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^{1}H-NMR Studies on Nucleotide Binding to the Sarcoplasmic Reticulum Ca^{2+}ATPase

Determination of the Conformations of Bound Nucleotides by the Measurement of Proton-Proton Transferred Nuclear Overhauser Enhancements

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The glycosidic bond torsion angles and the conformations of the ribose of Mg^{2+}ATP, Mg^{2+}ADP and Mg^{2+}Ado*PP*[NH]*P* (magnesium adenosine 5′-[β,γ-imido]triphosphate) bound to Ca^{2+}ATPase, both native and modified with fluorescein isothiocyanate (FITC), in intact sarcoplasmic reticulum have been determined by the measurement of proton-proton transferred nuclear Overhauser enhancements by ^{1}H-NMR spectroscopy. This method shows clearly the existence of a low-affinity ATP binding site after modification of the high-affinity site with FITC. For all three nucleotides bound to both the high-affinity (catalytic) site and the low-affinity site, we find that the conformation about the glycosidic bond is *anti*, the conformation of the ribose 3′-*endo* of the N type and the conformation about the ribose C4′-C5′ bond either *gauche-trans* or *trans-gauche*. The values for the glycosidic bond torsion angles χ (O4′-C1′-N9-C4) for Mg^{2+}ATP, Mg^{2+}ADP and Mg^{2+}Ado*PP*[NH]*P* bound to the low-affinity site of FITC-modified Ca^{2+}ATPase are $\approx 270°$, $\approx 260°$ and $\approx 240°$ respectively. In the case of the nucleotides bound to the high-affinity (catalytic) site of native Ca^{2+}ATPase, χ lies in the range $240-280°$.

The binding of nucleotides by the Ca^{2+}ATPase of sarcoplasmic reticulum has been the subject of several investigations and interpretations (for a recent review see [1]). Kinetic and binding measurements [2, 3] have shown the presence of a high-affinity site for Mg^{2+}ATP ($K_d = 2\ \mu M$), present of the extent of 4 nmol/mg protein. The steady-state kinetics of ATP hydrolysis, however, do not follow simple Michaelis-Menten kinetics [2–4]: the reciprocal plots are non-linear showing an activation of the hydrolysis at high ATP concentration ($K_{0.5} \approx 0.4$ mM). The observed steady-state kinetics can be explained by three alternative models: (a) weak binding of ATP at a second site [3, 4]; (b) increased activity when two identical sites of a dimer, exhibiting negative cooperativity, are occupied [5]; (c) in a monomeric model, decreased affinity of the phospho-enzyme for ATP, the binding of which accelerates the dephosphorylation [6, 7].

Recent papers of Pick and coworkers [8–10] have shown that fluorescein isothiocyanate (FITC) reacts stoichiometrically in the neighbourhood of the ATP binding site, blocking high-affinity binding of ATP and inhibiting Ca^{2+}ATPase activity, without, however, preventing phosphorylation by inorganic phosphate. Moreover, it was claimed that the reaction of 1 mole of FITC was sufficient to inactivate 2 moles of Ca^{2+}ATPase [8, 10], thus supporting the suggestion of negative cooperativity. We have reinvestigated this (N. M. G. and C. M., unpublished data) and find that these results were probably caused by the presence of inactive Ca^{2+}ATPase in the preparation, and that 1 mole of Ca^{2+}ATPase reacts with 1 mole of FITC. Since it possesses no high-affinity binding site, the FITC-modified Ca^{2+}ATPase can be used to study the putative low-affinity ATP binding site.

