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^{1}H-NMR studies on nucleotide binding to the catalytic sites of bovine mitochondrial F_1-ATPase

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The conformation of adenine nucleotides bound to bovine mitochondrial F_1-ATPase was investigated using transfer nuclear Overhauser enhancement measurements. It is shown that all nucleotides investigated adopt a predominantly anti conformation when bound to the catalytic sites. Furthermore, the experiment suggests that 8-azido-ADP and 8-azido-ATP, which are predominantly in the syn conformation in solution, are in the anti conformation when bound to F_1 catalytic sites.

F_1-ATPase; Nucleotide; Bound conformation; NOE transferred

1. INTRODUCTION

The conformations of free 5′-adenine ribonucleotides in solution have been extensively studied by proton NMR spectroscopy [1–3]. A favored anti orientation of the base ring with respect to the ribose ring was found (fig.1). However, it was shown that bulky substituents (Br, CH_3S–) at the 8 position of the adenine ring shift the normal anti/syn equilibrium towards a predominantly syn form (fig.1) [4]. An important step in the understanding of an enzymatic mechanism is the elucidation of the substrate conformation when it is bound to the enzyme. In the case of bovine mitochondrial H^+-F_1-ATPase, a knowledge of the adenine nucleotide conformation when bound to the enzyme is of particular interest since the results of photolabelling experiments obtained either with 8-N_3ATP [5] or with 2-azido-ADP and 2-azido-ATP [6], have been interpreted in terms of hypothetical conformations of bound nucleotides at the F_1 catalytic sites [6]. Three exchangeable nucleotide binding sites (or catalytic sites) are present on F_1 [7]. We used proton–proton TRNOE measurements to study the nucleotide conformations of various adenine nucleotides bound at these sites. The TRNOE technique involves the extension of classical NOE measurements to exchanging systems such as protein-ligand complexes [8,9], and is ideally suited to study the conformations of ligands bound to very large proteins such as the F_1-ATPase (M_r 371135) [10].

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Abbreviations: AdoPP(NH)P, adenosine 5′-[β,γ-imido]triphosphate; NOE, nuclear Overhauser enhancement; TRNOE, transferred nuclear Overhauser enhancement

2. MATERIALS AND METHODS

2.1. *Chemicals*

ATP, ADP, and AdoP(NH)P were purchased from Boehringer Mannheim, 8-azido-ADP and 8-azido-ATP from Sigma.

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All other chemicals were of the highest purity commercially available.

2.2. *Preparation of mitochondrial F_1-ATPase*

Beef heart mitochondrial F_1 was prepared according to the method of Knowles et al. [11] modified by Klein et al. [12], and was stored at 4°C as an ammonium sulfate precipitate.

2.3. *Samples for NMR spectroscopy*

Nucleotides and nucleotide analogs were lyophilized twice to reduce the concentration of H_2O.

The F_1 suspension was centrifuged; the pellet was solubilised in a buffer consisting of 25 mM potassium phosphate, 50 mM KCl, 10% methanol, final pH 7.5, and desalted by chromatography on an ACA 202 column (IBF) equilibrated with 10 mM potassium phosphate, 20 mM KCl, 10% methanol, final pH 7.5. Methanol was introduced in the medium to stabilize the enzyme in the absence of any added nucleotide (Lunardi, personal communication). Desalted F_1 contained 3.5 ± 0.3 mol of nucleotides (ATP plus ADP) per mol of enzyme. 2 ml fractions were lyophilized separately, solubilized with D_2O and lyophilized again. The ATPase activity was tested with an ATP regenerating medium at 30°C: after the two lyophilization steps it still ranged between 55 and 75 μmol of ATP hydrolyzed per mg of F_1 per min. The final concentrations in the samples (final volume 500 μl) used for NMR were 55 μM protein, 4 mM nucleotide, 55 μM potassium phosphate, 40 mM KCl and either 2 mM Mg^{2+} or 2 mM EDTA.

2.4. *NMR spectroscopy*

^{1}H-NMR spectra were recorded at 500 MHz on a Bruker AM 500 spectrometer at 25°C. The TRNOEs were observed by directly collecting the difference free induction decay by interleaving 8 transients after saturation for a time t of a given resonance with 8 transients of off-resonance saturation applied for the same length of time. The spectra were recorded with a 90° observation pulse, a 0.5 s acquisition time and a 1 s relaxation delay. The irradiation power used was sufficient to be in the high power limit, ensuring that saturation was effectively instantaneous whilst preserving selectivity. 800 transients were collected for each difference spectrum.

