTPAmNS was fully released on phosphodiesterase treatment (Fig. 1B, trace d), which drastically reduced as the free fluoro-

¹ The abbreviations used are: AmNS, 1-aminonaphthalene-5-sulfonate; PPAmNS, pyrophosphate adduct of AmNS; UDPAmNS, uridine-5'-diphospho- β -1-(5-sulfonic acid) naphthylamidate; UTPAmNS, uridine-5'-triphospho- β -1-(5-sulfonic acid) naphthylamidate.

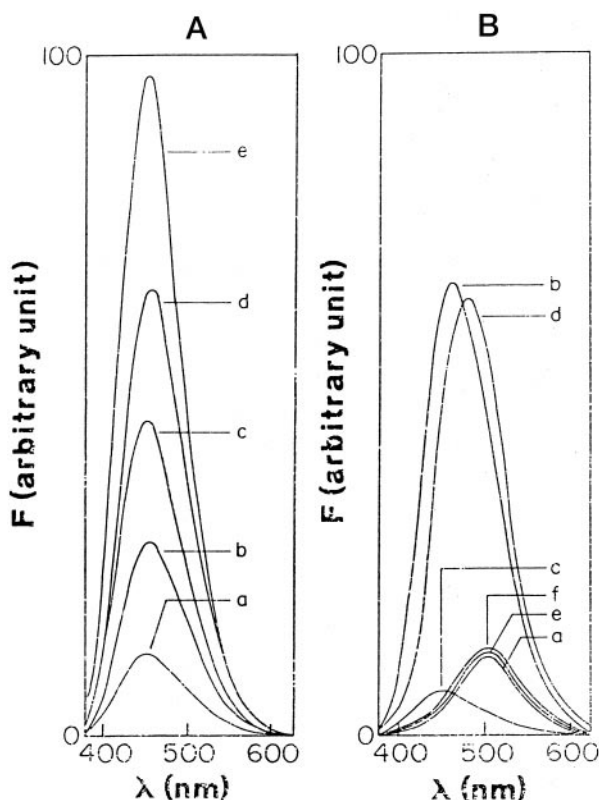


FIG. 1. A, dependence of fluorescence of UDPAmNS on solvent polarity. Equal amounts of UDPAmNS ($9.3 \mu\text{M}$) were taken in water and in varying concentrations of isopropanol and the fluorescence emission scanned. *a*, water; *b*, 25%; *c*, 50%; *d*, 75%; and *e*, 100% isopropanol. Excitation wavelength is 360 nm. B, relative fluorescence diagram. *a*, AmNS ($9.3 \mu\text{M}$); *b*, PPAmNS ($9.3 \mu\text{M}$); *c*, UDPAmNS ($9.3 \mu\text{M}$); *d*, after adding snake venom phosphodiesterase ($2 \mu\text{g}$) to *c* and incubation for 10 min; *e*, after adding alkaline phosphatase ($10 \mu\text{g}$) to *d* and incubation for 20 min; *f*, after incubating UDPAmNS ($9.3 \mu\text{M}$) in 0.5 N HCl at 37°C for 3 h. All incubations were done in 50 mM Tris-HCl , pH 8.0, containing 10 mM MgCl_2 and 0.1 mM dithiothreitol. The excitation wavelength used was 360 nm.

phore (AmNS) was generated by further treatment with alkaline phosphatase (Fig. 1B, trace *e*) or by acid hydrolysis of UDPAmNS (Fig. 1B, trace *f*). The phosphodiesterase-treated product is presumably PAmNS. The difference in fluorescence emission maxima between traces *b* and *d* probably reflects the difference in spectrum of PPAmNS and PAmNS. Taken together, these results suggested stacking interaction as the possible basis for quenching of fluorescence for UDPAmNS. As in the case of UDPAmNS, enhanced fluorescence could also be observed both for UMPAmNS and UTPAmNS in 100% isopropanol, the extent being 7- and 5-fold compared with that in water. ATPAmNS, GTPAmNS, and PPAmNS failed to show any significant enhancement under identical conditions. Most of the subsequent spectroscopic work reported here was carried out with UDPAmNS, because this molecule showed maximum enhancement of fluorescence and was also used as the probe for our subsequent study with UDPgalactose-4-epimerase, as reported in the following paper (32).

NMR Evidence for Stacking Interaction—When the ^1H NMR spectrum of UDPAmNS in D_2O was compared with that of free UDP, a large upfield shift of the signals ($\sim 0.5 \text{ ppm}$) was observed for both the uracil protons at the 5 and 6 positions (Fig. 2, A and B). The signal for the 6-H, which overlaps with the naphthalene proton signals, was confirmed by the position of the cross-peaks between the 5-H and 6-H in the two-dimensional COSY spectrum and from proton-decoupling studies (data not shown). This type of symmetrical upfield shift can

best be interpreted if the uracil ring comes very close to the naphthalene ring with the two protons positioned symmetrically over the two naphthalene ring centers. The proximity of the two rings in aqueous solutions was also indicated by the nuclear Overhauser effect spectroscopy spectrum, which showed faint but clear cross-peaks between the 5-H of the uracil and the naphthalene proton signals (data not shown).