In the present paper we have measured the low-affinity binding of ATP to the FITC-inhibited Ca^{2+}ATPase, and have used a recently developed NMR technique, namely the measurement of proton-proton transferred nuclear Overhauser enhancements (TRNOE) [11], to determine the glycosidic bond torsion angles and ribose conformations of nucleotides bound to the low-affinity site of the FITC-modified Ca^{2+}ATPase in intact sarcoplasmic reticulum by NMR spectroscopy. In addition, conformational information on nucleotides bound to the high-affinity site has been obtained by comparison of the results of TRNOE measurements on native Ca^{2+}ATPase with those on FITC-modified Ca^{2+}-ATPase. The TRNOE technique involves the extension of classical nuclear Overhauser enhancement (NOE) measurements [12–20] to exchanging systems such as protein-ligand complexes [21–29] and is ideally suited to study the conformations of ligands bound to very large proteins including membrane-bound proteins such as the Ca^{2+}ATPase as (a) it does not require the observation of individual protein or bound ligand resonances, and (b) its normalised magnitude increases as the molecular weight of the protein or membrane-protein complex increases [11].

EXPERIMENTAL PROCEDURE

Chemicals

ATP, ADP and Ado*PP*[NH]*P* were purchased from Sigma Chemicals Co. Ltd and used without further purification. All other chemicals were of the highest purity commercially available.

Abbreviations. Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; FITC, fluorescein 5′-isothiocyanate isomer I; NOE, nuclear Overhauser enhancement; TRNOE, transferred nuclear Overhauser enhancement.

Enzyme. Ca^{2+}ATPase, calcium and magnesium-independent adenosine triphosphatase (EC 3.6.1.3).

Preparation of Ca^{2+}ATPase

Sarcoplasmic reticulum membrane containing the Ca^{2+}-ATPase was prepared from New Zealand white rabbit back and leg muscles by the method 2 of Meissner et al. [30] and stored at −20 °C in 11% sucrose at a concentration of 30 mg/ml protein.

Preparation of FITC-Modified Ca^{2+}ATPase

The FITC-modified Ca^{2+}ATPase was prepared as follows: 90 µl of 5 mM FITC (isomer I, Molecular Probes Inc., Plano, TX) in dimethylformamide was added to 2 ml of 15 mg/ml sarcoplasmic reticulum protein in 50 mM bicine, 0.5 mM $CaCl_2$, pH 8.8. Allowing for an assayed 70% purity of FITC (*unpublished* results), this corresponds to 10 nmol FITC/mg sarcoplasmic reticulum protein. After 20 min at room temperature, the protein, which showed no Ca^{2+}ATPase activity, was diluted to a volume of 8 ml with H_2O containing 0.1 M KCl and 0.1 mM dithiothreitol, pH 6.8. The vesicles were then spun down for 20 min at $180000 \times g$ and washed twice in this solution.

The FITC-modified Ca^{2+}-ATPase was shown to contain 7.4 nmol FITC/mg sarcoplasmic reticulum protein, using $A^{1\%}_{cm} = 12.0$ for the protein in 1% sodium dodecyl sulphate [31] and an absorption coefficient at 500 nm of 80 mM^{-1} cm^{-1} for FITC bound to protein in 1% sodium dodecyl sulphate and 0.1 M NaOH [32].

Equilibrium Binding of Nucleotides to the Native and FITC-Modified Ca^{2+} ATPase

Samples of the native and FITC-modified Ca^{2+}ATPase, prepared as above, were washed extensively in 50 mM Tris pH 6.9 before being diluted to 10 mg/ml protein in 50 mM Tris, 5 mM $MgCl_2$, pH 6.9. The appropriate nucleotide was then added to 100-µl aliquots of protein to give final nucleotide concentrations in the range 0.02−1 mM. The sarcoplasmic reticulum vesicles were then spun down for at least 60 min at $180000 \times g$ and the absorbance of the supernatant measured to give the final concentration of unbound nucleotide, using $\varepsilon_{260} = 15$ mM^{-1} cm^{-1} [33].

Samples for NMR Spectroscopy

Stock nucleotide solutions (≈ 50 mM) were prepared by dissolving nucleotides in D_2O, adjusting their pH* (meter reading uncorrected for the isotope effect on the glass electrode) to 6.8 using microlitre volumes of 0.1−1.0 M KOD or DCl [> 99 atom % D; Ciba (ARL) Ltd], and finally lyophilising the samples twice from D_2O prior to making up a final solution of ≈ 50 mM nucleotide in D_2O.

