5.293(1), $c = 13.741(3)$ Å, $\beta = 108.69(4)°$, $V = 885.3(5)$ Å^3, $Z = 2$, $\rho_{calcd} = 1.371$ g cm^{-3}, 4036 measured reflections, 2022 symmetry-independent reflections, of which 1858 were observed ($I = 2\sigma(I)$), $R = 0.046$, wR^2 (all data) = 0.096, 239 parameters, Flack parameter 0.01(3). Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-101627. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336033; e-mail: deposit@ccdc.cam.ac.uk).

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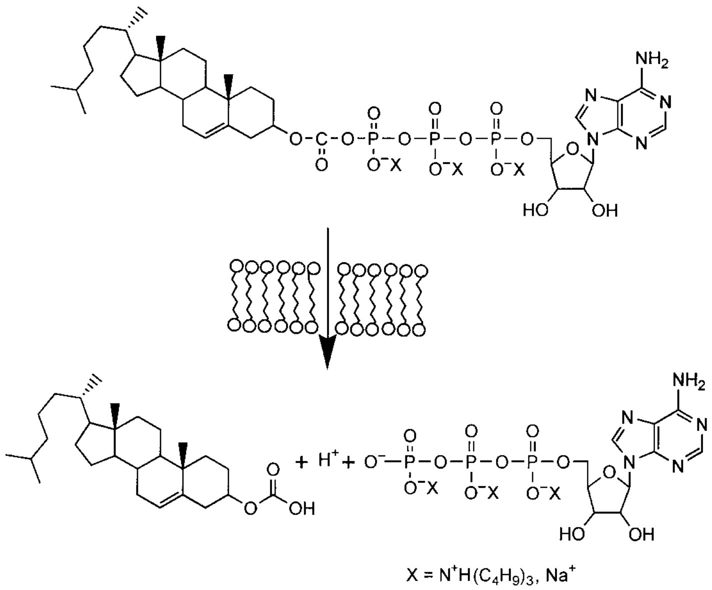
Transmembrane Transport of Adenosine 5′-Triphosphate Using a Lipophilic Cholesteryl Derivative**

Annett Kreimeyer, François André, Catherine Gouyette, and Tam Huynh-Dinh*



Scheme 1. Schematic representation of the transfer of Chol-ATP across the membrane and the release of ATP by hydrolysis.

Numerous studies to find prodrugs of nucleoside 5′-monophosphates have been reported.[1] However, little work has been described on synthetic carrier systems for nucleoside 5′-triphosphates.[2] To our knowledge no nucleotide derivatives currently exist that are capable of effecting the transfer across membranes with subsequent release of the parent nucleoside 5′-triphosphate. For instance a phospholipid ester of 3′-azido-3′-deoxythymidine (AZT) 5′-triphosphate has been shown to be an effective anti-HIV agent in vitro, but required liposomal formulation; its intracellular hydrolysis led to AZT 5′-monophosphate instead of AZT 5′-triphosphate.[3]

We have recently described the synthesis of acyl nucleoside 5′-di- and 5′-triphosphates as potential membrane-permeable prodrugs.[4, 5] Further development of this work led us to synthesize derivatives of adenosine 5′-triphosphate (ATP). Herein, we present the results of a ^{31}P NMR based study of the transfer of cholesteryloxycarbonyl-ATP (Chol-ATP) (Scheme 1) as a model of a lipophilic nucleotide.

Chol-ATP was synthesized in one step from ATP and cholesteryl chloroformate.[5] A suitable method to investigate the transmembrane transport of liponucleotide conjugates is ^{31}P NMR spectroscopy since it allows both the monitoring of ATP release after internalization of Chol-ATP into liposomes and the distinction between external and internal species in a well-compartmented system.[6, 7] Moreover, ^{31}P NMR resonances are highly sensitive probes of local pH changes.[8] Our approach consists of recording ^{31}P NMR spectra of Chol-ATP in the presence of small unilamellar vesicles (SUV) in a phosphate-buffered system using a pH gradient to distinguish between resonances of ATP molecules located inside and/or outside the liposomes.

