Conformation of ATPMg(II) bound at the specific site on bovine serum albumin: 1H-nuclear magnetic resonance study

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Bovine serum albumin (BSA) binds one molecule of adenosine 5'-triphosphate (ATP) at one of the fatty acid-binding sites. Interaction of ATPMg(II) with BSA and its conformation in the bound form has been investigated using 1H-nuclear magnetic resonance (NMR). Binding of nucleotide with albumin results in downfield shift of all the ligand proton resonances, indicating the involvement of adenine as well as ribose moiety in binding interactions. Measurements of change in chemical shifts of H2, H8 and H1' protons of the ATP in the bound form as a function of ligand concentration, has yielded a dissociation constant of $K_d = 2$ mM for ATPMg(II) with albumin. Two dimensional transferred nuclear Overhauser spectroscopy (TRNOESY) was used to determine the inter-proton distances in ATPMg(II) bound to BSA. In order to select a concentration range of the ligand where binding occurs exclusively at the specific site, we have measured the NOE intensities for H1'–H2' protons as a function of ATP concentration. For determining the conformation of bound ATPMg(II) at the specific site, NOE intensities have been measured for several mixing times ($T_m = 40$–200 ms). The resulting NOE buildup curves were analysed using complete relaxation matrix calculations to determine various inter-proton distances. Examination of amino-acid sequence of BSA has led to the identification of a region GVKGLSRS (aminoacyl residues 425–432) which is very similar to the consensus sequence present in several ATP-binding proteins. On the basis of observed similarities in the conformation of bound ATP with that of other ATP-utilizing enzymes and the presence of the nucleotide binding consensus sequence, we propose that the specific ATP binding site is located on Domain-III of BSA.

Materials and methods

Materials

Bovine serum albumin was purchased from Armour Pharmaceutical Company. ATP, 4.9 M – MgCl₂ solution and D₂O (99.96 atom% D) were obtained from Sigma. Chelex-100 was supplied by Bio-Rad. Tris(hydroxymethyl)aminomethane-d₁₁ (99.0 atom% D) (Tris-d₁₁) was purchased from ISOTEC Inc, USA. All other chemicals were of Analar grade and used without purification.

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Sample preparation

Bovine serum albumin was dissolved in 20 mM sodium phosphate, pH 7.5, and dialysed against the same buffer. The solution was treated with Chelex-100 to remove trace metal ions. H_2O was exchanged with D_2O by dialysing the sample against 20 mM sodium phosphate, pH 7.5, (without any isotopic correction) in a dialysis cell. At least ten changes of buffer were made for solvent exchange. This was lyophilized once and then dissolved in 99.96% D_2O. The concentration of BSA was determined spectrophotometrically with A_280 = 0.66 mg^{-1} ml cm^{-1} and molecular weight of 66,000 Daltons. Nucleotide solution was also passed through Chelex-100 column, lyophilized and dissolved in D_2O. The concentration of ATP was determined using A_278 = 15.4 mM^{-1} cm^{-1}.

NMR measurements

\(^1\)H-NMR measurements were made on a Bruker-AMX500 NMR spectrometer. In all the measurements, sample temperature was maintained at 10°C. Chemical shifts were measured relative to the internal standard, sodium trimethylsilyl propionate-2,2,3,3-d_4 (TSP). 1D-\(^1\)H NMR spectra were recorded with 16 K data points, 6024 Hz spectral width, 2 s relaxation delay and 64 transients were averaged for each spectrum. NOESY time domain data were acquired with 256 t_s increments and 2 K t_2 points in the phase sensitive mode with the use of TPPI. Sixty-four scans were averaged for each t_s increment. Measurements were made with mixing times 40, 80, 120, 160 and 200 ms. The carrier frequency was placed at the HDO resonance in all the experiments. The solvent resonance was suppressed by irradiation using the decoupler channel during intervals of relaxation delay, the t_s period, and the mixing time. The acquisition parameters were, 1.5 s relaxation delay, 0.17 s acquisition time, and 6024 Hz sweep width.

