Two-Dimensional Transferred Nuclear Overhauser Effect Spectroscopy (TRNOESY) Studies of Nucleotide Conformations in Creatine Kinase Complexes: Effects Due to Weak Nonspecific Binding†

N. Murali, Gotam K. Jarori,‡ Steven B. Landy, and B. D. Nageswara Rao*

Department of Physics, Indiana University—Purdue University at Indianapolis (IUPUI), 402 North Blackford Street, Indianapolis, Indiana 46202-3273

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ABSTRACT: The conformations of the adenosine moiety of MgADP and MgATP bound to rabbit muscle creatine kinase were investigated by two-dimensional transferred nuclear Overhauser effect spectroscopy (TRNOESY). The effects arising from adventitious binding of the ligands to the enzyme on the measurements were delineated. It was shown that, with sample protocols typically used thus far with the TRNOE method (enzyme, ~1 mM; ligand, ~10 mM), the TRNOESY pattern for the nucleotides with creatine kinase is similar to that with γ-globulin and bovine serum albumin, which do not have specific nucleotide binding site(s). Measurements of NOE between the H1′–H2′ proton pair as a function of ligand concentrations with the enzyme-ligand ratio kept constant at 1:10 showed that, for ligand concentrations over about 3–4 mM, weak nonspecific binding makes a significant contribution to the observed NOE. Thus the NOE values relevant for the determination of the nucleotide conformation at the active site were measured at nucleotide concentrations of about 1.5 mM. The TRNOE buildup curves for all the ligand–proton pairs were analyzed using a complete relaxation matrix approach. The interproton distances derived from the NOE's were then used as constraints in elucidating the ligand structure by using the program CHARMm. The NOE-determined structures of both MgADP and MgATP bound to creatine kinase correspond to an *anti* conformation with the glycosidic angle (O'_4–C'_1–N_9–C_8) $\chi = 51 \pm 5°$. The ribose pucker nominally representative of these data is a ${}^{O}_{4'}T$ with a phase angle of pseudorotation (p) of 70.5°.

Enzymes for which nucleotides are substrates occur in a variety of biochemical pathways and are an attractive group for structural studies aimed at elucidating enzyme mechanisms. The conformation of nucleotides in enzyme complexes of ATP[1]-utilizing enzymes is a subject of continued interest. A number of papers (Vasak et al., 1979; Rosevear et al., 1981, 1983, 1987; Gronenborn et al., 1984; Banerjee et al., 1985; Landy et al., 1992) appeared in recent years on the conformation of the adenosine moiety in these complexes investigated by the use of the proton transferred nuclear Overhauser effect (TRNOE) experiments (Albrand et al., 1979; Clore & Gronenborn, 1982, 1983). It is considered an advantage that these experiments are typically performed on samples containing a significant excess (10-fold or more) of substrate over the enzyme. In the two-dimensional NOESY spectrum (Anil Kumar et al., 1980) of such a sample, the diagonal peaks arise predominantly from the free substrate whereas the cross-peaks representing the TRNOE arise primarily from the enzyme-bound substrates in exchange with the free. The buildup of all the NOE's is monitored by varying the mixing time in the NOESY algorithm. Methods are available to analyze the buildup curves in a NOESY experiment and to derive interproton distances by the use of complete relaxation matrix incorporating either fast- (Clore & Gronenborn, 1982, 1983; Landy & Nageswara Rao, 1989; Campbell & Sykes, 1991) or slow-exchange conditions (Ni, 1992; London et al., 1992; Nirmala et al., 1992) as required for the complex under investigation. The structures compatible with the NOE-determined distances are deduced by utilizing energy minimization routines provided by molecular modeling programs, such as QUANTA.

In this paper a TRNOESY determination of the conformation of MgATP and MgADP bound to rabbit muscle creatine kinase is presented. The experiments explicitly examine the effect of adventitious nucleotide binding to the protein on the observed NOE. This question significantly impinges on the reliability of the TRNOESY-determined structures because the customary sample protocol of a large excess of substrate used in these experiments might allow appreciable weak nonspecific binding of the substrate. The observed NOE will then be a superposition of that from the substrate molecules bound at the specific and the nonspecific sites in exchange. The deduced substrate conformation may not represent the conformation at the active site. While the possibility of weak nonspecific binding influencing the TRNOE-determined structures is not entirely unrecognized (Williams & Rosevear, 1991), to the best of our knowledge this question has not been addressed in detail thus far in the published literature.

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* Author to whom correspondence should be addressed.

‡ On sabbatical leave from the Tata Institute of Fundamental Research, Bombay 400 005, India.

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TRNOE, transfer nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; TRNOESY, transfer NOESY; Tris-d_{11}, Tris(hydroxymethyl)aminomethane (deuterated—d_{11}); ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; BSA, bovine serum albumin; ESR, electron spin resonance; ppm, parts per million.

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Rosevear et al. (1987) have previously reported the conformation of MgATP bound to creatine kinase on the basis of one-dimensional TRNOE experiments. The NOE measurements were made by the method of monochromatic saturation and were analyzed by two-spin approximation applied to each pair. The results presented in this paper include a comprehensive analysis of the NOE data for all the spin pairs provided by the 2D-NMR methodology, in addition to delineating the effects of nonspecific binding. Furthermore, the conformation of bound MgADP has also been determined.

EXPERIMENTAL PROCEDURES

Materials. ADP, ATP, creatine and rabbit muscle lactate dehydrogenase, γ-globulin (Bovine Cohn fraction II), and bovine serum albumin were obtained from Sigma Chemical Co. Rabbit muscle pyruvate kinase was obtained from Boehringer Mannheim GmbH. Tris(hydroxymethyl)aminomethane (deuterated—d_{11}) and 99.99% D_2O were supplied by Research Organics Inc. All other chemicals used were of analytical reagent grade.