Protein samples were prepared by washing the sarcoplasmic reticulum vesicles containing either the native or the FITC-modified Ca^{2+}ATPase four times in D_2O containing 0.1 M KCl, 0.1 mM dithioerythritol, pH* 6.8.

The final concentrations in the samples used for NMR were 10.3 mg/ml protein, 8 mM nucleotide, 57 mM KCl, 57 µM dithioerythritol, 0.1 mM EGTA and 1 mM dioxan, pH* 6.8.

NMR Spectroscopy

^{1}H-NMR spectra were obtained at 270 MHz using a Bruker WH270 spectrometer opearing in the Fourier-transform mode with the pulse sequence $(t_1 - t_2 - \pi/2 - AT)_n$ where the selective irradiation at a chosen frequency was applied during the time interval t_1 (0.5 s), t_2 is a short delay (2 ms) to allow for electronic recovery after removal of the selective irradiation, and AT is the acquisition time (0.487 s). 60 transients, obtained by quadrature detection with 4096 data points for a spectral width of 4.2 kHz, were averaged for each spectrum. Prior to Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz. TRNOE measurements were carried out systematically at 20-Hz intervals throughout the entire sugar proton region of the spectrum. Chemical shifts are expressed relative to internal (1 mM) dioxan (3.71 ppm downfield from 2,2-dimethyl silapentane-5-sulphonate). All experiments were carried out at 20 °C.

RESULTS

Equilibrium Nucleotide Binding

The results of equilibrium binding of ADP to native Ca^{2+}-ATPase and ADP and ATP to FITC-modified Ca^{2+}ATPase are shown in Table 1. The spectroscopic determination of free nucleotide was complicated by light scattering resulting from the presence of residual protein in the supernatant causing large errors at the lowest nucleotide concentrations (< 200 µM). Further, at high nucleotide concentrations, the determination of the concentration of bound nucleotide by difference was also subject to large errors. We nevertheless used the centrifugation method because its speed minimised uncertainty due to hydrolysis of ATP or disproportionation of ADP which might have been significant in an equilibrium dialysis experiment. Bearing these errors in mind, the data show the abolition of high-affinity nucleotide binding clearly seen in native Ca^{2+}ATPase [K_d(ADP) ≈ 25 µM] when the Ca^{2+}ATPase was modified by FITC. The data also show the presence of a low-affinity binding site for ATP and ADP in the FITC-modified Ca^{2+}ATPase, although the magnitude of K_d was uncertain (0.1−0.5 mM). No binding of Ado*PP*[NH]*P* to the FITC-modified Ca^{2+}ATPase was detectable below 1.5 mM nucleotide.

Transferred Nuclear Overhauser Enhancement (TRNOE) Measurements

The proton-proton NOE is the most direct NMR method of conformational analysis in solution as it can be used to demonstrate the proximity in space of two protons and to determine the distance between them [12−20]. The TRNOE involves the extension of NOE measurements to exchanging systems such as protein-ligand complexes [11,21−29]. The theory, principles and applications of the TRNOE have recently been dealt with in detail by Clore and Gronenborn [11] and only the pertinent points will be summarised here.

The basis of the TRNOE involves the use of chemical exchange between the free and bound states of the ligand to transfer magnetic information concerning cross-relaxation between bound ligand protons from the bound state to the free state so that easily detectable negative TRNOE values can be detected on free or averaged ligand resonances folowing irradiation of other free, bound or averaged ligand resonances. For an exchanging ligand-protein system with the protein in the spin diffusion limit ($\omega\tau_c \gg 1$), a negative TRNOE will be observed on the free or averaged resonance of ligand proton i following irradiation of the free, bound or averaged resonance of a ligand proton j providing $k > 10\,\varrho_{iF}$ and $|a\sigma_{i_F j_F}|$

Table 1. *Equilibrium binding of (a) ADP to native Ca^{2+}ATPase, (b) ADP to FITC-modified Ca^{2+}ATPase and (c) ATP to FITC-modified Ca^{2+}-ATPase*