Data were processed on an Aspect-X32 computer with Bruker software. All the data were processed identically with a two-dimensional Fourier transformation along both dimensions with a Gaussian apodization and a zero filling in t_s to obtain 1 K (F1) x 2 K (F2) real data points. The spectra were phased to pure absorption mode. The volume of the cross peaks was determined by integration. Percentage of NOEs was derived by dividing the observed cross peak volume by the diagonal peak volume of H_1' extrapolated to zero mixing time and then multiplying it by 100. In experiments where measurements were made only for a single mixing time, the diagonal intensity of H_1' in the same spectrum was used to determine the per cent NOE.

Determination of binding constant

In an equilibrium between a macromolecule E and ligand L,

\[ E + L \rightleftharpoons EL, \]

the dissociation constant is given by

\[ K_d = \frac{[E][L]}{[EL]}, \]

For a given value of K_d, E_T and L_T, where E_T and L_T are the total concentrations of the macromolecule and the ligand; the mole fraction of the bound ligand (P_m), i.e. \([EL]/[L_T]\) can be derived easily. Further, if the binding of L to E results in a change in chemical shift of L resonance by \(\Delta \omega\), then under conditions of fast exchange it can easily be shown that

\[ \Delta \omega = P_m \Delta \omega_m, \]

where \(\Delta \omega_m\) is the chemical shift of the fully bound form of the ligand with respect to its position in unbound form. Thus, the measurement of the change in the chemical shift where a fixed concentration of macromolecule is titrated with varying concentrations of the ligand, can be analysed by two parameter iterative fit to derive the values of K_d and \(\Delta \omega_m\).

Relaxation matrix calculations

Simulations of complete NOE buildup curves in a TRNOESY experiment are greatly simplified if the fast exchange condition prevails, i.e. the 'on' and 'off' rates of the ligand must be faster than the typical cross relaxation rates. In the limits of fast exchange, the intensity of the i \leftrightarrow j cross peak represents a polarization transfer from spin j to i, for a mixing time \(\tau_m\) and is given by

\[ m_{i \rightarrow j}(\tau_m) = (e^{-R\tau_m})_j M_{ij} \]

or

\[ = [1 - R\tau_m + \frac{1}{2} R^2 \tau_m^2 - \frac{1}{6} R^3 \tau_m^3 + \ldots]_j M_{ij}, \]

where M_{ij} is the equilibrium value of the j spin magnetization and R is an average relaxation matrix given by

\[ R = p_b W^b + p_f W^f, \]

where p_b and p_f are bound and free fractions of ligand each containing n-spins, W^b and W^f are the dipolar interaction terms between two protons in bound and free form respectively and are given by
\[ W_o = \frac{\gamma^2 \tau_i}{10 r_{ij}^2} \left[ 1 + \frac{6}{1 + 4 \omega^2 \tau_c^2} \right] \] (4)

and

\[ W_n = \frac{\gamma^2 \tau_c}{10} \left[ 1 + \frac{3}{1 + \omega^2 \tau_c^2} + \frac{6}{1 + 4 \omega^2 \tau_c^2} \right] \sum_{k \neq 1} r_{ik}^4, \] (5)

in which \( \gamma \) and \( \omega \) are the gyromagnetic ratio and Larmor frequency of the protons, \( r_{ij} \) is the distance between spins \( i \) and \( j \), and \( \tau_i \) is the isotropic rotational correlation time. Equations (4) and (5) assume that the spin system is in a single conformation characterized by distance \( r_{ij} \), and undergoing isotropic rotational diffusion characterized by \( \tau_c \). Equation (2) shows that, for short mixing times, the build-up of the intensity of a cross peak in a TRNOESY spectrum, given by \( m_{i-j} (r_{ij}) \) vs \( \tau_m' \), is a polynomial in \( \tau_m' \), and the initial slope of the build-up which is just the linear term, yields \( R_{ij} \). Usually \( \tau_m' \gg \tau_c \), \( p_{ij} / p_i \) is 0.1–0.15, the last term in equation (3) is negligible so that

\[ R_{ij} = P_{ij} W_{ij}. \] (6)