Enzyme Preparation. Creatine kinase was prepared from rabbit skeletal muscle, by method B of Kuby et al. (1954) and was subjected to Sephadex G-75 column chromatography as described by Jarori et al. (1985). The specific activity of enzyme at this stage was ~70 IU. Such a preparation of the enzyme was free from ATPase and adenylate kinase contamination. During the NMR measurements, which typically lasted for ~70 h, no hydrolysis of ATP was observed. For TRNOESY experiments, the enzyme was dialyzed against 1 mM NH_4OH, pH 9.0, lyophilized, and then dissolved in a D_2O solution of 50 mM Tris-d_{11}-Cl, pH 8.0. The sample was freeze-dried once more and redissolved in 99.99% D_2O. The specific activity at this stage was >55 IU. Protein and nucleotide concentrations were determined spectrophotometrically with $\epsilon_{280}^{mg/mL}$ = 0.894 cm^{-1} and a dimer molecular weight of 81 000 (Noda et al., 1954) for the enzyme and ϵ_{259}^{mM} = 15.4 cm^{-1} for ATP and ADP. The enzyme concentrations were defined in terms of a subunit molecular weight of 40 500. The pH values reported here correspond to the readings on a Beckman Altex Model 3500 digital pH meter and are not corrected for isotope effects.

NMR Measurements. ^1H NMR measurements at 500 MHz were made on a Varian Unity 500 NMR spectrometer. Composition of a typical sample on which measurements were made to determine the nucleotide structure: creatine kinase, 0.364 mM; ATP (or ADP), 1.47 mM; $MgCl_2$, 20 mM; Tris-d_{11}-Cl, 50 mM at pH 8.0 in a sample volume of 600 μL. This protocol was arrived at after a detailed investigation of the effects of nonspecific binding of the nucleotides to the protein which occurs at higher nucleotide concentrations (see Results and Analysis). The solutions were in D_2O, and the sample temperature was maintained at 10 °C. Magnesium ion concentration was adequate for complete saturation of the nucleotide, as evidenced by the coalescence H5′ and H5″ resonances in the spectrum. The numbering of the relevant protons is illustrated in Figure 1.

NOESY time-domain data were collected in the hyper complex mode (States et al., 1982) with 256 t_1 increments and 2K data points during the acquisition period (t_2 dimension), for mixing times of 40, 80, 120, 160, 200, and 300 ms. Thirty-two scans were averaged for each t_1 increment, and the zero-quantum interference was suppressed by random variation of the mixing time (up to 10% of its value) between different t_1 increments (Macura et al., 1981). A relaxation delay of 2 s

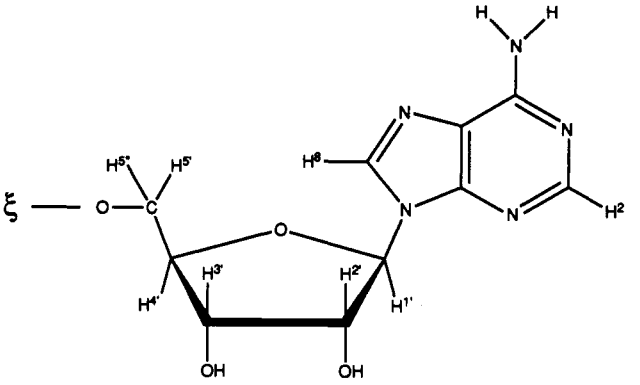

FIGURE 1: Adenosine moiety showing the numbering system used for the relevant protons.

was used, and the carrier frequency was placed at the HDO resonance in all the experiments. The solvent resonance was also suppressed by monochromatic irradiation using the decoupler channel during intervals of the relaxation delay, the t_1 period, and the mixing period. Two-dimensional Fourier transformation was performed along both the dimensions with a Gaussian apodization and zero-filling to obtain a 2K (F1) × 4K (F2) data set. The spectra were phased to pure absorption mode. Normalized NOE's were determined by dividing the observed cross-peak volume by the diagonal peak volume of H1′ extrapolated to zero mixing time and then converted to percentage. In experiments where the measurements were made only for a single mixing time (80 ms), a control spectrum was recorded with zero mixing time. The diagonal intensity of the H1′ resonance from such a control measurement was used to normalize the NOE's.

Theoretical Details. It has been shown that in the limit of fast exchange the characteristic equation for TRNOE is given by

$$\frac{d}{dt}\mathbf{m} = -\mathbf{R}\mathbf{m} \tag{1}$$

where $\mathbf{m}$ is a magnetization vector for all the spins and $\mathbf{R}$ is an average relaxation matrix given by

$$\mathbf{m} = \mathbf{m}^b + \mathbf{m}^f \tag{2}$$

$$\mathbf{R} = p_b\mathbf{W}^b + p_f\mathbf{W}^f \tag{3}$$

In eqs 2 and 3, $\mathbf{m}^b$ and $\mathbf{m}^f$ are n-component magnetization vectors, $\mathbf{W}^b$ and $\mathbf{W}^f$ are $n \times n$ relaxation matrices, and p_b and p_f are population fractions respectively for the bound and free ligands, each containing n spins (Landy & Nageswara Rao, 1989; Koning et al., 1990; Campbell & Sykes, 1991). The relaxation matrix elements of $\mathbf{W}^b$ and $\mathbf{W}^f$ are given by standard expressions for the case of the dipolar interaction (Abragam, 1961; Noggle & Schirmer, 1971; Kalk & Berendson, 1976; Keepers & James, 1984; Landy & Nageswara Rao, 1989)

$$W_{ij} = W_{ji} = \frac{\gamma^4\hbar^2\tau_c}{10r_{ij}^6}\left[-1 + \frac{6}{1+4\omega^2\tau_c^2}\right] \tag{4}$$

and

$$W_{ii} = \frac{\gamma^4\hbar^2\tau_c}{10}\left[1 + \frac{3}{1+\omega^2\tau_c^2} + \frac{6}{1+4\omega^2\tau_c^2}\right]\sum_{k\neq i} r_{ik}^{-6} \tag{5}$$

in which γ and ω are the gyromagnetic ratio and Larmor frequency of the protons, r_{ij} is the distance between spins i and j, and τ_c is the isotropic rotational correlation time. These parameters will correspond to the bound and free species,

depending on whether $\mathbf{W}^b$ or $\mathbf{W}^f$ is evaluated. The elements of $\mathbf{m}^b$ and $\mathbf{m}^f$ are similarly given by analogous expressions, e.g.