3. RESULTS AND DISCUSSION

The theory of the TRNOE has been described in detail [8,9] and only the pertinent points will be summarized here. The TRNOE is based on the use of chemical exchange to transfer information concerning cross-relaxation from the bound to the free state, thereby enabling effects from the bound state to be easily observed on the free or averaged ligand resonances. When exchange is fast on the chemical shift scale, the observed cross-relaxation rate between ligand protons in the presence of protein is simply the weighted average of the cross-relaxation rates in the free and bound states. The free ligand lies in the $\omega\tau_c < 1$ region (where ω is the spectrometer frequency and τ_c the correlation time) such that it exhibits positive NOEs in the free state. Because the values of cross-relaxation rates of the free ligand are very small it is a simple matter to obtain conditions where no positive NOEs are observed in the absence of protein (i.e. a short irradiation time in a one-dimensional experiment). The ligand-protein complex, on the other hand, lies in the $\tau_c \gg 1$ region, such that it exhibits negative NOEs. The cross-relaxation rates of the bound ligand are very large; thus, when ligand and protein are mixed with ligand in large excess, negative TRNOEs arising solely from cross-relaxation in the bound state, will be observed between averaged ligand resonances. Because the NOE is proportional to r^{-6} at short irradiation times, it follows that the TRNOE provides a sensitive probe of conformation of the bound ligand.

Because of the spectral simplicity of the nucleotides, we chose to perform the TRNOE experiments by simple one-dimensional methods, irradiating an averaged ligand resonance for a given period of time (0.2–0.3 s) and observing the effects on other averaged ligand resonances. Typically, the ratio of free to bound ligand was 25:1.

We systematically irradiated the proton's sugar resonances in turn and observed effects on the H8 and H2 base resonances. For all the nucleotides examined (ADP, ATP and AdoPP(NH)P), both in the presence and absence of Mg^{2+}, no TRNOEs could be observed on the H2 resonance following irradiation of the H1′, H2′, H3′, H4′, H5′, and H5′′ sugar resonances whereas noticeable TRNOEs were observed on the H8 resonance following irradiation of the H2′, H3′ and H5′/H5′′ sugar resonances (table 1, fig.2). These data indicate that the H8 proton has to be close to the H2′ and H3′ sugar protons, which is only the case for an anti conformation about the glycosidic bond (fig.1). The large size of the TRNOE between

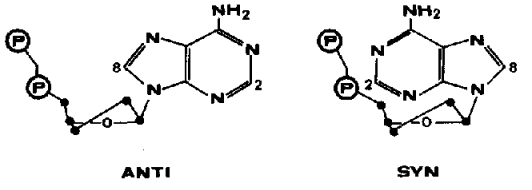

Fig.1. Schematic representation of the anti and syn conformations of ADP.