Experimental conditions: 50 mM Tris, 5 mM $MgCl_2$, pH 6.9; protein concentration: (a) 12.5 mg/ml, (b) 8.1 mg/ml and (c) 8.8 mg/ml

Ca^{2+}ATPase + ADP		FITC-Ca^{2+}ATPase + nucleotide		
total ADP	bound ADP	total nucleotide	bound ADP	bound ATP
μM				
19	19	48	15	0
48	48	137	24	–
96	59	150	–	34
143	53	180	46	24
187	73	360	–	31
375	99	450	–	48
470	123	540	36	57
750	136	720	59	83
940	210	900	65	145

$< |(1-a)\sigma_{i_B j_B}|$ where k is the chemical exchange rate between the free and bound states of the ligand, ϱ_{iF} is the total spin-lattice relaxation rate (including cross-relaxation terms) of the free ligand proton i_F, a is the mole fraction of free ligand, and $\sigma_{i_F j_F}$ and $\sigma_{i_B j_B}$ are the cross relaxation rates between protons i and j in the free and bound states respectively [11].

TRNOE measurements on the complexes of Mg^{2+}ATP, Mg^{2+}ADP and Mg^{2+}Ado*PP*[NH]*P* with the FITC-modified and native Ca^{2+}ATPase, carried out by systematic irradiation at 20-Hz (≡ 0.074 ppm) intervals throughout the sugar proton region of the spectrum, are shown in Tables 2 and 3 respectively. The ratio of the concentrations of nucleotide to protein employed in these experiments was approximately 100, corresponding to ratios of free to bound nucleotide concentrations of about 100 and 50 for the FITC-modified and native Ca^{2+}ATPase respectively. Specific negative TRNOE values from the H2′, H3′ and H5′/H5″ sugar protons to the H8 adenine proton were observed for all the cases, and these were maximal on irradiation at positions corresponding to those of the free H2′ (0.95 ppm), H3′ (0.88 ppm) and H5′/H5″ (0.43 ppm) resonances. This is illustrated in Fig. 1 for the Mg^{2+}ATP/native Ca^{2+}ATPase system: irradiation at the position of the free H2′ (0.95 ppm) results in a selective decrease in the intensity of the H8 signal of −26%. Thus chemical exchange between and bound nucleotides must be fast on the chemical shift scale with respect to the H2′, H3′ and H5′/H5″ sugar resonances for the nucleotide complexes with both FITC-modified and native Ca^{2+}ATPase. No TRNOE values were observed on either the adenine H2 or H8 resonances following irradiation of the H1′ resonance, and none were observed on the H2 resonance following irradiation of any of the sugar proton resonances.

In the absence of protein or in the presence of denatured protein in 3 M guanidinium chloride, no NOE values could be detected on either the H2 or H8 resonances following irradiation of any of the sugar proton resonances. It should be noted that positive steady state NOE values from the sugar proton resonances to both the H8 and H2 resonances have been reported for free nucleoside monophosphates [37, 38] under conditions where the expected sum of the steady state NOE values on both the H8 and H2 resonances is at its maximum value of +0.5, namely the use of a low (60-MHz) magnetic field strength to ensure that $\omega\tau_c \ll 1$ and the use of degassed samples containing EDTA to remove any dissolved oxygen and trace paramagnetic impurities which may contribute to the spin-lattice relaxation of the H8 and H2 protons. The latter is particularly important in NOE measurements on

Table 2. *Normalised magnitude of the TRNOE values measured on the resonance of the H8 proton of the adenine ring following irradiation of the resonances of the adenine sugar protons in the presence of FITC-modified Ca^{2+}ATPase together with the distance ratios, values of the glycosidic bond torsion angles and the conformation of the adenine ribose of bound nucleotides*