$$m_i^b = M_{zi}^b - M_{0i}^b \quad (6)$$

where M_{zi}^b is the instantaneous value of the z component of the magnetization of spin i in the bound state and M_{0i}^b is its equilibrium value. Equations 4 and 5 assume that the spin system is in a single conformation characterized by the distances r_{ij} and undergoing isotropic rotational diffusion characterized by τ_c. The intensity of the $i \leftarrow j$ cross-peak in a two-dimensional TRNOESY experiment representing polarization transfer from j to i, for a mixing time τ_m, is given by

$$m_{i\leftarrow j}(\tau_m) = (e^{-\mathbf{R}\tau_m})_{ij} M_{0j} \quad (7)$$

$$= \left[\mathbf{1} - \mathbf{R}\tau_m + \frac{1}{2}\mathbf{R}^2\tau_m^2 - \frac{1}{6}\mathbf{R}^3\tau_m^3 + \ldots\right]_{ij} M_{0j} \quad (8)$$

Equation 8 shows that, for short mixing times, the buildup of the intensity of a cross-peak in a TRNOESY spectrum, given by $m_{i\leftarrow j}(\tau_m)$ *vs* τ_m, is a polynomial in τ_m, and the initial slope of the buildup given by the linear term yields R_{ij}. Since usually $\tau_c^b > \tau_c^f$, p_b/p_f is 0.1–0.25, and $(\omega\tau_c^b)^2 \gg 1$, it is readily seen from eqs 3 and 4 that

$$R_{ij} \approx p_b W_{ij} \approx -\frac{\gamma^4\hbar^2 p_b \tau_c^b}{10(r_{ij}^b)^6} \quad (9)$$

Thus the ratios of initial slopes for different spin pairs are related to the corresponding internuclear distances in the bound conformation as

$$(R_{ij}/R_{ik}) \approx (r_{ik}^b/r_{ij}^b)^6 \quad (10)$$

The distance r_{ij}^b can be estimated in terms of a calibration distance from eq 10 if such a distance can be identified within the spin system. Implicitly, the calibration distance allows the evaluation of τ_c^b (more precisely $p_b\tau_c^b$). Depending on the specific spin system of interest, and the accuracy and completeness of the experimental data, a comprehensive analysis of the data may then be made using eq 7 to obtain an iterative fit between theory and experiment. It may be seen from eqs 3, 4, and 8 that the cross-peak intensities in a TRNOESY spectrum for a ligand in fast exchange between bound and free forms are similar to those of an intact system with an effective correlation time given by $p_b\tau_c^b$. Multiple-spin (or spin-diffusion) effects on the observed intensities arise from quadratic and higher-order terms in eq 8.

Molecular Modeling and Energy Calculations. Molecular modeling and energy calculations were carried out using the CHARMm program (Brooks et al., 1983) in the software package QUANTA running on a Silicon Graphics computer system. The calculations were performed on an AMP molecule in vacuum. The distances derived from NOE's were given as constraints having a force constant of 20 kcal/(mol Å^2) and allowing a range of variation of ±10% in distances. The energy was minimized using Powell's method available with the package.

RESULTS AND ANALYSIS

Nonspecific Binding of the Nucleotides to the Protein. Figure 2A shows the proton TRNOESY spectrum of MgADP in the presence of creatine kinase for a mixing time of 120 ms. The spectrum clearly shows all the interproton NOE's in the adenosine moiety of MgADP. The substrate and enzyme

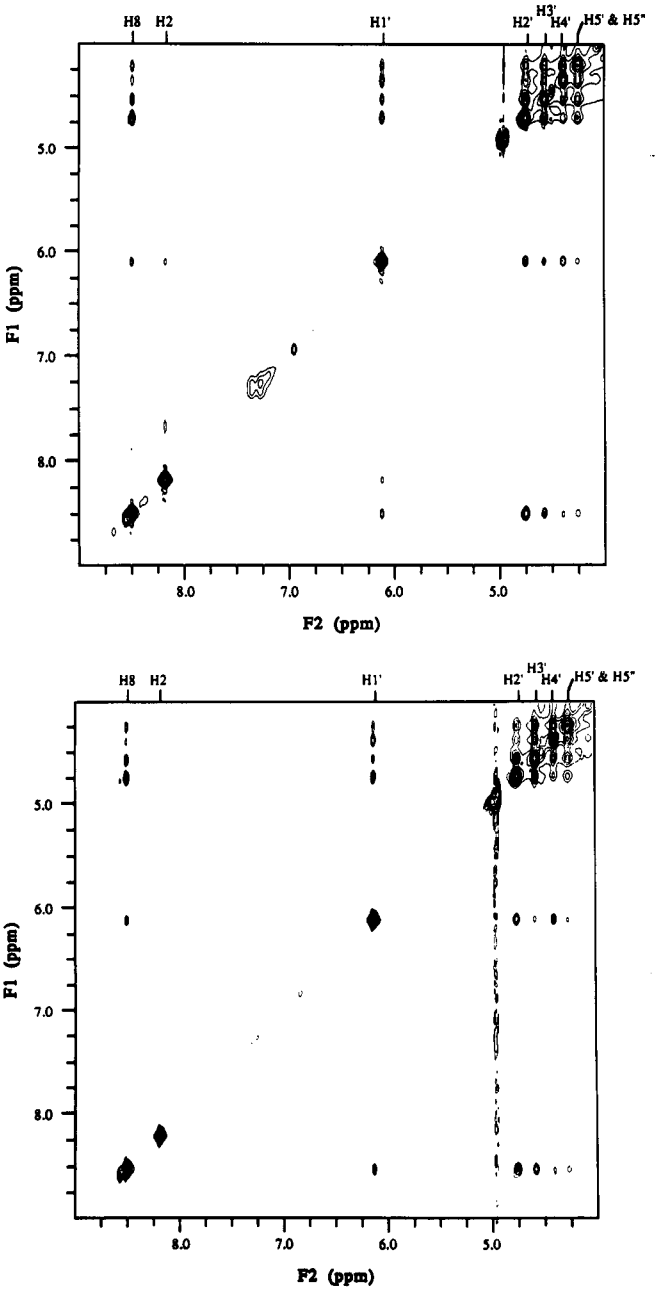

FIGURE 2: (A, Top) 500-MHz proton TRNOESY spectrum of a creatine kinase·MgADP complex in D_2O solution at 10 °C and pH 8.0. The sample contained creatine kinase (1.03 mM), ADP (10.18 mM), and $MgCl_2$ (20 mM); 50 mM Tris-d_{11}-Cl buffer was used to maintain the pH at 8. The sample volume was 600 μL. NOESY time domain data were collected with 256 × 2 t_1 increments and 2K points during each accumulation during t_2; 32 transients were collected for each t_1. The mixing time was 120 ms, and a relaxation delay of 2 s was used. Two-dimensional Fourier transformation was performed along both the dimensions with a Gaussian apodization and zero-filling to obtain a 2K (F1) × 4K (F2) data set. The spectra were phased to pure absorption mode. (B, Bottom) 500-MHz proton TRNOESY spectrum of a γ-globulin·MgADP complex in D_2O solution at 10 °C. Concentrations: γ-globulin, 0.45 mM; ADP, 10.18 mM; $MgCl_2$, 20 mM. The NOESY accumulation and processing parameters are same as described for part A.