Essential requirements of such an experiment are that the compound of interest does not act as a detergent damaging the liposomal structure, and that its hydrolysis kinetic is compatible with the incubation time in the presence of the vesicles.[9] In order to observe the resonances of ATP at different pH values, the liposomes must be able to maintain the proton gradient (ΔpH) across the membrane within the required acquisition time of the ^{31}P NMR spectrum (one hour). To monitor Chol-ATP transport through membranes and the subsequent ATP release, the specific pattern of a ^{31}P NMR spectrum of ATP entrapped in liposomes was first characterized. For this purpose, we prepared vesicles containing ATP. Figure 1 shows ^{31}P NMR spectra of ATP (internal concentration 50 mM) entrapped in liposomes prepared in a phosphate-buffered medium recorded at different times. The comparison of these spectra provided three important pieces of information: a) The appearance of an inorganic phosphate peak, at a resonance frequency identical to the initial step before the pH jump, indicates the preservation of the pH 5 in the inner volume of the liposomes during the acquisition time. This, and the fact that no extravesicular ATP resonances occurred, demonstrates the integrity of the vesicles (Figure 1b). b) After the pH jump the integral value for the two inorganic phosphate resonances gives an accurate determination of the distribution between the inner (6%) and outer volume (94%). As a result, the concentration of internal ATP was equivalent to 3 mM with respect to the total volume. c) A direct comparison of the lineshapes of intra- and extravesicular ATP resonances, while

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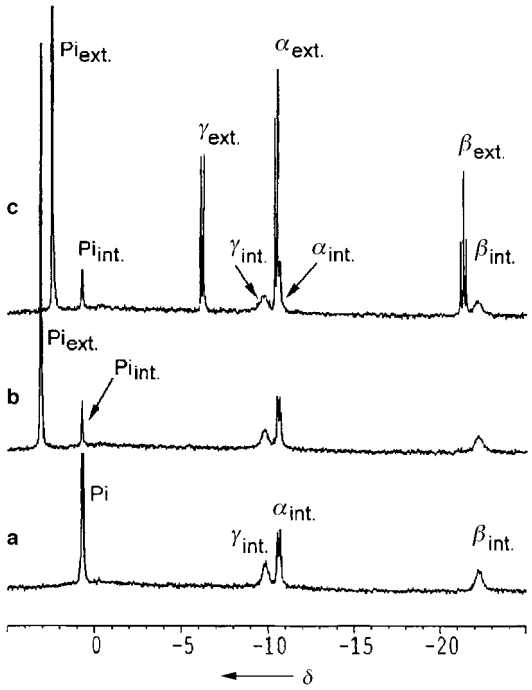

Figure 1. ^{31}P NMR spectra (121.49 MHz) of free ATP, entrapped in liposomes in a phosphate-buffered solution at 25 °C: a) Equilibrated at pH 5; b) after addition of aqueous NaOH, external pH set at pH 8—no phosphorus lines of external ATP were detected; c) after addition of 3 mM ATP extravesicular. The addition of stock ATP modified the extravesicular pH, responsible for a slight shift of the corresponding inorganic phosphate resonance. int. = intravesicular; ext. = extravesicular; Pi = inorganic phosphate.

