Gentamicin Nucleotidyltransferase
STEREOREGICAL INVERSION AT PHOSPHORUS IN ENZYMATIC 2'-DEOXYADENYLYL TRANSFER TO TOBRAMYCIN*

Jean E. Van Pelt†, Radha Iyengar‡, and Perry A. Frey
From the Institute for Enzyme Research, Graduate School, and the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53705

Gentamicin nucleotidyltransferase-catalyzed reaction of (S₉)·(a·¹⁷⁰)₂⁻dATP with tobramycin produced 2'-(2'-deoxyadenosine 5'·(¹⁷⁰)phosphoryl)tobramycin. The configuration at phosphorus in this product was shown to be Rₛ by chemical degradation to chiral [¹⁷⁰,¹⁰]₀DAMP using a stereocchemically defined procedure, and determination of the configuration at phosphorus in this product. Periodate-base treatment of 2'-(2'-deoxyadenosine 5'·(¹⁰)phosphoryl)tobramycin followed by NaBH₄ reduction produced (2'-glyceryl)-[¹⁰]₀DAMP, which upon snake venom phosphodiesterase-catalyzed hydrolysis in H₂O produced [¹⁰,¹⁰]₀DAMP. The configuration at phosphorus in this product was shown to be S by enzymatic phosphorylation to [¹⁰,¹⁰]₀ATP, adenyl cyclase (Bordetella pertussis)-catalyzed cyclization to 3',5'-cyclic [¹⁰,¹⁰]₀DAMP, and ³¹P NMR analysis of the ethyl esters. Since snake venom phosphodiesterase-catalyzed hydrolyses proceed with retention of configuration at phosphorus, (S₉)·(¹⁰)₀DAMP must have been produced from (Rₛ)·(2'-glyceryl)-[¹⁰]₀DAMP; and since the chemical degradation to the latter compound did not involve cleavage of any bonds to phosphorus, the initial enzymatic product must have been (Rₛ)·2'-(2'-deoxyadenosine 5'·(¹⁰)phosphoryl)tobramycin. Therefore, nucleotidyl transfer catalyzed by gentamicin nucleotidyltransferase proceeds with inversion of configuration at phosphorus, and the reaction mechanism involves an uneven number of phosphotransfer steps. Inasmuch as this is an uncomplicated two-substrate, two-product nucleotidyltransferase reaction, inversion strongly implies a mechanism involving a one-step, direct group transfer between bound substrates; whereas retention implies a two-step, double displacement mechanism mediated by a covalent nucleotidyl-enzyme as a compulsory intermediate (7). We here report stereoregional inversion at phosphorus in the gentamicin nucleotidyltransferase-catalyzed reaction of (S₉)·(¹⁰)₀DATP with tobramycin, consistent with a single displacement mechanism.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

In preliminary experiments, we investigated the possibilities for determining the stereochemical course of nucleotidyl transfer by using either (S₉)- or (Rₛ)-ATP·OS as the nucleo-

\[ \text{Gentamicin + MgATP} \rightarrow \text{AMP-gentamicin + MgPP} \]

(1–4). The resulting nucleotidylaminoglycosides are inactive as drugs for combating Gram-negative bacteria. The enzyme can be purified by affinity chromatography and is known to follow a sequential kinetic pathway (5, 6).

One means of obtaining information about the number of nucleotidyl transfer steps in the mechanism, and therefore about the possible involvement of a covalent nucleotidyl-enzyme intermediate, is to determine the stereochemical consequences of nucleotidyl transfer using a nucleotidyl donor substrate having a center of chirality at P'. If the configuration at this center is inverted in the product, it can be concluded that the mechanism involves an uneven number of phosphotransfer steps, whereas if the configuration is retained, an even number of steps is involved (7, 8). In uncomplicated two-substrate, two-product nucleotidyltransferase reactions, inversion strongly implies a mechanism involving a one-step, direct group transfer between bound substrates; whereas retention implies a two-step, double displacement mechanism mediated by a covalent nucleotidyl-enzyme as a compulsory intermediate (7). We here report stereoregional inversion at phosphorus in the gentamicin nucleotidyltransferase-catalyzed reaction of (S₉)·(¹⁰)₀DATP with tobramycin, consistent with a single displacement mechanism.