concentrations in this sample were 10.18 mM, MgADP, and 1.03 mM, creatine kinase (sites). Other details regarding the sample are given in the figure caption. The sample protocol is similar to those used in most published TRNOE experiments on MgATP bound to ATP-utilizing enzymes (Vasak et al., 1979; Rosevear et al., 1981, 1983, 1987; Gronenborn et al.,

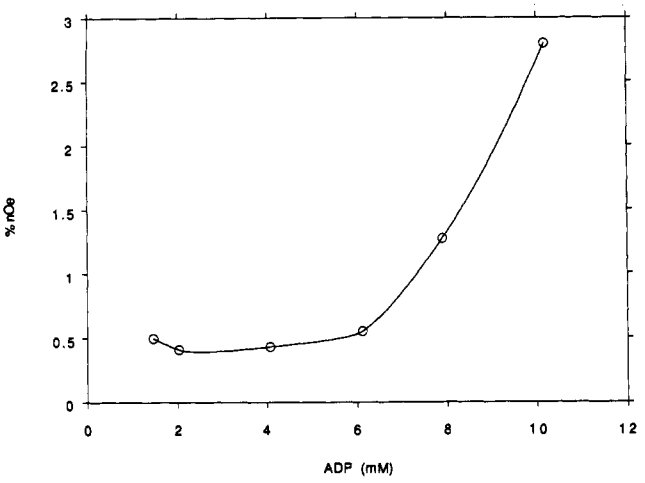

FIGURE 3: Concentration dependence of the percentage NOE of the H1′–H2′ proton pair in a creatine kinase·MgADP complex for a mixing time of 80 ms. The ligand concentration was varied between 1.5 and 10 mM, with the ligand:enzyme concentration ratio kept constant at 10:1. $MgCl_2$ was added in sufficient amount to saturate the ligand. The experimental points are represented by circles, and the solid curve represents interpolation through the experimental points.

1984; Banerjee et al., 1985; Landy et al., 1992). In order to investigate the possible contribution of adventitious binding of the nucleotide to the observed NOE's, a similar experiment was performed on a sample containing 10.18 mM MgADP and 0.45 mM γ-globulin, which is not known to have a specific binding site for MgADP. The results of the TRNOESY experiment on this sample, shown in Figure 2B, bear a striking similarity to those in Figure 2A. The negative NOE's for MgADP in Figure 2B indicate that the ligand which is predominantly free in solution is undergoing exchange with slowly tumbling macromolecular complexes. The complexes of MgADP with γ-globulin arise due to nonspecific binding. A third experiment was performed in which γ-globulin was replaced by BSA. BSA is also not known to have a specific binding site for MgADP, and a TRNOE pattern (data not shown) similar to that in Figure 2B was obtained, substantiating the view that nonspecific binding of ADP contributes to the observed TRNOE. The measurements on the γ-globulin sample were repeated after the addition of 100 mM KCl to the sample. This resulted approximately in 25% reduction in the observed NOE's, indicating that binding of MgADP to the protein does occur and is diminished in the presence of high salt concentration.

In order to assess the extent of weak nonspecific binding in the creatine kinase–MgADP sample and to determine the sample protocol that will minimize this adventitious binding, the following measurements were made. With the ratio of ligand (MgADP) to enzyme concentration kept constant at a value of 10:1, several samples were prepared in which the ligand concentration was varied from 1.5 to 10 mM. The TRNOE was measured in all these samples for a single mixing time of 80 ms. The normalized NOE observed for the H1′–H2′ pair is plotted as a function of ligand concentration in Figure 3. This plot clearly shows that the NOE is practically unchanged for ADP concentrations up to ~6 mM and then starts to increase rapidly with increasing ligand concentration, going up by a factor of nearly 5 for 10 mM ADP. Since the H1′–H2′ distance is considered to be invariant in any conformation of adenosine (DeLeeuw et al., 1980; Rosevear et al., 1983), the increase in NOE at high concentrations must arise from an increase in p_b because τ_c^b is not expected to change with concentrations in this range.[2] On the basis of the known dissociation constants (Reed et al., 1970), the occupation of the active site changes at the most from 95% to 99% with the increase in concentrations. The increase in p_b by nearly a factor of 5 must, therefore, be due to adventitious binding of the nucleotide to the enzyme.

