The Role of a Second Magnesium Ion in APH(3')-IIIa

Paul Benjamin Pruett

University of Tennessee - Knoxville

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Appendix D - UNIVERSITY HONORS PROGRAM
SENIOR PROJECT - APPROVAL

Name: Paul Pruett

College: Arts & Science
Department: BCMB

Faculty Mentor: Engin H. Serpentsu

PROJECT TITLE: The Role of a Second Magnesium Ion in APH(S')-IIa

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Engin Serpentsu, Faculty Mentor

Date: 5/9/00

Comments (Optional):
The Role of a Second Magnesium Ion in APH(3′)-IIIa

Paul B. Pruett
Faculty Mentor: Dr. Engin H. Serpersu

Abstract

Due to the overprescription and misuse of antibiotics, an environment has been created which has allowed a number of resistant organisms to flourish. These organisms mediate their resistance through a variety of means. Those organisms which have become resistant to the aminoglycoside class of antibiotics carry out their resistance through one of three classes of modifying enzymes. These classes are phosphotransferases, nucleotidyl transferases, and acetyl transferases. The aminoglycoside phosphotransferases (APH) detoxify an aminoglycoside by adding a phosphate to the antibiotic from an ATP molecule. APH(3′)-IIIa is one of the most well studied phosphotransferases due in fact to its crystal structure having been solved. In this study, we make use of some of the exchange-inert metal-ATP analogues. Using these, we have shown that a second metal ion in the crystal structure of APH(3′)-IIIa does not play a role in catalysis or ATP binding.
Introduction

The resistance to antibiotics is a growing problem in this country and throughout the world. Through such means as conjugation and transfection, bacteria are able to transfer R-plasmids that carry genes for various resistance enzymes. This has allowed for the rapid spread of resistance enzymes through a number of organisms. It is critical that scientists understand how these enzymes work in order to combat their action.

The aminoglycoside antibiotics are carbohydrate-based molecules that kill bacteria by terminating protein synthesis. These antibiotics mediate this by binding to the 30S subunit of the ribosome and thereby preventing the initiation of protein synthesis (Tanaka, 1982). Unfortunately, soon after these new antibiotics were discovered, resistant organisms soon appeared. It has now been characterized that there are three classes of enzymes that detoxify aminoglycoside antibiotics by modifying them. These classes are the nucleotidyl transferases (ANT), acetyl transferases (AAC), and the phosphotransferases (APH) (Shaw et al., 1993).

The enzyme of focus in this study is APH(3')-IIIa. As indicated by the nomenclature, this enzyme catalyzes the transfer of the γ-phosphate of an ATP molecule to the 3' hydroxyl of an aminoglycoside. This enzyme is well-studied due to the fact that Hon and colleagues (1997) have solved the crystal structure for this enzyme. This has given researchers a type of road map to direct future research. In the case of this study, crystallography determined that there are two magnesium ions in the active site of APH(3')-IIIa. One of the magnesium ions is coordinated to the β and γ phosphates of ATP. The role of the second metal ion was unknown. This study uses the aid of exchange-inert Metal-ATP complexes to elucidate the role of this second magnesium ion.
The magnesium, the ion normally coordinated to an ATP molecule at normal physiological conditions, exchanges at a very high rate and is spectroscopically very uninteresting. In order to study the role metal ions play in certain enzymes, magnesium can be replaced with a metal ion that does not exchange its ligands at a high rate (Dunaway-Mariano and Cleland, 1980). These exchange-inert Metal-ATP complexes have been used to study a number of enzymes in this and other labs (Serpersu et al., 1992; Pappu et al., 1994; Dunaway-Mariano and Cleland, 1980). By using these complexes in this study, one can specify one of the two metal ions present. Then, the second ion can be added, and the effect on inactivation or activity can be ascertained.

This study makes use of both CrATP and RhATP. CrATP was used in this study to look at the role of the second magnesium in MeATP binding. Previous unpublished research in this lab has shown that CrATP inactivates APH(3')-IIIa in a pseudo-first order reaction. This inactivation can be modeled to the normal binding of ATP to the enzyme. Any effects added magnesium has on inactivation can be connected to its effects on ATP binding. The paramagnetic nature of chromium precludes its use in NMR. This is why RhATP, a diamagnetic Metal-ATP complex, was used. RhATP was used as a substrate in an NMR tube to follow the reaction of APH by $^{31}$P NMR. This sensitive technique would elucidate any effect added magnesium has on catalysis.

**Materials and Methods**

1. **Materials**

CrATP was prepared by the method of Dunaway-Mariano and Cleland (1980). The concentration of CrATP was determined spectrophotometrically by measuring the absorbances at 427 nm ($\varepsilon$=30 M$^{-1}$ cm$^{-1}$), 606 nm ($\varepsilon$=25 M$^{-1}$ cm$^{-1}$), and 258 nm ($\varepsilon$=15,800
M\(^{-1}\) cm\(^{-1}\)). RhATP was prepared by first synthesizing rhodium (III) perchlorate hexahydrate by the method of Ayres and Forrester (1957) and then following the procedure of Lu et al. (1988). RhATP was prepared as a 50/50 mixture of β,γ-bidentate and α,β,γ-tridentate RhATP by heating the reaction mixture for ten minutes at 80°. The presence of a 50/50 mixture was confirmed by \(^{31}\)P NMR. Concentration of RhATP was determined spectrophotometrically at 258 nm (ε=15,800 M\(^{-1}\) cm\(^{-1}\)). APH(3')-IIIa was overexpressed in *Escherichia coli* (BL-21 strain) and purified as described by McKay et al. (1994). All chemicals used were of the highest grade available.