TRNOE measurements were carried out systematically at 20-Hz (≡ 0.074-ppm) intervals throughout the entire sugar proton region of the spectrum. The ratio of the concentration of nucleotide to protein was approximately 100, corresponding to a molar ratio of free to bound nucleotide of about 100. The total concentrations of nucleotide and protein employed were 8 mM and 10.3 mg/ml respectively. Other experimental conditions are given in Experimental Procedures. Note that no TRNOE values were detected on the resonance of the H2 proton following irradiation of any of the sugar proton resonances. The resonances of the H5′ and H5″ protons are superimposed so that no distinction can be made as to whether the TRNOE to the H8 proton arises from the H5′ or H5″ proton. In calculating the distance ratio $r_{H8-H2'}/r_{H8'-H5'/H5''}$ we have assumed that the conformation about the C4′-C5′ bond exists in only one conformation so that the TRNOE arises from only one of these two protons. The only conformations about the C4′-C5′ bond which are consistent with the observation of a TRNOE on the H8 resonance following irradiation of the H5′/H5″ resonance, are the *gauche-trans* and *trans-gauche* conformations. In the *gauche-gauche* conformation both the H5′ and H5″ protons are too far away (> 0.4 nm) from the H8 proton for a TRNOE to be observed. The notation $N_{H8}(j)$ refers to the TRNOE observed on the resonance of the H8 proton following irradiation of the resonance of proton j. The distance ratios were calculated using Eqn (1). The justification for the use of Eqn (1) is discussed in the text. The errors on the distance ratios are calculated on the basis of a ± 3% error on the TRNOE values. The error on the estimation of χ is about ± 5°. The convention used for defining χ is the standard one adopted by Davies [35]: χ = O4′-C1′-N9-C4. χ is related to the angle χ' defined by Arnott and Hukins [36] and adopted by most crystallographers by the relation $\chi = 360° - \chi'$. From model building it can be shown that for adenine ribose in the 2′-*endo* conformation the ratio $r_{H8-H2'}/r_{H8-H3'}$ has a maximum values of ≈ 0.5 at $\chi \approx 225°$. For a value of 0.5 for this ratio and a value of −17% for the TRNOE on the resonance of the H8 proton following irradiation of the resonance of the H2′ proton, the value of the TRNOE on the resonance of the H8 proton following irradiation of the resonance of the H3′ proton would be −0.27% which is below the limit of detectability under our experimental conditions

Irradiated resonance	Observed resonance	TRNOE of		
		Mg^{2+}ATP	Mg^{2+}ADP	Mg^{2+}Ado-*PP*[NH]*P*
		%		
H1′	H8	0	0	0
H2′	H8	−15	−17	− 6
H3′	H8	−13	−12	−13
H5′/H5″	H8	− 8	− 8	− 8
$\Sigma N_{H8}(j)$		−36	−37	−27
Other parameters				
$r_{H8-H2'}/r_{H8-H3'}$		$0.98^{+0.07}_{-0.08}$	$0.94^{+0.07}_{-0.06}$	$1.14^{+0.18}_{-0.14}$
$r_{H8-H2'}/r_{H8-H5'/H5''}$		$0.90^{+0.09}_{-0.10}$	$0.88^{+0.08}_{-0.09}$	$1.05^{+0.21}_{-0.15}$
χ(O4′-C1′-N9-C4)		~ 270°	~ 260°	~ 240°
Adenine ribose		3′-*endo*	3′-*endo*	3′-*endo*

Table 3. *Normalised magnitudes of the TRNOE values measured on the resonance of the H8 of the adenine ring following irradiation of the resonances of the adenine sugar protons in the presence of native Ca^{2+} ATPase*
TRNOE measurements were carried out systematically at 20-Hz (≡ 0.074-ppm) intervals throughout the entire sugar proton region of the spectrum. The ratio of the concentration of nucleotide to protein was approximately 100, corresponding to a molar ratio of free to bound nucleotide of about 50. The total concentrations of nucleotide and protein employed were 8 mM and 10.3 mg/ml respectively. Other experimental conditions are given in Experimental Procedures. $N_{H8}(j)$ is defined in Table 2