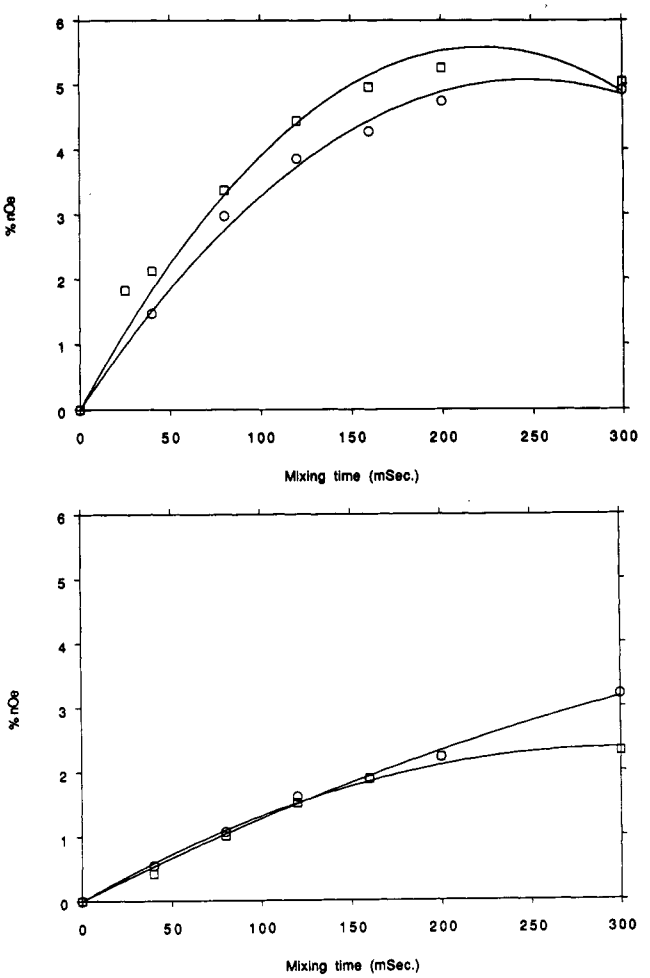

FIGURE 4: (A, Top) Comparison of percentage NOE buildup for H1′–H2′ in (□) MgATP and (○) MgADP complexes of creatine kinase at high concentration. The high-concentration-sample conditions were identical to those for Figure 2A. (B, Bottom) Comparison of percentage NOE buildup for H1′–H2′ in (□) MgATP and (○) MgADP complexes of creatine kinase at low concentrations. The sample conditions for the low concentration were identical to those for Figure 5.

Since the nucleotides have a negatively charged phosphate chain, adventitious association with the proteins is expected to occur in the vicinity of positively charged regions on the surface of the protein. This view is substantiated by the buildup curves shown in Figure 4 for H1′–H2′ pairs in MgADP and

[2] The assumption that τ_c does not increase with protein concentration is based on NMR data from studies of enzyme-bound substrates of creatine kinase (Nageswara Rao & Cohn, 1981; Jarori et al., 1985; Nageswara Rao & Ray, 1992). In these experiments, enzyme concentrations up to about 6 mM were used and no evidence of protein aggregation was found from the line widths of the resonances of enzyme-bound substrates. A related question is whether τ_c^f changes and becomes comparable to τ_c^b at high nucleotide concentrations possibly due to nucleotide stacking. Although some stacking of the free Mg-nucleotides cannot be ruled out at concentrations of about 10 mM, NOE measurements on free nucleotides at these concentrations with and without Mg(II), and TRNOE measurements on other enzyme–nucleotide complexes in which adventitious binding effects appear to be negligible, suggest that nucleotide stacking effects are not a significant factor in the NOE measurements on high-concentration samples.

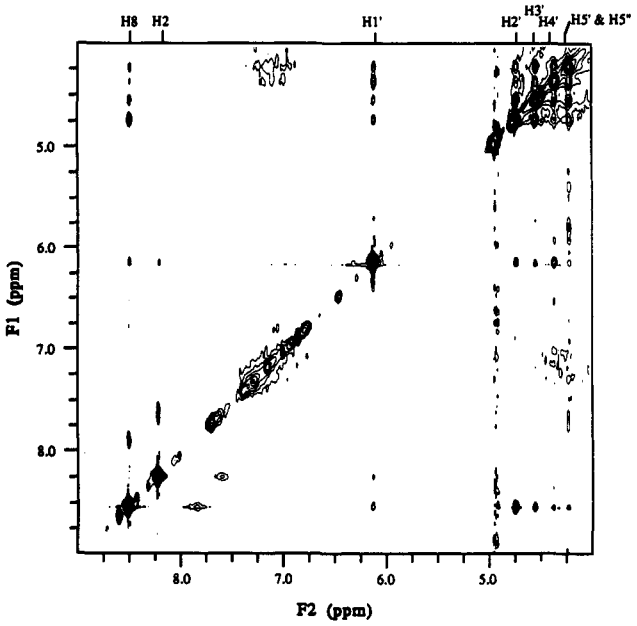

FIGURE 5: 500-MHz proton TRNOESY spectrum of a creatine kinase-MgADP complex in D_2O solution at 10 °C and pH 8.0. Concentrations: creatine kinase, 0.364 mM; ADP, 1.47 mM; $MgCl_2$, 20 mM in 50 mM Tris-d_{11}. The NOESY accumulation and processing parameters are same as described for Figure 2A.

MgATP bound to creatine kinase determined at both high- and low-concentration sample protocols. It may be noted that the normalized NOE is consistently larger for MgATP than for MgADP at high concentrations, whereas at low concentrations, at which the weak nonspecific association is negligible, the normalized NOE for the two nucleotides is identical within experimental error. This result shows that the adventitious binding is larger for MgATP, which at pH 8.0 carries two negative charges while MgADP carries only one, consistent with the notion that electrostatic interactions are a major cause for the nonspecific association.

TRNOE Measurements for Structure Determination. Since the results presented above unequivocally establish the presence of considerable weak nonspecific binding of the nucleotides to the protein at high nucleotide concentrations, the TRNOE measurements used for structure determinations of the adenosine moiety at the active sites were performed at low concentrations of about 1.5 mM and with ratios of 1:4 between the enzyme (sites) and the substrate concentrations (see details of sample conditions given under Experimental Procedures). The low nucleotide concentrations, dictated by the need to minimize the effects of adventitious binding, reduce the sensitivity of the TRNOE measurements. In order to offset this loss of sensitivity, the enzyme–substrate concentration ratio is increased to 1:4 from 1:10 used in the measurements for Figure 3, thereby increasing the NOE observed. The ratio 1:4 is found to be nearly optimal for this protein. The NOE's involving H8 and H1′ provide the most accurate data because these resonances occur in isolated regions in the spectrum with no appreciable overlap from the protein resonances. In contrast, the resonances of H2′, H3′, H4′, and H5′ and H5″ ride a broad feature from the protein and the NOE's between these protons are less accurate. Increasing the enzyme concentration any further leads to larger signals from the protein on which the signals of the nucleotides overlap, and thus the accuracy of the NOE measurement is even more impaired. A typical TRNOESY spectrum of creatine kinase–MgADP sample from which measurements were made for structure determination is shown in Figure 5.