* This research was supported by Grant GM 30480 from the National Institute of General Medical Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Present address: Dept. of Biochemistry, University of Minnesota, St. Paul, MN 55108.
‡ Present address: Dept. of Applied Biological Sciences, Massachusetts Inst. of Technology, Cambridge, MA 02138.
Inversion at Phosphorus in 2'-Deoxynucleotidyl Transfer
d
15996

We readily found (S)-ATP to be an excellent substrate, whereas the (R)-epimer did not react. However, we were unable to establish the configuration at phosphorus in the product, since it proved not to be a substrate for snake venom phosphodiesterase or nuclease S1, which have been shown to exhibit high selectivities for the R<sub>s</sub> and S<sub>s</sub> configurations, respectively, in thiophosphodiester substrates (15, 16, 18). AMP-tobramycin also proved to be refractory to nuclease action; and neither acetylation nor succinylation of the amino groups in AMP-tobramycin, which destroyed the positive charges, altered this refractoriness. Therefore, [α-<sup>17</sup>O]<sup>18</sup>O] ATP was not considered to be a practical alternative substrate since the configuration of the product at phosphorus could not readily be established by nuclease degradation to [α-<sup>17</sup>O]<sup>18</sup>O] AMP. We chose (S)-[α-<sup>17</sup>O]dATP as the substrate for this stereocchemical study because dAMP-tobramycin (see Structure 1) has a single periodate cleavage site (C<sup>4'</sup>-C<sup>3'</sup>), and the cleavage product can be degraded by alkaline elimination to a substrate for snake venom phosphodiesterase without affecting the configuration at phosphorus.

**Synthesis of (S)-[α-<sup>17</sup>O]dATP**—The procedure outlined in Scheme I was followed for preparing this substrate. Michelson phosphoanhydride coupling of dAMPs with AMP produced the epimer mixture (S<sub>p</sub> + R<sub>p</sub>)-P<sup>1</sup>-5'-d(2'-dAdo)-P<sup>2</sup>-5'-Ado-1'-thiodiphosphate, which was separated by chromatography into the pure (R<sub>p</sub>)-epimer and a fraction highly enriched in the (S<sub>p</sub>)-epimer. Desulfurization of the (R<sub>p</sub>)-epimer by reaction with BrCN in H<sub>2</sub>17O produced (S<sub>p</sub>)-P<sup>1</sup>-5'-d(2'-dAdo)-P<sup>2</sup>-5'-Ado-[1-<sup>17</sup>O]diphosphate in a reaction known to proceed with inversion of configuration at phosphorus (19). Removal of the adenosyl moiety by periodate cleavage and alkaline elimination produced (S<sub>p</sub>)-[α-<sup>17</sup>O]dADP, which was enzymatically phosphorylated to (S<sub>p</sub>)-[α-<sup>17</sup>O]dATP.

**Enzymatic Production of [α-<sup>17</sup>O]dAMP-tobramycin and Degradation to [17O]<sup>18</sup>O]dAMP**—(S<sub>p</sub>)-[α-<sup>17</sup>O]dATP was used as the nucleotidyl donor substrate with gentamicin nucleotidyltransferase and tobramycin as the acceptor to produce [17O] dAMP-tobramycin. To prepare the compound for stereocchemical analysis at phosphorus, it was degraded to [17O]<sup>18</sup>O]dAMP by the procedure outlined in Scheme II. Cleavage of the C<sup>3'</sup>-C<sup>4'</sup> bond by 1 eq of periodate produced the corresponding dialdehyde which, upon treatment with base, underwent a series of elimination processes to the 2-hydroxymalonialdehyde ester of dAMP. Reduction by NaBH<sub>4</sub> produced the 2-glyceryl ester of dAMP, which proved to be a substrate for snake venom phosphodiesterase. The nonenzymatic phase of this procedure did not involve cleavage of bonds to phosphorus and so could not have affected the configuration at this center. The enzymatic phase, snake venom phosphodiesterase-catalyzed hydrolysis in H<sub>2</sub>18O, proceeded with P-O bond cleavage to replace the 2-hydroxyl group of glycerol with <sup>18</sup>O. Snake venom phosphodiesterase reactions have been shown to proceed with retention of configuration at phosphorus, as illus-