Irradiated resonance	Observed resonance	TRNOE of		
		Mg^{2+}ATP	Mg^{2+}ADP	Mg^{2+}Ado-*PP*[NH]*P*
		%		
H1′	H8	0	0	0
H2′	H8	−26	−24	−22
H3′	H8	−21	−27	−21
H5′/H5″	H8	−15	−11	−12
$\sum_j N_{H8}(j)$		−62	−62	−55

small molecules ($\omega\tau_c < 1$) where the cross-relaxation rates are slow, but is of no significance in TRNOE measurements involving large molecules ($\omega\tau_c \gg 1$) where the cross-relaxation rates are fast [11]. In our experiments a high (270-MHz) magnetic field was used, the samples were not degassed and the radiofrequency field was only applied for 0.55 not allowing sufficient time for a steady state NOE to be built up, thus accounting for the absence of any observed positive NOE values for the free nucleotides in the absence of native protein.

DISCUSSION

Conformation of Nucleotides Bound to the FITC-Modified Ca^{2+} ATPase

The results of both the equilibrium binding studies and TRNOE measurements clearly demonstrate the presence of a low-affinity nucleotide binding site on the FITC-modified Ca^{2+}ATPase and the abolition of the high-affinity site. The loss of the high-affinity site on the FITC-modified Ca^{2+}ATPase has also been shown by Andersen et al. [34]. However, we cannot ascertain from our data whether the low-affinity site is a regulatory site whose occupancy by ATP modulates the activity of the Ca^{2+}ATPase.

In the presence of the FITC-modified Ca^2ATPase, the TRNOE values obtained are characteristic of the *anti* conformation about the glycosidic bond, namely from the H2′, H3′ and H5′/H5″ sugar protons to the H8 proton. To obtain a quantitative estimate of the glycosidic bond torsion angles χ (O4′-C1′-N9-C4) for the bound nucleotides we have calculated the distance ratios $r_{H8-H2'}/r_{H8-H3'}$ and $r_{H8-H2'}/r_{H8-H5'/H5''}$ using the equation

$$r_{i_B k_B}/r_{i_B j_B} = [N_i(j)/N_i(k)]^{1/6} \quad (1)$$

where $r_{i_B k_B}$ and $r_{i_B j_B}$ are the distances from proton i to protons j and k respectively in the bound ligand, and $N_i(j)$ and $N_i(k)$ are the normalised magnitudes of the TRNOE values observed on the resonance of proton i following irradiation of the resonances of protons j and k respectively. The

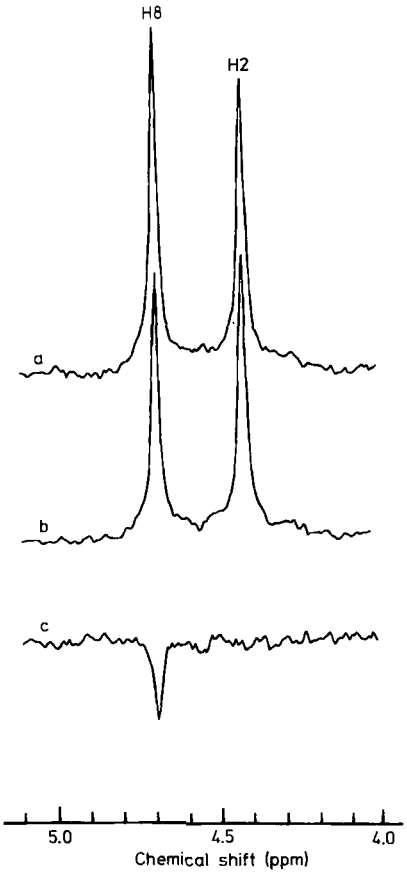

Fig. 1. *The aromatic region of the 270-MHz ^{1}H-NMR spectrum of 8 mM Mg^{2+}ATP in the presence of 10.3 mg/ml native Ca^{2+}ATPase.* The concentrations correspond to a ratio of free to bound nucleotide of about 50. (a) Control irradiation at −0.15 ppm; (b) irradiation of the H2′ sugar resonance at 0.95 ppm; (c) spectrum (b) minus spectrum (a). The free and bound states of Mg^{2+}ATP are in fast exchange on the chemical shift scale so that there is only a single set of averaged ligand resonances whose positions, under these experimental conditions, are at the position of the corresponding resonances of free Mg^{2+}ATP. Other experimental conditions are given in Experimental Procedures. Chemical shifts are expressed relative to internal (1 mM) dioxan which is 3.71 ppm downfield from 2,2-dimethyl silapentane-5-sulphonate