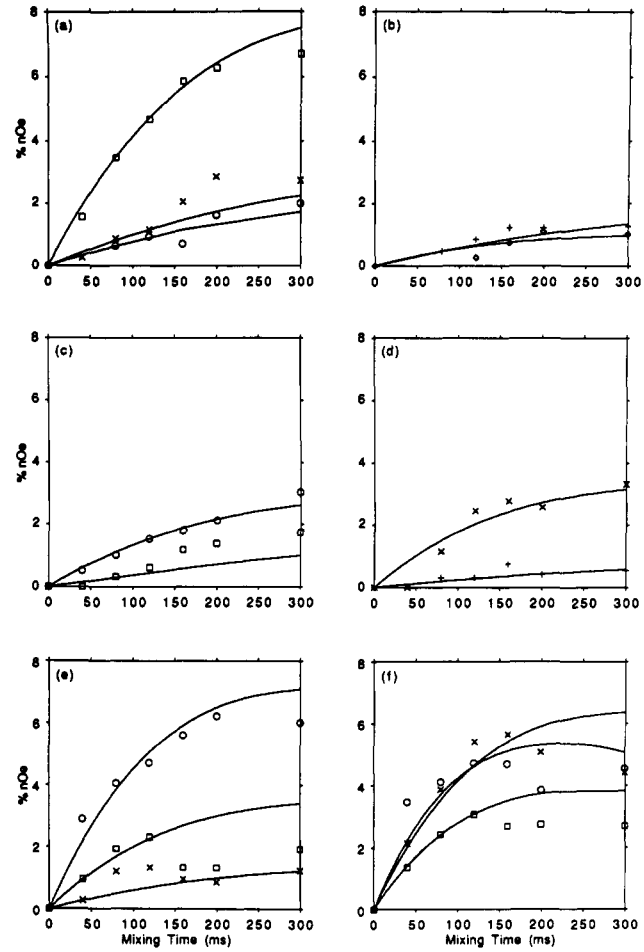

FIGURE 6: Percentage NOE buildup curves for a creatine kinase-MgADP complex. The sample contained 0.36 mM creatine kinase, 1.46 mM ADP, and 20 mM $MgCl_2$ in 50 mM Tris-d_{11}-Cl at pH 8.0. Protons involved: (a) (○) 8–1′, (□) 8–2′, (×) 8–3′; (b) (+) 8–4′, (◇) 8–5′/5″; (c) (○) 1′–2′, (□) 1′–3′; (d) (×) 1′–4′, (+) 1′–5′/5″; (e) (○) 2′–3′, (□) 2′–4′, (×) 2′–5′/5″; (f) (○) 3′–4′, (□) 3′–5′/5″, (×) 4′–5′/5″. The solid curves represent theoretically simulated buildup curves based on the NOE matrix distances given for the creatine kinase-MgADP complex given in Table I. External relaxation rates for H8, H2, H1′, H2′, H3′, H4′, H5′, and H5″ used in the fitting routine were 1, 1, 1, 1.25, 1, 1.25, 2.5, and 2.5 s^{-1}, respectively. The rotational correlation time used for the bound ligand was 7.4 ns.

The experimental data of normalized NOE's are plotted in three different groups in Figure 6, along with the theoretical curves generated as described below.

Analysis of Data. The normalized NOE's for each pair shown in Figure 6 were fit with a second-order polynomial in τ_m, and the initial slope of the buildup curve was determined for each pair. In performing the fit, the (0,0) point was included as part of the data in order that the leading term be linear in τ_m and the constant term in the polynomial be minimized. Furthermore, emphasis was placed on fitting the data up to 160 ms, because the correlation times ultimately deduced (see below) show that a second-order polynomial is sufficiently accurate up to this value of τ_m. Due to the overlap of the resonances of H5′ and H5″, the two protons were treated as a single spin. Hence the observed NOE's involving H5′ and H5″ were divided by a factor of 2 before fitting them by a second-order polynomial. The interproton distance calculated from such an NOE is, therefore, an effective distance which yields half the observed NOE and does not represent the distance of the third proton from either H5′ or H5″. Using a value of 2.9 Å for the H1′–H2′ distance and the initial slopes obtained for different pairs along with eq 10, a set of

Table I: Interproton Distances in MgADP and MgATP Bound at the Active Site of Rabbit Muscle Creatine Kinase[a]

	distance (Å)		
proton pair	E·Mg(II)·ADP	E·Mg(II)·ATP	energy minimized
8–1′	3.25	3.23	3.56
8–2′	2.40	2.42	2.55
8–3′	3.12	2.95	3.13
8–4′	3.45	3.28	4.35
8–5′	3.35	3.57	2.88
8–5″	3.35	3.57	4.23
1′–2′	2.90	2.90	2.92
1′–3′	3.78	3.19	3.99
1′–4′	2.75	2.85	3.00
1′–5′	4.00	3.89	4.41
1′–5″	4.00	3.89	4.87
2′–3′	2.33	2.33	2.39
2′–4′	2.70	2.95	3.97
2′–5′	3.40	3.19	4.02
2′–5″	3.40	3.19	4.42
3′–4′	2.37	2.52	3.00
3′–5′	2.50	2.61	2.29
3′–5″	2.50	2.61	2.56
4′–5′	2.30	2.28	3.01
4′–5″	2.30	2.28	2.52

[a] The distances obtained from the complete relaxation matrix fit of the NOE data are given in column 2 for the creatine kinase·MgADP complex and column 3 for the creatine kinase·MgATP complex. The last column represents the energy-minimized distance for both the complexes.

distances were obtained for the bound conformation. This calibration using the H1′–H2′ distance also yields a value of $p_b\tau_c^b$ (see eq 9). Using these parameters, the complete relaxation matrix equation is set up and cross-peak intensities for each spin pair are calculated as a function of mixing time (τ_m), according to the prescription of eqs 1–8. In order to fit the experimental data for larger mixing times (>160 ms), contributions from any external relaxation mechanisms were added as a leakage term to each diagonal element of the relaxation matrix (see caption of Figure 6). For free MgATP, a set of distances representing an energy-minimized structure was used (Landy et al., 1992). The buildup curves thus calculated are compared with the experimental data. At this stage, any of the parameters (the correlation times, distances, and the leakage term) may be adjusted to improve the agreement between the calculated buildup curves and the experimental data. The correlation times finally chosen were τ_c^b = 7.4 ns and τ_c^f = 0.3 ns (assuming complete saturation of the enzyme active sites). The solid curves in Figure 6 are calculated for the best fit obtained. The scatter in the experimental data involving the spins of H4′–H5′ and H5″, H3′–H5′ and H5″, and H3′–H4′ is mainly due to the fact that these resonances ride on a broad feature from the protein protons and thus the fit to these data appear relatively poorer (Figure 6f). The interproton distances obtained for MgADP and MgATP complexes from this TRNOE analysis are listed in Table I.