From equations (4) and (5) it can easily be shown that the ratio of initial slopes for different spin pairs is related to the corresponding internuclear distances in the bound conformation as

\[ \left( \frac{R_{ij}}{R_{ak}} \right) = \left( \frac{r_{ij}^4}{r_{ak}^4} \right)^{1/4}. \] (7)

Here \( r_{ij}^b = 2.90 \pm 0.2 \) Å is the calibration distance between H1' and H2'. This distance is known to be independent of the nucleotide conformation. The inter-proton distances derived using equation (7) were used as initial distances and a comprehensive analysis of data was done using equations (1) and (2) to obtain an iterative fit between theory and experiment.

**Energy minimization and molecular modelling**

Molecular modelling and restrained energy minimization were performed using Insight II (V2.3.0) in the software package, Discover (V2.9.5) supplied by Biosym Technologies. The software was run on Iris Indigo workstation supplied by Silicon graphics computer systems. The calculations were performed on adenosine molecule in vacuum. The inter-proton distances derived after complete relaxation matrix calculations were used as restraints allowing ±10% variation in the distance. The energy was minimized using steepest descent method followed by conjugate gradients method available with the software package.

**Results and analysis**

**Binding of ATPMg(II)**

In 1H-NMR spectrum of ATPMg(II), resonances due to H2, H8, H1' (see Figure 1 for nomenclature of various protons in ATP) are observable at 8.51, 8.22 and 6.14 ppm respectively. Binding of ATPMg(II) to BSA results in downfield shift of all the three resonances. The H2 proton resonance of adenine ring exhibits the largest shift. A sample containing 0.50 mM BSA in 25 mM Tris-d1Cl, pH 7.5, and 25 mM MgCl2, was titrated with ATP in the concentration range of 0.43–4.73 mM. The observed change in the chemical shift of a proton was measured in Hz, from the corresponding resonance of ATPMg(II) in the absence of BSA. Largest downfield shift was observable at the lowest concentration of ATP. With increasing concentration of ATP, the resonances tend to shift towards the unbound ATP positions. Such behaviour is characteristic of a fast exchange condition where the rate of exchange of a ligand between bound and free environment is faster than that of the difference in chemical shift in two states. The data from this experiment have been analysed (as described above) to determine the dissociation constant for ligand binding \( (K_d) \) and the change in chemical shift for various protons in the bound form \( (\Delta \omega_M) \). Figure 2 presents the observed change in chemical shift measured from the respective free ligand resonance as a function of mole fraction of the bound ligand. The dissociation constant \( (K_d) \) derived for ATPMg(II) is 2.00 ± 0.15 mM. Downfield shift of H2, H8 and H1' resonances in bound form with respect to free form are 191 ± 12 (0.382 ppm), 76 ± 6 (0.158 ppm), and 66 ± 5 (0.132 ppm) Hz respectively. In a simple case of two site exchange, if \( \omega_A \) and \( \omega_B \) are the resonance frequencies at the two sites (free and bound respectively) in the absence of exchange and \( \tau_A \) and \( \tau_B \) are the corresponding lifetimes at the two sites when exchange occurs, the fast exchange condition can be defined by

![Figure 1. Schematic diagram of adenosine moiety of ATP, indicating the proton numbering system used here.](image)
\[ \omega_A + \omega_B = \Delta \omega_M < \tau_A^{-1}, \tau_B^{-1}. \] Since the \( \tau_B \) is the lifetime of ligand in EL complex, \( \tau_A^{-1} \) represents the rate of dissociation of ligand \( (k_{dA}) \) from the complex. Thus, it can be shown that under fast exchange condition, \( \tau_B^{-1}(k_{dA}) > \Delta \omega_M (2\Delta \omega) \). The largest change in chemical shift of H2-proton in the bound form allows us to set the lower limit for the rate of dissociation of ligand from the complex \( (k_{dA}) \), that is \( k_{dA} \approx 1200 \text{ s}^{-1} \).