use of Eqn (1) to calculate distance ratios from TRNOE values is justified providing:

$$|a\,\sigma_{i_F x_F}| \ll |(1-a)\,\sigma_{i_B x_B}| \quad (2)$$

where $\sigma_{i_F x_F}$ and $\sigma_{i_B x_B}$ are the cross-relaxation rates between protons i and x in the free and bound states (and x is used to represent protons j and k) and a is the mole fraction of free ligand [11]. As under our experimental conditions no positive NOE values were observed on the H8 resonance following irradiation of any of the sugar proton resonances, either on the free nucleotides or on the nucleotides in the presence of denatured protein in 3 M guanidinium chloride, this relation is satisfied. From the values of the distance ratios, the value of χ (O4′-C1′-N9-C4) can be estimated by simple model building. The values of χ obtained in this manner are ≈ 270°, ≈ 260° and ≈ 240° for Mg^{2+}ATP, Mg^{2+}ADP and Mg^{2+}Ado*PP*[NH]*P*, respectively (see Table 2). In addition, the distance ratios obtained (see Table 2) are only consistent with a 3′-*endo* conformation of the N type for the ribose. The observation of a TRNOE from the H5′/

H5″ protons to the H8 proton also indicates that the conformation about the C4′-C5′ bond is either *gauche-trans* or *trans-gauche*; we cannot distinguish between these two possibilities as the resonances of the H5′ and H5″ protons are superimposed.

These results contrast with the situation for 5′-nucleotides in free solution which exist as a mixture of various conformers with a predominance of the *anti* conformation about the glycosidic bond, the 2′-*endo* conformation of the S type for the ribose and the *gauche-gauche* conformation about the C4′-C5′ bond [35].

Conformations of Nucleotides Bound to Native Ca^{2+}ATPase

A comparison of the normalised magnitudes of the TRNOE values observed on the nucleotides in the presence of native Ca^{2+}ATPase (Table 3) with those observed in the presence of the FITC-modified Ca^{2+}ATPase (Table 2) shows that the values of the former are 1.5–2 times as large as those of the latter, due to the presence of an additional site, namely the catalytic site. As in the case of the FITC-modified Ca^{2+}ATPase, the only TRNOE values observed are characteristic of the *anti* conformation, namely from the H2′, H3′ and H5′/H5″ proton to the H8 proton. A quantitative estimate of the glycosidic bond torsion angle χ for the nucleotides bound in the catalytic site, however, cannot be obtained, as the contributions of the nucleotides bound in the two sites, the catalytic and low affinity sites, are not strictly additive. Nevertheless, a range of χ between 240° and 280° for the nucleotides bound in the catalytic site can be estimated on the basis that when the TRNOE values on the H8 resonance obtained with the FITC-modified Ca^{2+}ATPase are subtracted from those with the native Ca^{2+}ATPase we find that the differences on irradiation of successive sugar proton resonances decrease in the order H2′ $\gtrsim$ H3′ > H5′. Moreover, the increase in the magnitude of the TRNOE from the H3′ to the H8 proton in the presence of native Ca^{2+}ATPase relative to that obtained in the presence of FITC-modified Ca^{2+}ATPase indicates that the conformation of the ribose for the nucleotides bound in the catalytic site is also 3′-*endo*. Similarly, from the increase in the magnitude of the TRNOE from the H5′/H5″ proton to the H8 proton in the presence of native Ca^{2+}-ATPase relative to that obtained in the presence of the FITC-modified Ca^{2+}ATPase we deduce that the conformation of the C4′-C5′ bond for the nucleotides bound in the catalytic site is either *gauche-trans* or *trans-gauche*.

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