Energy Minimization Calculations. In order to examine the energy implications of the NOE-determined distances on bound nucleotide structure by using the CHARMm program, the distance constraints were applied in a sequence of five steps starting from H8–H2′ and H8–H3′ distances. These two distance constraints are the most significant determinants for the glycosidic torsion angle. In the second step, additional constraints from H8–H1′ and H1′–H4′ distances were used. The H1′–H4′ distance constraint altered the sugar pucker, and the glycosidic torsion was also altered to include the H8–H1′ distance constraint along with the earlier ones. Next, H1′–H2′, H1′–H3′, H1′–H4′, H2′–H3′, H2′–H4′, and H3′–H4′ distance constraints were added to get the overall sugar pucker. This step typically disturbed some of the previously optimized distances involving H8 and the other sugar protons, and the following final two steps were introduced to iteratively fine-tune such discrepancies. In step 4, along with those constraints applied in step 2, additional constraints such as H1′–H2′, H2′–H3′, and all distances involving the H5′ and H5″ protons were included. As mentioned above, because of the overlap of H5′ and H5″ resonances, the NOE measurements yield an effective distance rather than the actual distances to these protons and, therefore, these distances were allowed to vary over a range of ±25% in the calculation. This step fixed the H5′ and H5″ orientation with respect to the H8 proton. In the last step, the constraints on H8–H2′, H8–H3′, H8–H1′, H8–H4′, H1′–H2′, H1′–H3′, and H1′–H4′ distances were all brought back in order to refine the structure and minimize the distance deviations that arose from constraints applied in step 3. The final set of distances, obtained by energy calculations, compatible with the NOE-determined distances (Table I) yielded a structure with an energy of about 5 kcal over the structure without any distance constraints. The distances in the final structure correspond to a glycosidic torsion angle (χ) of 51 ± 5°, and the various sugar torsions are listed in Table II. The phase angle of pseudorotation (p) is 70.5° for both the MgATP and the MgADP complexes. This pseudorotation angle corresponds to a $^{O}_{4'}T$ sugar pucker (Altona & Sundaralingam, 1972; DeLeeuw et al., 1980). The orientation of the adenine ring with respect to the ribose for the MgATP complex is depicted in Figure 7.

Table II: Various Torsion Angles and the Pseudorotation Phase Angle p for the Ribose at the Active Site of Creatine Kinase[a]

torsion	angle (deg)
$\chi(O'_4–C'_1–N'_9–C_8)$	51
$\nu_0(C'_4–O'_4–C'_1–C'_2)$	−22
$\nu_1(O'_4–C'_1–C'_2–C'_3)$	6
$\nu_2(C'_1–C'_2–C'_3–C'_4)$	9
$\nu_3(C'_2–C'_3–C'_4–O'_4)$	−22
$\nu_4(C'_3–C'_4–O'_4–C'_1)$	28
$\gamma(O'_5–C'_5–C'_4–C'_3)$	−165

$$p = \tan^{-1}\{[(\nu_4 + \nu_1) - (\nu_3 + \nu_0)]/[2\nu_2(\sin 36° + \sin 72°)]\} = 70.45°$$

[a] The definitions used for various torsion angles are the same as described by Sanger (1984).

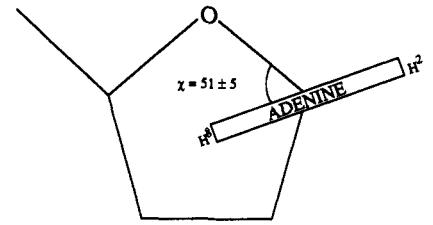

FIGURE 7: Schematic drawing showing the glycosidic torsion of the adenine base with respect to the ribose sugar.

NOE's between Nucleotide and Enzyme Protons. The 2D spectrum of the low-concentration sample, shown in Figure 5, shows rather prominent cross-peaks between the protons of the enzyme and H8, H2, and H1′ of ADP. These cross-peaks are not due to exchange between bound and free ligands because no measurable chemical shifts exist between the two species. Therefore, these intermolecular cross-peaks indicate proximity between these nucleotide protons and the corresponding enzyme protons. Among the TRNOE experiments on such systems published thus far, examples of those that showed significant protein–ligand NOE's are few and far

between (James, 1976; Anglister et al., 1989). It may be noted that these intermolecular cross-peaks are not readily seen in the spectrum of the high-concentration sample shown in Figure 2A, since the fractional NOE's are much weaker for the intermolecular NOE's due to the higher ligand and enzyme concentration ratio. The possibility of observing ligand proton NOE at a ligand concentration of 4:1 was proposed in an earlier study of creatine kinase–nucleotide complexes by James (1976). The chemical shifts of the enzyme protons, viz. one at 7.85 ppm close to H8, one at 7.6 ppm close to H2, and another at 6.95 ppm close to H1′, suggest a tryptophan residue. A similar observation was made by Vasak et al. (1979).

DISCUSSION

The special feature of TRNOESY spectra that the diagonal peaks arise from the free substrate and the cross-peaks which represent the NOE arising from the bound substrate is due to the fact that under fast-exchange conditions the average relaxation matrix elements are dominated by the bound substrate so long as $p_b\tau_c^b \gg p_f\tau_c^f$ (see eq 9). In obtaining this condition, there is sufficient latitude in the choice of p_f/p_b depending on the ratio τ_b/τ_f. Since the sensitivity as well as the ease of experimental measurement is optimized by choosing as large a value of p_f as this condition allows, substrate concentrations exceeding that of the enzyme (sites) by a factor of 10 or more were used in a number of previously published TRNOE measurements on various enzyme complexes. For substrates such as the nucleotides, which contain charged phosphate groups, and are thereby likely to associate due to electrostatic interactions, the large excess of free nucleotide facilitates adventitious binding at the positively charged regions on the protein surface. The extent of such weak nonspecific binding, the multiplicity of such sites, and the relative dissociation constants depend on the particular enzyme–substrate complex and the sample protocol. However, since the experimental NOE's in such samples will now be due to exchange between the substrate bound at the (specific) active site and the nonspecific site(s), a significant contribution from weak nonspecific binding is potentially capable of vitiating the structures deduced from these measurements.