**Dependence of NOE on ligand concentration**

Figure 3 presents a typical NOESY spectrum of BSA-ATPMg(II), where several ligand inter-proton NOEs are observable as off diagonal peaks. To observe the site-specific NOEs arising due to interaction of ligand at the site of interest, appropriate sample conditions must be determined\(^8\). We have measured TRNOESY spectra with a mixing time of 120 ms at several different concentrations of the ligand. In each case the ratio of protein to ligand was maintained at 1 : 10. Note that protein concentration will also vary along with the ligand. Separate samples were prepared for each ATP concentration to avoid errors due to dilution. Figure 4 shows a plot of per cent NOE for H'-H2' (distance between these two protons is invariant, irrespective of the nucleotide conformation) as a function of ATP concentration. Initial increase in per cent NOE to \( \approx 2\% \) for a ligand concentration of 5 mM is indicative of gradual saturation of specific nucleotide-binding site. Observed increase in per cent NOE at ligand concentrations >5 mM may arise due to weak binding of ATPMg(II) at some adventitious site(s). Similar behaviour has been observed in case of rabbit muscle creatine kinase\(^8\) and yeast pyruvate kinase\(^9\). From these data it is clear that for the determination of bound nucleotide conformation, measurements must be performed on a sample with a ligand concentration <5 mM.

**Structure-dependent intramolecular NOE measurements**

For the determination of conformation of adenosine moiety bound to BSA, measurements were made on a sample containing 4.0 mM ATP. Such sample composition maximizes the binding of ligand at the specific site and restricts interaction at low affinity non-specific sites.

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*Figure 2. A plot of observed downfield change in chemical shift (Hz) of H2, H8 and H1' protons of ATP measured from the respective free resonances, as a function of bound ATP mole fraction ([BSA-ATPMg(II)]/([ATP]), Sample contained 0.50 mM BSA; 20 mM MgCl\(_2\); 25 mM Tris-(d\(_{3})\)-HCl pH 7.5 and the ATP concentration was varied from 0.43 to 4.73 mM. The spectra were recorded at 500 MHz, 10°C with a digital resolution of 0.35 Hz. The points are experimental and the solid curves are computed with a \( K = 2.0 \text{ mM} \) for BSA-ATPMg(II) complex and the difference in chemical shift for bound and free resonances to be 191 ± 12, 76 ± 6 and 66 ± 5 Hz for H2, H8 and H1' protons respectively.*

*Figure 3. TRNOESY spectrum of BSA-ATPMg(II) complex recorded at 500 MHz and 10°C. The sample contained 0.572 mM BSA; 4.0 mM ATP; 20 mM MgCl\(_2\), in 20 mM sodium phosphate, pH 7.5. The sample volume was 600 \( \mu \)l. NOESY time domain data were collected with 256 \( \times \) 4 increments and 2 K points during each accumulation in \( r_2 \). The mixing time of 160 ms and a relaxation delay of 2 s were used for the spectrum presented here. Two dimensional Fourier transformation was performed along both the dimensions with a Gaussian apodization and zero-filling to obtain 1 K (F1) \( \times \) 2 K (F2) data set. The spectra were phased to pure absorption phase.*
The mole fraction of bound ligand \( (P_b) \) was also within the range \((-0.1-0.25)\) as suggested by Campbell and Sykes\(^{33} \) on the basis of theoretical considerations. The final sample composition chosen for NOE measurements was 0.572 mM BSA, 4.0 mM ATP and 20 mM MgCl\(_2\) in 20 mM sodium phosphate, pH 7.5. Under such sample condition and for a \( K_m = 2 \) mM, \( P_b \) is \(-0.1\). The experimentally measured NOE buildup curves are shown in Figure 5, where per cent NOE is plotted as a function of mixing time for different proton pairs. The points represent experimentally measured values, while the solid curves were theoretically simulated as described below.