**Structure 1. dAMP-tobramycin.**

**Scheme I.**

**Scheme II.**
Inversion at Phosphorus in 2'-Deoxynucleotidyl Transfer

The configurations at phosphorus, shown in Scheme II mainly for illustrative purposes, were retrospectively chosen to be in accord with the stereochemical facts, as elucidated by configurational analysis of the [\(^{17}O\),\(^{18}O\)]dAMP ultimately isolated from the degradation.

Configurational Analysis of [\(^{17}O\),\(^{18}O\)]dAMP—The configurational analysis of [\(^{17}O\),\(^{18}O\)]dAMP has been described by Gerlt and co-workers (20). We have used this procedure, which is outlined in Scheme III. Enzymatic phosphorylation of [\(^{17}O\),\(^{18}O\)]dAMP by phosphoenolpyruvate in the presence of adenylate kinase, pyruvate kinase, and a catalytic amount of dATP produced a randomly pyrophosphorylated mixture of [\(^{\alpha-17}O\),\(^{18}O\)]dATP stereoisomers. Adenylylcyclase-catalyzed cyclization produced the corresponding three stereoisomers of 3',5'-cyclic dAMP, one of which shown in Scheme III contained \(^{18}O\) but no \(^{17}O\). Species containing \(^{17}O\) were produced but are not shown because they were NMR-silent in the analysis. Alkylation by diazoethane produced a mixture of axial and equatorial ethylated 3',5'-cyclic dAMP which was subjected to \(^{31}P\) NMR analysis for bridging and nonbridging \(^{18}O\).

The configurational analysis of 3',5'-cyclic [\(^{18}O\)]dAMP is based on the differential effects of doubly and singly bonded \(^{18}O\) on the \(^{31}P\) chemical shifts in the axial and equatorial isomers. For the configuration shown in Scheme III, the \(^{18}O\) is singly bonded to phosphorus in the equatorial isomer and doubly bonded in the axial isomer. The reverse would be the case when \(^{18}O\) is in the axial position of 3',5'-cyclic [\(^{18}O\)]dAMP. The configuration is revealed by integration of the resolved \(^{18}O\)-shifted signals for doubly and singly bonded \(^{18}O\) in the equatorial and axial isomers of ethyl 3',5'-cyclic [\(^{18}O\)]dAMP. The analysis is complicated by the fact that positions shown as \(^{17}O\) in [\(^{\alpha-17}O\),\(^{18}O\)]dATP are only 51.7% enriched by \(^{17}O\) and also contain 37.0% \(^{18}O\), whereas the position shown as \(^{18}O\) is 88% enriched. Therefore, each set of spectra consists of \(^{31}P\) NMR lines corresponding to \(^{18}O_2\) (one singly and one doubly bonded), doubly bonded \(^{18}O\), singly bonded \(^{18}O\), and no \(^{17}O\). The relative signal intensities reveal the configuration at phosphorus.