The TRNOESY measurements presented in this paper using high- and low-nucleotide-concentration protocols, and the experiments with γ-globulin and BSA, clearly show that, in samples containing 1 mM creatine kinase (sites) and a 10-fold excess of nucleotide, adventitious binding exceeds the binding at the active site. The concentration dependence of the TRNOE of the H1′–H2′ pair (Figure 3) shows that the active-site-bound nucleotide predominates for nucleotide concentrations below 5 mM. For different enzyme–substrate systems this demarcation in the concentration is likely to vary and the choice of optimal sample protocol for structure measurements at the active site needs to be determined by deliberate experimentation. For the creatine kinase complexes, a nucleotide concentration of approximately 1.5 mM was used with a 1:4 enzyme (site):substrate ratio. This choice has led to the circumstance that a number of NOE's used for structure determination are less than 2%. Nevertheless, since the adventitious binding is negligible at the low substrate concentration chosen, the structure deduced is representative of the substrate at the active site. It may be noted that the separation of the NOE arising due to the specific active-site binding from that due to adventitious binding became possible because the latter is considerably weaker. The dissociation constant of MgATP binding is known to be 50–100 μM (Reed et al., 1970), which according to the data in Figure 3 suggests that the adventitious binding occurs at dissociation constants larger than about 2 mM.

Analyses of NOE data for structure determinations of macromolecular systems and complexes are based on theories that assume an isotropic reorientation characterized by a single correlation time. The complexity of NOE theories in the presence of internal motions and/or anisotropic reorientation make the analysis of data for distance determination almost intractable. Attempts have been made to argue that the errors in the distances determined by ignoring the internal motions may not be serious. Such rationalizations are not entirely general. However, in the case of nucleotides bound to ATP-utilizing enzymes, ^{13}C NMR line shapes of [2-^{13}C]ATP bound to a number of these enzymes, including creatine kinase, are accounted for reasonably by an isotropic correlation time appropriate for the molecular mass of the protein (Nageswara Rao & Ray, 1992). This conclusion implies that the glycosidic rotation of the adenosine moiety, which persists unimpeded even at high solvent viscosities (2000 cP), is nevertheless arrested in the enzyme–nucleotide complexes. Thus the use of a single correlation time for the bound complexes in the analysis of TRNOE data appears justified, whereas such a procedure for the free nucleotides is decidedly an approximation. This approximation is, however, not likely to be serious in the calculation of the relaxation matrix elements because the contribution of the free substrate to these elements is relatively small.

The correlation time deduced for the bound complexes by fitting the NOE data used for structure determination is about 8.0 ns. This value is almost 4–5-fold smaller than the 30–40 ns appropriate for a protein of the size of creatine kinase. Correlation times in the range 2.4–12 ns for complexes of protein molecular mass in the range 40–64 kDa were also deduced from previously published TRNOE measurements (Rosevear et al., 1987; Williams & Rosevear, 1991). If a calibration distance, such as the H1′–H2′ distance in the nucleotides, is used in the calculation, the distances determined are unaffected, because the actual value of the correlation time does not affect the distance ratios (see eq 10). The lower value of the correlation time deduced indicates that the normalized NOE values are severalfold smaller than those expected on the basis of the size of the protein. Part of this lowering of the NOE is likely to be due to the fact that dipolar interactions with the protein protons are not included in the calculation. Thus the magnetization shared by the protein protons is not properly taken into account. This problem merits further investigation, and its resolution may well include the acquisition of some information regarding the protein protons in the environment of the substrate protons at the active site.

It may be noted that when the NOE data from the samples with the high nucleotide concentrations (Figure 2A) were used for the analysis by ignoring the nonspecific binding effects entirely, we also found a preferred *anti* conformation for the adenosine and the correlation time τ_c^b of 42 ns. This suggests that although the value of τ_c^b appears reasonable for the protein size, its value is inflated by a factor of about 5 by using a p_b value appropriate for saturating just the active site. In actuality, the nonspecific binding enhances p_b by that factor.

The use of energy minimization calculations to refine the NOE-determined distances seems to compensate for some of the inaccuracies in the latter. For example, the deviations in the distances involving H5′ and H5″ and in distances such as

H8–H4′ and H2′–H4′ appear to be due, at least in part, to inaccuracies in the NOE-determined distances, which arose in turn from the scatter in the experimental data caused by the broad protein feature underlying the resonances arising from H3′, H4′, H5′, and H5″ of the ribose. There could be other factors, such as the interference from protein protons, which may also cause uncertainties in the NOE-determined ligand interproton distances. This question can be fully addressed when more information on the protein protons at the active site is available. Nevertheless, the energy-minimized distances in Table I appear to characterize the most acceptable structure compatible with the distances provided by the TRNOE method. The structures of both MgADP and MgATP bound to creatine kinase correspond to an *anti* conformation with the glycosidic angle $\chi = 51 \pm 5°$. This value differs from $78 \pm 10°$ previously deduced by Rosevear et al. (1987) for MgATP. The ribose pucker nominally representative of these data is a ${}^{O}_{4'}T$ with a pseudorotation angle of 70.45° compared to the O1′-*endo* or C4′-*exo* pucker suggested by Rosevear et al. (1987). On the basis of the results of this paper, with the sample protocol used by Rosevear et al. (1987) (creatine kinase, 1.3 mM; ATP, 14.8 mM), the NOE values measured should have substantial contributions from nonspecific binding. It is not clear why a value of $\tau_c^b =$ 2.4 ns was obtained by Rosevear et al. (1987) although nonspecific binding was ignored by them. It may be recalled that their analysis of data is based on a two-spin approximation.

The fact that the glycosidic torsion angle of the adenosine in MgATP and MgADP bound to creatine kinase is qualitatively described by an *anti* conformation by taking into account the nonspecific binding as in this paper or by ignoring it as in previous publications suggests that a major proportion of Mg·nucleotide population at the nonspecific binding site also assume an *anti* conformation. This may not be a general feature, and should perhaps not be used as a rationalization for ignoring the nonspecific binding effects. In fact, it is not likely that the features of nonspecific binding sites will lend themselves to any generalization, and each enzyme system will require individual scrutiny to assess the contribution of these effects. Similar experiments on a number of other ATP-utilizing enzymes such as arginine kinase, 3-phosphoglycerate kinase, pyruvate kinase, and PRPP-synthetase are now nearing completion and will be reported in future.

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