NOE buildup curves (shown in Figure 5) were analysed to obtain the inter-proton distances. The data analysis was performed in a similar way as was done for creatine kinase and arginine kinase\(^{18,19} \). Initially, the experimental NOEs were fitted with a second order polynomial in \( r_{nm} \), including \((0,0)\) point as a part of data. Two distinct resonances are observed for H5' and H5'' of free ATP. However, in the presence of Mg(II), these two resonances overlap. In the analysis of data here, these two protons were treated as a single proton and observed cross peak intensities involving H5' and H5'' were divided by a factor of two before fitting them with the polynomial. Using a calibration distance of 2.9 Å for H1'H2' (refs 15, 20) in conjunction with equation (7) and the initial slopes obtained for different proton-pairs, a set of inter-proton distances were determined. NOE data for H1'-H2' with the calibration distance of 2.9 Å, also yields a value of \( P_b r_e \) [see equations (4) and (6)]. These two parameters were separated by determining the \( P_b \) from the known dissociation constant. The set of initial slope distances, \( P_b \) and \( r_e \), obtained in this manner were used as a starter for complete relaxation matrix calculations. The cross peak intensities were calculated for each spin pair as a function of mixing time \( (r_{nm}) \) according to equations (1), (3) and (4). To account for the contribution of free ATP-Mg(II) [see equation (3)], a \( r_e \) of 0.3 ns (ref. 38) and inter-proton distances from an energy minimized structure were used. To obtain a better fit for the data at larger mixing times, a leakage term was added to account for the contributions arising from any external relaxation mechanism (see legend to Figure 5).

The buildup curves computed theoretically on the basis of these parameters were then compared with the experimental data. At this stage any of the parameters, i.e. \( r_e \) and \( r_{nm} \) and leakage terms could be adjusted to obtain the best fit for the experimental data. The bound correlation time, \( r_e \), chosen for the best fit of experimental data was 53 ns. The inter-proton distances determined from the analysis of TRNOE data are given in Table 1 along with the initial slope distances. The solid curves were computed using this set of distances along with other parameters mentioned in the legend of Figure 5.

The distances derived from initial slope approximation and relaxation matrix calculations are plotted in Figure 6 a. It is interesting to note that initial slope approximation underestimates the inter-proton distances which are \(>3.5\) Å. However, distance \(<3.5\) Å shows a good agreement between the two methods.

**Bound nucleotide structure**

The inter-proton distances derived from analysis of NOE data (see Table 1) were used as restraints in energy minimization calculations using Discover (V2.9.5) in conjunction with Insight II (V2.3.0). During minimization, experimentally obtained distances were used as target distances and a variation of \(\pm 10\%\) was allowed. A symmetrical simple harmonic function with a force constant of 50 kcal mol\(^{-1}\) Å\(^{-2}\) was used to restrain the inter-atomic distances within the range. Since there were no distances determined in the triphosphate chain region of ATP, calculations were performed only on adenosine molecule. Distance restraints were applied sequentially\(^{21} \), starting with H8 and all other protons. In the second step, the distances between H1' and all other protons were used. In the third and the final step, all the distances were used. Each time, the energy minimization was performed using conjugate gradient method. The initial structure obtained in this manner fitted well with target distances. However, the energy of such structure was quite high compared to the energy of the free adenosine molecule. To further refine the initial structure, all the distances were used but the force constant was reduced to 25 kcal mol\(^{-1}\) Å\(^{-2}\). This resulted in a structure with significantly lower energy with minor changes in inter-proton distances. Inter-proton distances between