2. **APH(3')-IIIa Activity Assay**

Activity of APH(3')-IIIa was determined by using a coupled enzyme reaction. The ADP formed in the APH reaction reacts with phosphoenolpyruvate (PEP) to form ATP and pyruvate via pyruvate kinase (PK). The pyruvate is then used by lactate dehydrogenase (LDH) to oxidize NADH. One can follow the reaction by monitoring the rate of oxidation at 340 nm. This reaction is carried out in a mixture of 100 mM KCl, 20.4 mM MgCl\(_2\), 50 mM MES (pH 6.5), 0.34 mM NADH, 2.0 mM PEP (cyclohexylammonium salt), 0.5 mM Kanamycin A, 10.2 mM ATP, 90 units of PK, and 55 units of LDH. APH(3')-IIIa was added to a concentration of 112 μg/mL, and NADH oxidation was measured at 340 nm with a Beckman DU-70 spectrophotometer at 37°C.

3. **CrATP Inactivation Studies**

APH(3')-IIIa has previously been shown to be inactivated by incubation with CrATP (Cox, Ph.D. dissertation). The inactivation was carried out at 37°C in a 200 μL mixture of the following: 100 mM KCl, 3.7 mg mL\(^{-1}\) APH(3')-IIIa, 50 mM MES (pH 6.5), and 0.4 mM CrATP. In some experiments, either EDTA or MgCl\(_2\) was added to
obtain concentrations of 0.5 mM and 2 mM, respectively. At certain time intervals during the incubation, 25-μL aliquots were removed from the incubation mixture and assayed as described above to determine loss of activity.

3. RhATP NMR Studies

A 500-μL solution (10% D$_2$O) of 25 mM MES (pH 6.5), 3.4 mM RhATP, 0.84 mM Kanamycin A, and 0.89 mM APH(3')-IIIa was monitored by $^{31}$P NMR to study the catalytic cycle of APH. MgCl$_2$ in one experiment was added to 2 mM. Spectra were taken with a Bruker AMX-400 wide-bore spectrometer with a 5-mm quad-nuclear probe. Spectra were processed and analyzed using Felix 95.0 software from Biosym®.

Results

Inactivation of APH(3')-IIIa by CrATP has previously been shown to be a pseudo-first order reaction (Cox, Ph.D. dissertation). Inactivation experiments were
carried out as described in both the absence and addition of added MgCl₂ to determine the role of the second Mg²⁺ ion in MeATP binding. EDTA was added in one experiment to ensure there were no contaminating metal ions present in the enzyme. As can be seen from Figure 1, there was no significant effect on CrATP inactivation rates by the addition of MgCl₂.

RhATP ³¹P spectra were collected in both the absence and addition of MgCl₂ to study the role of the second Mg²⁺ ion in catalysis. The first set of spectra (Figure 2)

Figure 2 - Spectra with No Added Mg²⁺ shows the progression of the APH reaction without added MgCl₂. The top spectrum is the beginning of the reaction. Each spectrum is a collection of 4000 scans taken over a two-hour period. In Figure 3, one can see the set of spectra in which the reaction was run in the presence of added MgCl₂. Again, the top is the beginning of the reaction and each spectrum is composed of 4000 scans.
The chemical shifts of the $\alpha,\beta,\gamma$-bidentate phosphorus atoms are at points 6900, 5600, and 3700, respectively. Likewise, the chemical shifts of the $\alpha,\beta,\gamma$-bidentate phosphorus atoms are at points 4900, 5700, and 3650, respectively. As phosphorylated aminoglycoside is formed, its peak grows at 4900 underneath the $\alpha$-tridentate phosphorus resonance. Product formation is accompanied by the attenuation of the $\gamma$-bidentate phosphorus resonance at 5600. This is consistent with the known reaction. At point 3800, one can see the formation of a new peak. As Cr$^{3+}$ coordinates to the enzyme over time, it causes a change in chemical environment, and thereby chemical shift, for the $\gamma$-phosphorus atoms.

![Graph](product_formattion.png)

**Figure 4**

The integration of the product peak was divided by the integration of the $\beta$-phosphorus peak in order to standardize the integrations across the different spectra. These ratios were then plotted against time to determine the rate of product formation in both absence and addition of MgCl$_2$ (Figure 4). As can be seen, the presence of MgCl$_2$ does not significantly alter the rate of product formation.
Discussion

These studies with exchange-inert metal-ATP complexes have elucidated the role of the second metal ion found in APH(3')-IIIa. The result that added magnesium had no effect on CrATP inactivation suggests that the second metal ion does not play a role in MeATP binding. Likewise, the rate of product formation as determined by RhATP and $^{31}$P NMR showed no visible change with the addition of magnesium. This shows that the second magnesium ion does not play a role in catalytic activity. Possible roles for this second metal ion include the stabilization of the active site or the enzyme itself. One might argue that the second magnesium is bound to APH(3')-IIIa from expression and purification. This would definitely explain the similarity in the results between the absence and addition of Mg$^{2+}$. However, this is unlikely due to the fact the magnesium is exposed to the surface of the enzyme and, as previously mentioned, coordinates and dissociates at a very high rate.

There are a few implications for the information gathered in this study. The ultimate goal in understanding the mechanisms of the aminoglycoside modifying enzymes is to use the information in rational drug design. Due to its lack of activity in binding or catalysis, it is now known that the second magnesium ion will not be a good candidate for interactions with any designed inhibitor. Other targets for drug design in the enzyme will need to be found. Fortuitously, ongoing research in this lab has shown the presence of an essential tyrosine. Time will eventually elucidate this prime target for drug design.
References