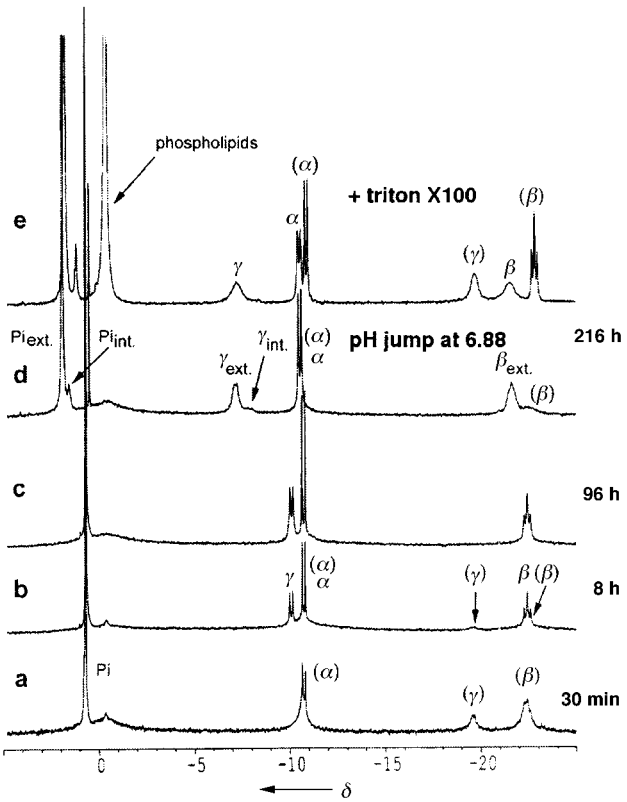

Figure 2. ^{31}P NMR spectra (121.49 MHz) of Chol-ATP incubating in a phosphate-buffered solution containing the liposomes (lipid content 32 mg mL^{-1}) recorded after a) 30 min incubation time at pH 5, b) 8 h at pH 5, c) 96 h at pH 5, d) pH jump to pH 6.88 after addition of aqueous NaOH at 216 h incubation time, and e) addition of Triton X100. Chol-ATP signals are quoted in parentheses; int. = intravesicular; ext. = extravesicular.

both species have been set at equivalent concentrations, revealed that the internal ATP phosphorus resonances were considerably broadened (Figure 1c). Moreover, the broadening was much more marked for β- and γ-phosphate resonances than for α ones. It is expected that the observation of free ATP internalized in liposomes will be very difficult.

To study the transmembrane transport of Chol-ATP the compound was incubated for nine days with SUV, which did not contain entrapped Chol-ATP, and monitored by ^{31}P NMR spectroscopy (Figure 2). The lifetime of the compound was also controlled by ^{31}P NMR spectroscopy in the same buffered medium (pH 5) in the absence of liposomes ($t_{1/2} = 69$ h). The total disappearence of the γ-phosphate signal of Chol-ATP after 96 h can be explained by an association of the molecule in the water–membrane interface. Moreover the absence of any sharp resonance of phospholipids provided evidence that there was no destruction of the liposomal structure due to Chol-ATP. When aqueous NaOH was added in the NMR tube the resonances of ATP, as well as of inorganic phosphate (both extravesicular), were shifted (Figure 2d). During the required acquisition time of the ^{31}P NMR spectrum the adjustment of the pH between the different compartments started. This induced a pH-dependent shift of the resonances of ATP and consequently they were broadened. Considering the typical large pattern of entrapped ATP, one can assume that under these conditions the corresponding signals could be broadened beyond detection. Despite these shortcomings, a weak signal centered at $\delta = -8.2$ was observed (Figure 2d). This resonance was in agreement with the predicted chemical shift of the γ-phosphorus of entrapped ATP, calculated through the pH value which was indicated by the resonance of the internal inorganic phosphate peak. Thus, the release of ATP from Chol-ATP, inside the liposomes, can be assumed. To confirm this result, we added the detergent Triton X100 to the solution to break the liposomal structure. Consequently, the signals of the phospholipids appeared, indicating their micellization. Hence, the ΔpH was neutralized and the resonances of extra- and intravesicular phosphorus coalesced. The areas of the signals of the β- and γ-phosphate groups of the total free ATP (Figure 2e) increased by 10% after the detergent addition, when compared to the area of the signal for free external ATP (Figure 2d); this increase can be considered as the amount of entrapped ATP. Concomitantly, the signal of the γ-phosphate of residual intact Chol-ATP (Figure 2e) reappeared.

As a further proof, we checked the evolution of the ΔpH between the compartments during Chol-ATP internalization. It is indeed expected that the hydrolysis of Chol-ATP leads to the release of protons (Scheme 1). In a control experiment, a pH jump in the same phosphate-buffered system containing only liposomes was applied and the development of the proton gradient was monitored by ^{31}P NMR spectroscopy over a long period (Figure 3A). In contrast to the decreasing ΔpH value in the control experiment, an initial increase of the