The configurations shown in Scheme III for illustrative purposes are those actually established by the analysis of (\(S_\text{r}\)-\([\alpha-17]O\),\(^{18}O\)]dAMP resulting from the degradation of \([\alpha-17]O\)]dAMP-tobramycin according to Scheme II. The \(^{31}P\) NMR integration data are given in Table II. (\(S_\text{r}\)-\([\alpha-17]O\),\(^{18}O\)]dAMP in Scheme III would arise from the degradation of (\(R_\text{r}\)-\([\alpha-17]O\),\(^{18}O\)]dAMP-tobramycin in Scheme II, and this in turn would be produced as the result of inversion of configuration at phosphorus in the nucleotidyltransferase-catalyzed reaction of (\(S_\text{r}\)-\([\alpha-17]O\)]dATP. As shown by Table II, the \(^{31}P\) NMR data are consistent with these configurations, that is, with inversion and not with retention as the stereochemical course of nucleotidyl transfer. Therefore, gentamicin nucleotidyltransferase catalyzes the reaction of (\(S_\text{r}\)-\([\alpha-17]O\)]dATP in Scheme I with the C2'-OH of tobramycin to form (\(R_\text{r}\)-\([\alpha-17]O\)]dAMP-tobramycin (Scheme II), i.e., with inversion of configuration.

Mechanism of Nucleotidyl Group Transfer—Inasmuch as the gentamicin nucleotidyltransferase reaction proceeds with inversion of configuration at \(P_1\) of dATP, the reaction mechanism must involve an uneven number of displacement steps. The simplest interpretation, and the one we favor, is that the mechanism involves the sequential binding of a nucleoside triphosphate and an aminoglycoside at adjacent binding sites of the enzyme to form a ternary complex, in which the C2'-

![Scheme III](image)

| Table II |
|-----------------------------------------------|
| \(^{31}P\) NMR intensities for the ethyl esters of 3',5'-cyclic [\(^{17}O\),\(^{18}O\)] dAMP |

The individual entries quote the percent contribution of each species to the total signal for the equatorial or axial triester. The theoretical values are calculated based on the isotopic composition of [\(^{17}O\),\(^{18}O\)]dAMP derived from the degradation of [\(^{17}O\)]dAMP-tobramycin according to Scheme II and assuming the configuration of [\(^{17}O\),\(^{18}O\)]dAMP to be \(S_\text{r}\) for inversion and \(R_\text{r}\) for retention of configuration in the nucleotidyl transfer. Experimental uncertainty in the tabulated values due to error is such that 10% epimerization at phosphorus would not be discerned in or ruled out by the data.

| Species | Equatorial triester | Axial triester |
|---------|---------------------|---------------|
|          | Observed values | Theoretical values | Observed values | Theoretical values |
| \(EXO-P=O\) | 25 | 19 | 19 | 22 | 19 | 19 |
| \(Et\(_2\)O-P=O\) | 38 | 43 | 24 | 21 | 24 | 43 |
| \(EO-P=O\) | 22 | 24 | 43 | 41 | 43 | 24 |
| \(Et\(_2\)O-P=O\) | 15 | 14 | 16 | 14 | 14 | 14 |
OH of the aminoglycoside is positioned near P1 of the nucleotide. Direct in-line displacement of pyrophosphate from P1 by the C2'-OH produces the nucleotidylaminoglycoside in a single step accompanied by inversion of configuration at phosphorus. This mechanistic pathway is consistent with both our stereochemical result and the fact that the kinetics for the reaction is sequential. If in the sequential kinetic pathway the conversion of the substrate ternary complex to the product ternary complex had involved an enzyme-mediated double displacement with an intervening covalent nucleotidyl-enzyme intermediate, the nucleotidyl transfer would have taken place with overall retention of configuration at phosphorus.

The present work extends the list of two-substrate, two-product phosphotransferases that follow sequential kinetics and proceed with inversion of configuration at phosphorus. Thus far, all two-substrate, two-product phosphotransferase reactions that proceed with overall retention at phosphorus follow ping-pong kinetic pathways (7). This pattern for Bi Bi reactions is extensively documented and has been rationalized on the basis that the role of covalent intermediates in the simplest Bi Bi phosphotransferases is to protect the phos- phofr extrane phosphoanhydrohride bond energy during the changeover of acceptors by dissociation and association at a common binding site in ping-pong pathways (7). Such a role for the enzyme is unnecessary in the sequential pathways since the donor and acceptor substrates occupy adjacent sites in the ternary complexes and group transfer is a direct, single step process.