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**Figure 4.** Concentration dependence of per cent NOE for H1'-H2' proton pair in BSA.ATP-Mg(II) complex for a mixing time of 120 ms. The ATP concentration was varied between 2 and 10 mM. The ATP: BSA ratio was kept at 10:1. The sample contained 2–10 mM ATP: 0.2–1 mM BSA; 20 mM MgCl\(_2\) in 20 mM sodium phosphate, pH 7.5. The experimental points are represented by circles and the solid curves represent smoo MED interpolation through the experimental points.
Figure 5. Per cent NOE for different pairs of ATP protons as a function of mixing time for HSA:ATP:Mg(II) complex (64 μl) of the sample containing 0.572 mM BSA, 4.0 mM ATP, 20 mM MgCl₂ in 20 mM sodium phosphate, pH 7.5 was used. Spectral acquisition and processing parameters are described in 'Materials and methods'. The points represent experimentally measured cross peak volumes and the solid curves are the simulations based on the relaxation matrix distances given in Table 1. External relaxation rates for H8, H2, H1', H2', H3', H4', H5 and H5' used in the fitting routine are 4.6, 2.0, 3.33, 3.33, 3.33, 2.86, 4.0 and 4.0 s⁻¹ respectively. The rotational correlation time used for the bound ligand is 53 ms.

Various pairs of protons in energy minimized structure are listed in Table 1. There are three violations in upper bound (between proton pairs H2'-H4', H2'-H5' and H3'-H5') and two in lower bound distances (between proton pairs H2'-H3' and H1'-H5') (see Figure 6 b). Note that in these five violations, three involve H5' whose distance from other protons is difficult to determine accurately. The sum of upper bound violations (SUV) is 1.03 Å and sum of lower bound violations (SLV) is 0.14 Å. Thus the distances in energy minimized structure match reasonably well with those determined by NOE's. The structure of the nucleotide bound to BSA derived above is schematically represented in Figure 7. It has a glycosidic torsion angle \( \chi = 48 \pm 5^\circ \), indicating an anti configuration of the adenine ring with respect to ribose. The values of various dihedral angles for the sugar ring are given in Table 2. In the notation of Altona and Sunderalingam, the phase angle of pseudorotation is about 150° corresponding to an unsymmetrical C2'-endo-C1'-exo twist (\( \phi_T \)) sugar pucker. The amplitude of the sugar pucker, \( \tau \) is about 16°.

Discussion

Binding of ATP to BSA at a specific site was first reported by Bauer et al. Using appropriate spin labelled nucleotide analogues, these authors determined the binding constant for ATP to be in the range of 50-100 μM. Competitive displacement of bound spin probes by stearic acid suggested that the nucleotide binds at the site where the fatty acids interact, BSA is known to have about five fatty acid sites, with a binding constant \( \approx 10^8 \) - \( 10^9 \) M⁻¹. Differential nature of these sites is evident.
Table 1. Inter-proton distances determined for ATPMg(II) bound to bovine serum albumin. The uncertainty in distances is about \( \pm 0.20 \) Å. Also note that distances of H5’ and H5” from any other proton are not determined separately as both protons resonate at the same frequency. Only in restrained energy minimization, this differentiation is made and separate distances are obtained.