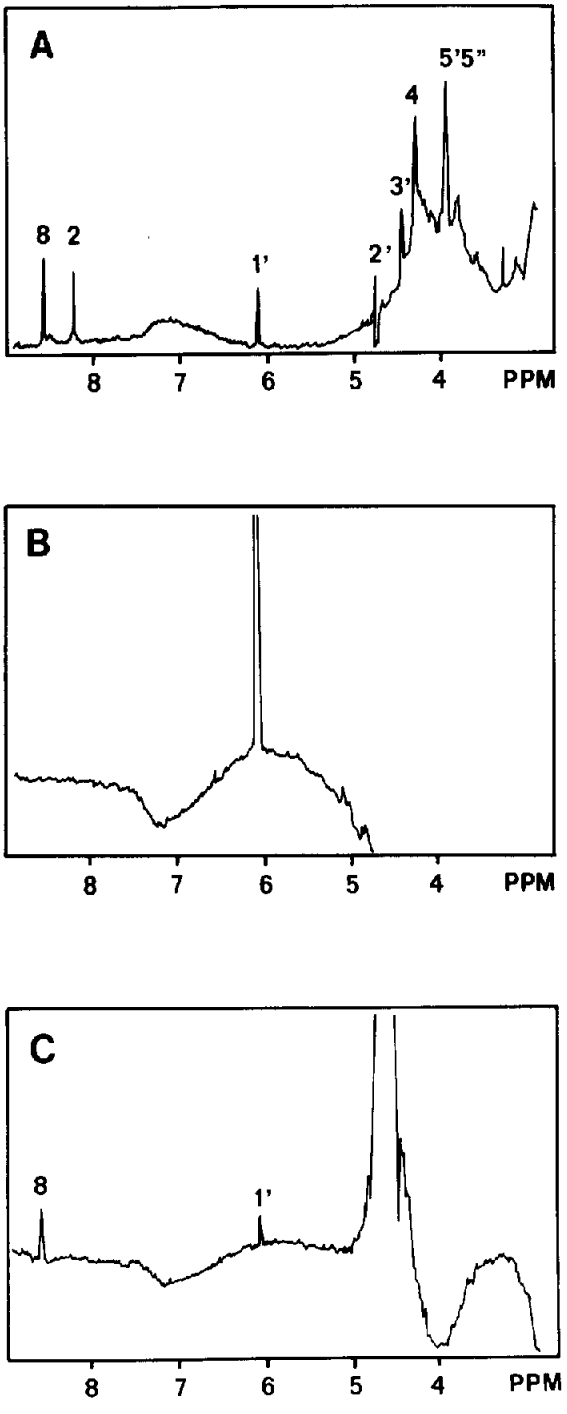

Fig.2. (A) Proton NMR spectrum of MgADP in the presence of mitochondrial F_1-ATPase. (B) Difference spectrum after irradiation of the H1′ resonance. (C) Difference spectrum after irradiation of the H2′ resonance.

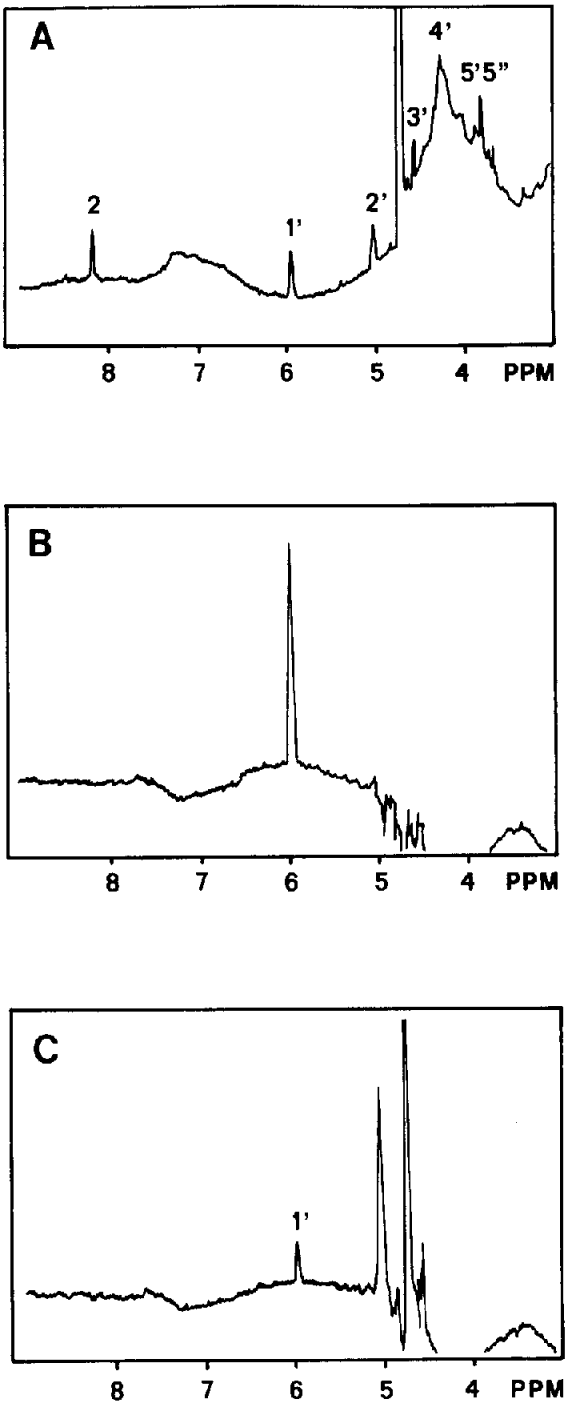

Fig.3. (A) Proton NMR spectrum of Mg/8-azido-ADP in the presence of mitochondrial F_1-ATPase. (B) Difference spectrum after irradiation of the H1′ resonance. (C) Difference spectrum after irradiation of the H2′ resonance.