Ter Ter and Ter Bi reactions such as ATP-dependent phosphotransferases will not necessarily follow this rule, however, since additional roles (such as group translocation between substrates for covalent intermediates) can be expected to be important within quaternary complexes. Stereochemical analysis of such reactions may uncover these alternative roles for covalent intermediates.

Acknowledgments—We are grateful to Professor N. D. Goldberg and Dr. D. Confer of the Department of Pharmacology, University of Minnesota for providing us with the B. pertussis extract and to Professor D. B. Northrop and Dr. C. A. Gates of the School of Pharmacy, University of Wisconsin, for the gentamicin nucleotidyl-transferase.

REFERENCES

1. Davies, J., and Smith, D. I. (1978) Annu. Rev. Microbiol. 32, 469-518
2. Beneveniste, R., and Davies, J. (1971) FEBS Lett. 14, 293-296
3. Yagisawa, M., Naganawa, H., Kondo, S., Hamada, M., Takeuchi, T., and Umizawa, H. (1971) J. Antibiot. (Tokyo) 24, 911-912
4. Yagisawa, M., Naganawa, H., Kondo, S., Takeuchi, T., and Umizawa, H. (1972) J. Antibiot. (Tokyo) 25, 492-494
5. Van Pelt, J. E., and Northrop, D. B. (1984) Arch. Biochem. Biophys. 230, 252-263
6. Gates, C. A., and Northrop, D. B. (1985) Fed. Proc. 44, 1810
7. Frey, P. A. (1982) in New Comprehensive Biochemistry (Neuburger, L. M., ed.) Stereochimy (Tamm, Ch., volume ed) Vol. 3, pp. 201-248
8. Frey, P. A. (1982) Tetrabedron 38, 1541-1567
9. Richard, J. P., and Frey, P. A. (1982) J. Am. Chem. Soc. 104, 3476-3481
10. Agard, G. (1969) Carbohydr. Res. 11, 119-123
11. Richard, J. P., Prasher, D. C., Ives, D. H., and Frey, P. A. (1979) J. Biol. Chem. 254, 4339-4341
12. Lowe, G., Tansley, G., and Cullis, P. M. (1982) J. Soc. Chem. Commun. 595-598
13. Ho, H.-T., and Frey, P. A. (1984) Biochemistry 23, 1978-1983
14. Michelson, A. M. (1964) Biochem. Biophys. Acts 91, 1-13
15. Bryant, F. R., and Benkovic, S. J. (1979) Biochemistry 18, 2825-2828
16. Burgers, P. M. J., Eckstein, F., and Hunningen, D. H. (1979) J. Biol. Chem. 254, 7476-7478
17. Arndt, F. (1963) Organic Syntheses, Collective Vol. II, pp. 165-167, Wiley-Interscience, New York
18. Potter, B. V. L., Romaniuk, P. J., and Eckstein, F. (1983) J. Biol. Chem. 258, 1770-1780
19. Sammons, R. D., and Frey, P. A. (1982) J. Biol. Chem. 257, 1138-1141
20. Codere, J. A., Mehdi, S., and Gerlt, J. A. (1981) J. Am. Chem. Soc. 103, 1872-1875
21. Gerlt, J. A., Cordero, J. A., and Wolin, M. S. (1980) J. Biol. Chem. 255, 331-334
22. Eckstein, F., Romaniuk, P. J., Heideman, W., and Storm, D. R. (1981) J. Biol. Chem. 256, 9118-9120

Supplemented by: "STEREOCHEMICAL INVERSION AT PHOSPHORUS IN PHOSPHOPHOSPHORUS TRANSFERS: A DISCUSSION OF THE NEED FOR A NEW CONVENTION" by Jean E. Van Pelt, J. E., Consumer, D. E., and J. E. Frey.


data.