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from the fact that ethanol binds only at three of these sites. Ethanol binding fatty acid sites appear to be the same where 1-anillino-8-naphthalenesulfonate (1,8-ANS) also interacts\(^{24,40,41}\). Further differences in binding determinants at these fatty acid sites are evident from the fact that ATP binds at only one of these sites. This region on the surface of BSA is likely to be amphipathic, i.e. aminoacyl residues present at this site are likely to be apolar and cationic. In the experiments presented here, we have determined the dissociation constant for binding of ATPMg(II) with BSA which is about an order of magnitude larger than that of free ATP (ref. 6). At pH > 7, free ATP has four negative charges whereas ATPMg(II) complex has only two. Adenosine 5’-monophosphate (AMP), which has two negative charges (resembles ATPMg(II) in this respect), also binds weakly with a dissociation constant in the millimolar range\(^6\). Binding affinity of a ligand to fatty acid binding region of BSA seems to be critically dependent on the number of negative charges on the lipid molecule. The observation of significant changes in the chemical shifts (see Figure 2) of the adenine and the ribose ring protons, indicate that these two rings along with triphosphate chain are involved in direct interaction with the BSA. Although binding of nucleotide to BSA has no known physiological function, interaction of all three regions of ATP with protein suggests that binding domain may resemble other ATP utilizing proteins. We examined the amino-acid sequence of BSA for the presence of a consensus sequence found in several ATP-binding pro-"
The measurement of TRNOE between the H1'-H2' proton pair of ATPMg(II) as a function of ligand concentration (Figure 4) shows that at the higher concentration of ligand, large contributions to observed NOE may arise due to binding at adventitious site(s). In this respect BSA behaves similar to creatine kinase\textsuperscript{18} and pyruvate kinase\textsuperscript{20}. Experimental evaluation of the extent of non-specific binding is essential for selection of proper sample composition where site-specific TRNOE measurements could be made. Theoretical analysis of TRNOE data collected under appropriate sample conditions (to maximize specific site binding) implicitly involve the following assumptions. Firstly, the fast exchange conditions prevail, i.e. the inverse of the life time of the ligand in the macromolecular complex should be larger than the typical proton–proton cross relaxation rates. Secondly, it is assumed that all proton pairs have a single effective rotational correlation time ($\tau_\text{p}$). It is, therefore, quite instructive to estimate relaxation rates and compare with the ‘off’ rate for ATPMg(II) from the protein complex. The ‘off’ rate for bound ATPMg(II) obtained for BSA. ATPMg(II) complex from chemical shift measurements is $\gg 1200$ s\textsuperscript{-1}. Bovine serum albumin, having a molecular weight of 66000 Daltons, may have a rotational correlation time as large as 60–70 ns. The cross relaxation rate between the two protons is given by $\gamma_\text{H}^2 \tau_\text{p} / 10 \rho$ [see equation (4)]. For an inter-proton distance of 1.90 Å and $\tau_\text{p} = 70$ ns, the cross-relaxation rate is 85 s\textsuperscript{-1}. The cross-relaxation rate computed here is much less than the ‘off rate’ ($k_\text{off}$) of the ligand from the protein complex. Thus, our analysis of TRNOE data using the formulation evolved for fast exchange condition is valid. Further, use of a single rotational correlation time, $\tau_\text{p} = 53$ ns for all the proton pairs is justified by the fact that the binding of nucleotide with protein involves all three regions of ATP. This will rule out any differential segmental motion for the ligand. The correlation time, $\tau_\text{p} = 53$ ns which gives the best fit for the experimental data, is in close agreement with the value computed on the basis of Stokes equation ($\sim 60$–$70$ ns). In most of the ATP utilizing enzymes, $\tau_\text{p}$ obtained from the analysis of TRNOE data are much smaller (by a factor of 2–10) compared to Stokes equation estimates\textsuperscript{18,21,44,45} and this has been attributed to unaccounted spin diffusion in bound complexes.

The final structure determined for ATPMg(II) bound to BSA has a glycosidic torsion angle $\chi = 48 \pm 5^\circ$. This value is in general agreement with that obtained for several ATP-utilizing enzymes, viz. creatine kinase ($\chi = 51 \pm 5^\circ$)\textsuperscript{18}, arginine kinase ($\chi = 50 \pm 5^\circ$)\textsuperscript{19}, pyruvate kinase (active site $\chi = 44 \pm 5^\circ$, ancillary site $\chi = 46 \pm 5^\circ$)\textsuperscript{20}, phosphoribosylpyrophosphate synthetase ($\chi = 50 \pm 5^\circ$)\textsuperscript{21}, Mut T ($\chi = 53 \pm 9^\circ$)\textsuperscript{23} (for AMPPCP). The sugar pucker, which is described by the phase angle of pseudorotation, $p = 149.9^\circ$, determined here is unsymmetrical C2'-endo-
Cl',exo twist (\(\varepsilon\))T. The value of \(\rho\) differs greatly for different enzymes, e.g., 42.4\(^\circ\) for pyruvate kinase (active site), 70.5\(^\circ\) for creatine kinase, 114.9\(^\circ\) for phosphoribosyl pyrophosphate synthetase, 130.8\(^\circ\) for arginine kinase. Although the binding of ATPMg(II) to BSA has no known physiological function, it is interesting to note that the bound nucleotide has a conformation very similar to that at the active site(s) of several ATP-utilizing enzymes.


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