Table 1

Normalized TRNOE values for various nucleotides bound to mitochondrial F_1 (all TRNOE values are normalized to H1′–H2′ = 1)

	ADP/Mg^{2+}	ADP/EDTA	ATP/EDTA	$8N_3$ADP/Mg^{2+}	$8N_3$ATP/EDTA	AdoPP(NH)P/Mg^{2+}
H1′–H8	0.0	0.0	0.2	0.0	0.0	0.4
H2′–H8	3.3	3.7	0.9	0.0	0.0	0.7
H3′–H8	1.2	1.4	0.7	0.0	0.0	0.9
H5′/H5′′–H8	1.0	1.1	0.2	0.0	0.0	0.7
H1′–H2′	1.0	1.0	1.0	1.0	1.0	1.0
H1′–H4′	0.8	1.5	0.9	1.0	1.0	0.9
H1′–H2	0.0	0.0	0.0	0.0	0.0	0.0
H2′–H2	0.0	0.0	0.0	0.0	0.0	0.0
H3′–H2	0.0	0.0	0.0	0.0	0.0	0.0
H5′–H5′′	0.0	0.0	0.0	0.0	0.0	0.0

the H2′ and the H8 resonances for ADP is indicative of a 2′-endo sugar pucker conformation, while in the case of ATP and AdoPP(NH)P both 2′-endo or 3′-endo conformations are possible. The same conformation of bound ADP was found for TF_1, the enzyme of the thermophilic bacterium PS_3.

It is of interest to recall that adenine nucleotides have also been shown to bind in an anti conformation on several enzymes including sarcoplasmic reticulum Ca^{2+} ATPase, muscle adenylate kinase, pyruvate kinase and creatine kinase [13–16].

In solution, the photoactivable adenine nucleotide analog 2-azido-ADP is reported to adopt preferentially an anti conformation [17] as the natural nucleotides do [2]. The fact that 2-azido-ADP binds to the exchangeable nucleotide binding sites of mitochondrial F_1 with a high affinity (K_d = 5 μM) similar to the affinity of ADP (K_d = 2–4 μM) [18] is consistent with the preferential adoption of the anti conformation by the photoprobe in solution. It was, however, not possible to carry out meaningful TRNOE measurements on 2-azido nucleotides since several tautomeric forms are present under the conditions used (tetrazolo forms, [17]).

Free 8-azido-ADP, on the other hand, most likely adopts a syn conformation in solution (fig.1) in analogy to other nucleotides containing a bulky group at the 8 position of the adenine ring [4]. Thus, it was of interest to investigate the conformation of 8-azido-ADP/ATP bound at the catalytic sites of mitochondrial F_1. No NOEs were observed on the H2 resonance following irradiation of any of the sugar protons (table 1, fig.3), making it most probable that 8-azido-ADP and 8-azido-ATP are also bound in the anti conformation despite their propensity to adopt a syn conformation in solution. This can easily occur if the sugar pucker conformation is 2′-endo [19]. This possibility would explain two previous findings. (i) The low affinity of the F_1 catalytic sites for 8-azido-ADP under turnover conditions [20] could be due to the necessity to select the unfavored anti conformation from the predominant syn conformation upon binding since the catalytic sites of F_1 are highly specific of the anti conformation. (ii) The labelling of different regions of F_1 by 8-azido-ATP (β Tyr 345 region [6]) may not necessarily be attributed to different conformations adopted by the two photoprobes in the catalytic crevice; on the contrary, it may well be that both 8-azido-ATP and 2-azido-ADP/ATP bind in the same anti conformation, but the different localisation of the azido reactive group on the adenine ring (either the 8 or the 2 position) leads to the specific labelling of two opposite walls of the F_1 catalytic crevice. Thus, the TRNOE results and their explanation support the previously proposed model for the structure of the F_1 catalytic site [6].

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