The chemical shifts in D_2O were found to be independent of concentration, which ruled out any major intermolecular stacking interactions. When the ^1H NMR spectrum was taken in $d_6\text{-Me}_2\text{SO}$, the signals for 5-H and 6-H shifted downfield, and their chemical shifts were almost identical to those of UDP, confirming destacking of UDPAmNS under this condition (Fig. 2C). Finally, the extent of stacking interaction as indicated by the extent of the peak shift for 5-H was found to vary with the intermediate phosphoryl chain length. Excellent correlation was observed with the corresponding enhancement of fluorescence in 100% isopropanol. Of the three uridine nucleotide analogs, the UDP analog was found to have the maximum stacking interaction and is the most quenched (Fig. 2, inset).

Energetics of Stacking—To study the temperature dependence of the stacking phenomenon, the 5-H peak was monitored because it was distinct and isolated. Fig. 3 shows that after increasing the temperature from 30 to 80°C , a gradual downfield shift of 5-H peak was observed, which approached the chemical shift for the proton for UDP. This indicates that the equilibrium constant is changing with rising temperature to favor the extended form. Because no separate signals for the folded and unfolded forms were recorded, a rapid interconversion between the two forms could be assumed. The interconversion is faster with respect to the NMR time scale, and the observed value is actually a weighted average of the two forms. In their classic conformational study of NAD in aqueous solution using NMR techniques, Jardetzky and Wade-Jardetzky (4) observed a similar gradual shift of the proton signals on increasing the temperature. Using similar calculations as done by Jardetzky and Wade-Jardetzky (4) for NAD, the thermodynamic parameters at 30°C were calculated to be $\Delta H = -2.3 \text{ Kcal/mol}$, $\Delta S = -7.7 \text{ entropy units}$, and $K_{\text{eq}} = 0.75\text{--}1.0$, and the percentage of stacked form was 43–50%. These values are close to those obtained experimentally for other stacked systems such as NAD (4), etheno-NAD (9), and FAD (34). The theoretical value for stabilization energy in the benzene-naphthalene system as recently calculated by Jorgensen and Severance (35) is -3.2 to -3.5 Kcal/mol , which is sufficiently close to that obtained for our system.

Fluorescence Lifetime Measurements—To understand the molecular basis of quenching, fluorescence lifetime measurements were carried out at 25°C . The lifetime of UDPAmNS in water was found to be 2.5 ns , which increased to 16 ns on phosphodiesterase treatment. The fluorescence lifetime of PPAmNS in water was determined to be 15 ns . The lifetime of UDPAmNS in Me_2SO was 11 ns . Such a greatly reduced lifetime of UDPAmNS in aqueous solution strongly suggests collisional interaction between the two stacked rings to be the cause for the quenching of fluorescence.

DISCUSSION

Although a significant amount of both theoretical and experimental work has been done in recent years, the relative contribution of forces that drive aromatic-aromatic interactions in aqueous solutions is not clear yet. Thermodynamic signatures for self-association of purine and pyrimidine derivatives in aqueous solution (enthalpically favorable but entropically unfavorable) have been interpreted to imply that these associations are driven by intrinsic attractions between the heterocyclic rings, rather than by their mutual exclusion from water.