References

Adenosine nucleotide concentrations were calculated from

\[
\frac{A_{259}}{A_{259}} \times \left( \frac{1}{99} + \frac{1}{54} \right)
\]

Kemephalosphorothioates with terminal phosphonoester groups were determined in column effluents by qualitative analysis using t.l.c. (Braden) and dimethylformamide as described (19). Isotopic composition was monitored as described in detail in the preceding report.

High performance liquid chromatography was performed using a Hitachi model 600 system equipped with an ultraviolet and refractive index detectors and a Waters model 555 reverse phase column (1.9 mm 4.6 mm).

ECHELON REGIONAL MEASUREMENTS—13C NMR spectra were recorded as 2D using a Hitachi model 100-10 spectrometer operating at 50 MHz with quadrature detection. The spectrometer was field frequency locked on the resonance of deuterium in the sample. Chemical shifts were measured relative to external tetramethylsilane. Chemical shifts were determined by comparison with high resolution 13C spectra obtained by deconvolution of high-resolution 1H NMR spectra. The resonances for the methyl and methylene protons were determined by cleavage of the epoxide linkage by treatment with D2O. The results obtained at these obtained by manual integration of high-resolution 1H NMR spectra were compared with those obtained using a Hitachi model 500A spectrometer operating at 200 MHz. NMR spectra were obtained using a Hitachi model 200 spectrometer operating at 200 MHz.

Yamada, S., and Smith, D. I. (1978) J. Biol. Chem. 253, 9118-9120
Inversion at Phosphorus in 2'-Deoxynucleotidyl Transfer

Synthesis of \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\): This reaction was used to verify the stereochemical course of the \(\text{P}_{2}\) in the \(\text{P}_{2}\) of the phosphatidylserine. The reaction was catalyzed by the following reaction:

\[ \text{H}_{2}\text{PO}_{4}\text{(OH)}_{2} + \text{H}_{2}\text{PO}_{4}\text{(OH)}_{2} \rightarrow \text{H}_{4}\text{PO}_{3}(\text{OH})_{3} + \text{H}_{2}\text{O} \]

The reaction was monitored by high-resolution NMR spectroscopy. The product was isolated by chromatography through a 3.2 x 2 cm column of \(\text{C}_{18}\) reverse-phase silica gel. The product was characterized by high-resolution mass spectrometry and NMR spectroscopy.

Intermediate (\(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\)): This intermediate was isolated by column chromatography on silica gel. The intermediate was characterized by UV-visible spectroscopy and NMR spectroscopy.

Product (\(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\)): The product was isolated by chromatography through a 3.2 x 2 cm column of \(\text{C}_{18}\) reverse-phase silica gel. The product was characterized by high-resolution mass spectrometry and NMR spectroscopy.

Results: The reaction was monitored by high-resolution NMR spectroscopy. The product was isolated by chromatography through a 3.2 x 2 cm column of \(\text{C}_{18}\) reverse-phase silica gel. The product was characterized by high-resolution mass spectrometry and NMR spectroscopy.

Chemical Deposition of \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\): The \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\) was deposited on a glass slide by evaporation of a \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\) solution. The deposited material was characterized by UV-visible spectroscopy and XPS.

Table 1: NMR data on nucleotide phosphates

| Nucleotide | Chemical Shift (ppm) | δ (ppm) |
|------------|----------------------|---------|
| \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\) | -44.59 | -11.09 |
| \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\) | -43.01 | -5.62 |
| \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\) | -45.60 | -9.40 |
| \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\) | -46.20 | -9.05 |

Reaction of intermediates with \(\text{H}_{2}\text{PO}_{4}\text{(OH)}_{2}\): The reaction mixture was monitored by high-resolution NMR spectroscopy. The product was isolated by column chromatography through a 3.2 x 2 cm column of \(\text{C}_{18}\) reverse-phase silica gel. The product was characterized by high-resolution mass spectrometry and NMR spectroscopy.