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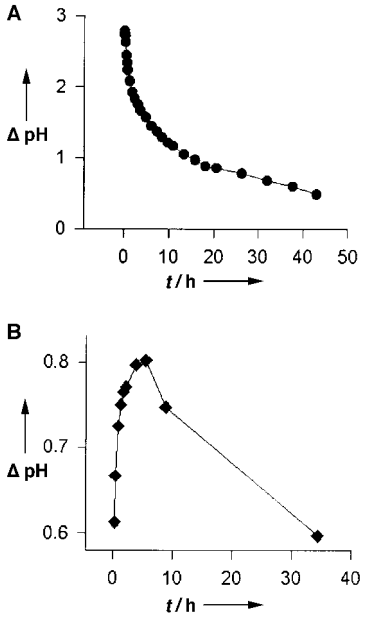

Figure 3. Evolution of the proton gradient between the two compartments after a pH jump in solutions (A) containing only liposomes and (B) containing liposomes and Chol-ATP.

proton gradient in the presence of Chol-ATP was observed, reflecting the hydrolysis of Chol-ATP and thus the release of the intravesicular ATP (Figure 3B).

In summary, we have demonstrated that ATP bearing a cholesteryl moiety at the γ-phosphate group can be transported across the membrane bilayer. It is now of interest to evaluate these model drug carriers of adenosine and various therapeutic nucleosides in integrated systems or living cells.

Experimental Section

Cholesteryloxycarbonyl-adenosine 5′-triphosphate: White powder. The ^{1}H NMR spectrum (D_2O) showed the characteristic signals of adenosine; protons of the cholesteryl moiety were observed between $\delta = 2.05$ and 0.57. ^{31}P NMR (D_2O, proton-decoupled, pH 7.1): $\delta = -10.35$ (d, α-P, $J_{PP} = 19.8$ Hz), -18.82 (br, γ-P), -21.51 (br, β-P). Mass spectrum (electrospray): calcd for $C_{38}H_{60}N_5O_{15}P_3(NBu_3)_3$: 919.33; found 919.4. HPLC (gradient: 5–70% acetonitrile in 0.01M triethylammonium acetate, pH 7): t_{ret} 17.04 min. Chol-ATP ($HNBu_3$ or Na salt) is freely soluble in water and organic solvents (dichloromethane, acetonitrile, alcohols).

Vesicles preparation: SUV of defined size were prepared by reverse-phase evaporation using a mixture of egg phosphatidylcholine and phosphatidic acid (mole ratio 9:1) according to Rigaud and Pitard.[10] The phosphate-buffered solution referred to in the text contained KH_2PO_4 (20mM), K_2SO_4 (15mM), and Na_2SO_4 (15mM) at pH 5.0 (90% H_2O/10% D_2O). After extrusion through 200-nm nucleopore membranes, the final lipid concentration was about 30 mg mL^{-1}. For the preparation of liposomes with entrapped ATP, the same procedure was applied in the presence of ATP 50mM, and external ATP was finally thoroughly removed by filtration of the SUV through pD 10 columns (Pharmacia).

^{31}P NMR experiments: ^{31}P NMR spectra were recorded on a Bruker DMX-300 operating at 121.49 MHz, and referenced relative to external H_3PO_4 85%. Two levels of broad band proton decoupling were applied for all the experiments. Samples were prepared immediately prior to use. In a glass vial, Chol-ATP (9 mg) was directly dissolved in the SUV preparation (550 µL, lipid concentration 30 mg mL^{-1}, phosphate-buffered solution pH 5.0, 90% H_2O/10% D_2O). After quick introduction into the spectrometer, an initial ^{31}P NMR spectrum ($t = 0$) was recorded (90° pulse 6 µs, relaxation delay 5 s, 16 scans). In general, successive short spectra (16 to 64 scans) were acquired at the beginning of the incubation to monitor the pH gradient development. Better signal-to-noise spectra (512 scans) were then recorded to monitor the slow kinetics of internalization and hydrolysis of Chol-ATP. The pH jump was performed by injection of a few microliters of NaOH 1N into the NMR tube.

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Ca_2AuN: A Nitride Containing Infinite Zigzag Gold Chains**

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Previous investigation of the calcium–gold–nitrogen ternary phase field[1] resulted in the characterization of Ca_3AuN,[2] which crystallizes with the cubic perovskite structure. This structural type is well represented in nitride chemistry, for example Ca_3XN (X = P, As, Sb, Bi, Ge, Sn, and Pb).[3] The nitrogen atom in these compounds is surrounded

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