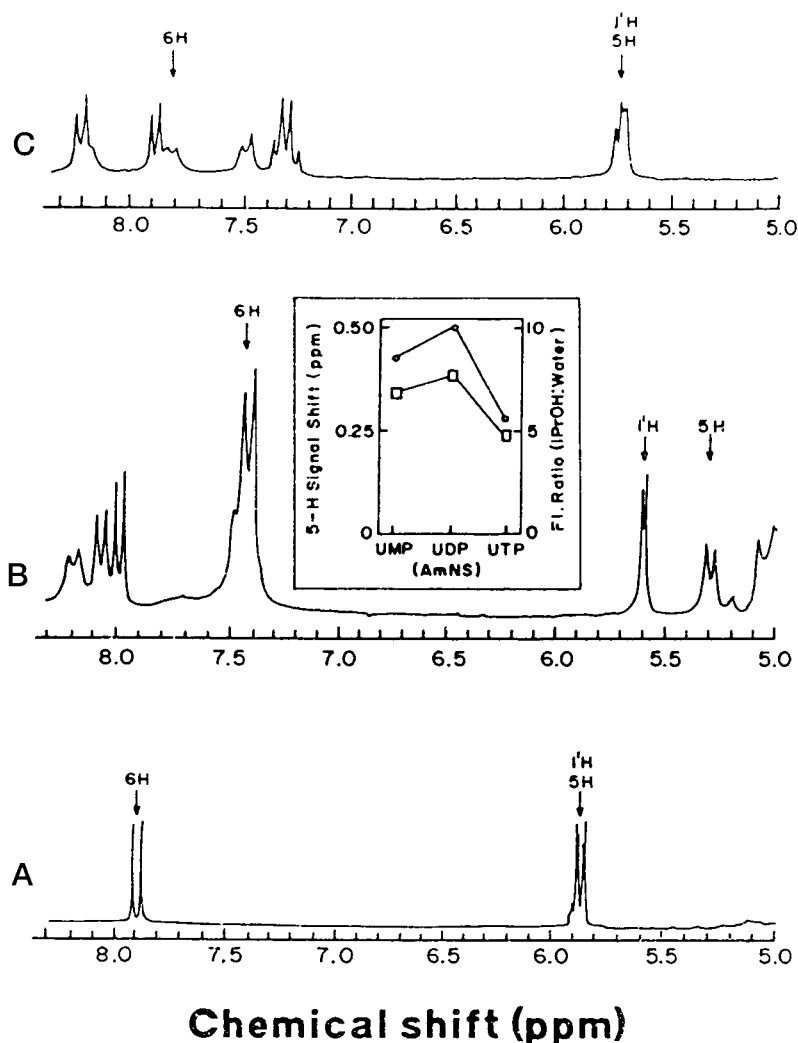


FIG. 2. ^1H NMR spectra of UDP and UDPAmNS. A, UDP in D_2O ; B, UDPAmNS in D_2O ; C, UDPAmNS in $d_6\text{-Me}_2\text{SO}$. The signals for 5-H, 6-H and 1'-H are indicated by arrows. The position of the 6-H signal in UDPAmNS was confirmed by the ^1H , ^1H -COSY spectrum from the position of the cross-peaks between 5-H and 6-H. Inset, correlation between the 5-H NMR signal shift in D_2O for the uridine nucleotide derivatives on stacking and the enhancement of fluorescence in 100% isopropanol compared with that in water. $\circ$ and $\square$, 5-H signal shift and fluorescence ratio, respectively.

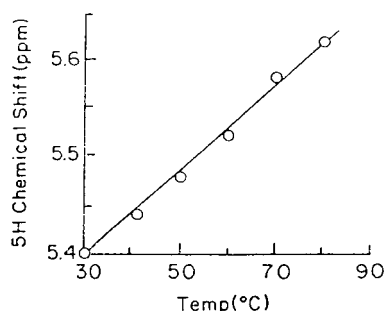


FIG. 3. Dependence of chemical shift on temperature. ^1H NMR UDPAmNS was taken in D_2O at different temperatures from 30 to 80 $^\circ\text{C}$. The chemical shift for the proton at the 5 position of the uracil ring was observed for this purpose.

The nature of the attraction between the heterocycles is uncertain; both dispersion forces and interactions between partial charges within the adjacent rings have been assumed (36, 37). In a recent study, involving naphthyl and adenine moieties connected through a carboxylated propylene linker, Newcomb and Gellman (26) suggested that attractive interactions between partial positive and negative charges along with a "non-classical" hydrophobic effect may be the main driving forces for stacking in aqueous solutions. Whatever may be the driving force for stacking, our work shows that all the uridine nucleotides have a significant population of molecules in stacked conformation that is in equilibrium with the relaxed conforma-

tion in aqueous solution. ATPAmNS does not undergo quenching of fluorescence in aqueous solution and has a long fluorescence lifetime of 20 ns in water. But contrary to our expectation, it was found to assume a stacked conformation in aqueous solution as evidenced from NMR spectroscopy.² Clearly, stacking is a necessary but not a sufficient condition for the designing of such quenched fluorophores. Dynamic fluorescence quenching as evidenced by very significant reduction in lifetime for UDPAmNS compared with that of free PPAms in aqueous solution suggests collisional interaction between the two rings. It is likely that replacement of the smaller pyrimidine ring by the more extended purine ring as in ATPAmNS introduces steric problems that still result in stacking, but the desired orientation and proximity of groups involved in quenching are not achieved. Aromatic stacking can take various conformations ranging from parallel to T type, although theoretical calculations suggest the T type to be energetically the preferred conformation in case of a benzene-naphthalene type interaction similar to our system (35). This remains an unanswered aspect of our present work. Needless to say, much more work needs to be done with different fluorophores and different nucleotides before the principles governing stacking that leads also to quenching can be understood. Our present work is progressing in that direction.

The usefulness of such stacked and quenched nucleotide fluorophores is easy to visualize. The quenched ligand can be

² G. Dhar and A. Bhaduri, unpublished data.

conveniently used as a probe to study its interaction with the target protein provided the fluorophore takes a stretched or unstacked conformation on the protein surface. Kinetic manipulations can then provide information regarding binding affinity, number of binding sites, and nature of cooperativity, if any. This was, in fact, essentially done for glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle with etheno-NAD as the probe (10). Folding studies can also be facilitated by using these probes to monitor the generation of ligand binding site during the folding process. In the following paper usefulness of UDPAmNS as a quenched fluorophore will be demonstrated taking UDPglucose-4-epimerase from *Escherichia coli* as the target enzyme (32).

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