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BOROHYDRIDE REDUCTION OF PERIODATE-OXIDIZED NUCLEOTIDES; ISOLATION AND STRUCTURE OF THE REDUCTION INTERMEDIATE

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ABSTRACT

The reduction of periodate-oxidized nucleotides with sodium borohydride proceeds *via* a reaction intermediate presumed to be a monoalcohol. The borohydride-reduction intermediate of periodate-oxidized ADP has been isolated by anion-exchange, liquid chromatography, and subjected to further reduction. Using sodium borohydride and sodium borodeuteride alternately in the two reduction steps, it was determined, by ^{1}H-n.m.r.-spectral analysis, that the two aldehyde groups are sequentially reduced in the order 3′ and 2′, and it was concluded that the isolated intermediate corresponds to the semi-reduced, 3′-alcohol, 2′-aldehyde derivative. This compound should be a useful analog for the study of enzymes and proteins that interact with nucleotides.

INTRODUCTION

Periodate-oxidized nucleotides are widely used as active-site reagents for the analysis of enzymes that employ nucleotides as substrates. These nucleotide analogs possess two reactive aldehyde groups, and can thus react with proteins in a variety of ways. This potential complexity could be lessened with the availability of nucleotides that possess only a single aldehyde group.

Reduction of these dialdehydes by borohydride to give the corresponding dialcohol proceeds in two steps *via* an intermediate presumed to be a monoaldehyde[1], but whether this intermediate is reduced at C-2′ or C-3′, or both, was unknown. Khym and Cohn[2] showed that periodate-oxidized nucleosides are selectively reduced at C-3′ under slightly acidic conditions; however, the mechanism that they proposed for this reaction requires a free 5′-hydroxyl group, and thus could not apply to 5′-nucleotides. In order to determine the structure of the borohydride-reduction intermediate of periodate-oxidized nucleotides, we established a procedure for purifying the reduction intermediate[3] of ADP-2′,3′-dialdehyde**, so that we were able to

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**The abbreviation ADP-2′,3′-dialdehyde is used to designate the periodate-oxidation product of ADP. Similarly, ADP-2′,3′-dialcohol denotes the product obtained on complete reduction of ADP-2′,3′-dialdehyde with sodium borohydride. ADP-semialdehyde designates the reaction intermediate formed during the course of that reduction.

effect the two reductions separately, using borohydride and then borodeuteride. ^{1}H-N.m.r.-spectral analysis of the various reduction products indicated that the two aldehyde groups are sequentially reduced in the order 3′ and then 2′, presumably because of the greater reactivity of the 3′-aldehyde group. We conclude from these observations that the intermediate that we have isolated is ADP-2′-semialdehyde. This compound should prove to be a useful analog for the study of enzymes and proteins that interact with nucleotides.

RESULTS AND DISCUSSION

The intermediate in the reduction of [β-^{32}P]GDP-2′,3′-dialdehyde with sodium borohydride was previously made visible on poly(ethyleneimine)cellulose as a transient, chromatographic species that appeared rapidly, and then decreased as the fully reduced compound GDP-2′,3′-dialcohol was formed[1]. We determined the rates for the two reaction steps by quantifying the three ^{32}P-labelled nucleotide species, following thin-layer chromatography at various times during the reduction. The pseudo-first-order rate-constant k_1 (3.6 min^{-1}) of the appearance of the intermediate was determined from the rate of disappearance of oxidized nucleotide. The second pseudo-first-order rate-constant k_2 (0.12 min^{-1}) was determined as previously described[4], to fit a two-step reaction-sequence.

The same type of reduction intermediate, presumed to be a semialdehyde, was observed for all of the nucleotide-2′,3′-dialdehydes tested (from ADP, AMP, CDP, GDP, GMP, and UDP). For practical reasons, the ADP derivative was chosen for structural characterization. This compound was obtained, as a mixture with the ADP-dialcohol, following partial reduction of ADP-dialdehyde with sodium borohydride. The reduction intermediate and ADP-dialcohol were separated by chromatography on Bio-Rad AG MP-1 ion-exchange resin by methods previously described[3].

If the reaction intermediate is a semialdehyde, its ^{1}H-n.m.r. spectrum should be quite complex, as this compound should exist as a hydrated form, as do periodate-oxidized nucleotides[5-7], and as a pair of diastereoisomeric hemiacetals. Such complexity was, indeed, observed in the ^{1}H-n.m.r. spectrum of the ADP-semialdehyde, although the same material gave a single spot in thin-layer chromatography. A series of coupled doublets (<1 H) were found in the δ 5–6 region, as would be expected for the 1′- and 2′-protons of the different, hemiacetal species.

The ^{1}H-n.m.r. spectrum of ADP-2′,3′-dialcohol was considerably simpler. The resonances of H-1′ and H-2′ were clearly visible at δ 6.2 and 4.2, respectively, but those of H-3′, H-4′, and H-5′, at δ 3.6–4.2, were not resolved.

Characterization of the intermediate by direct, n.m.r.-spectral analysis was not the route of choice in deducing its structure. Rather, we chose to characterize the intermediate indirectly by analyzing the spectra of fully reduced compounds. The intermediate could be isolated, and the two reduction steps could, therefore, be effected separately, with borohydride or borodeuteride, to generate mixed-isotope, reduced forms. The 1′- and 2′-proton resonances of these reduced compounds would

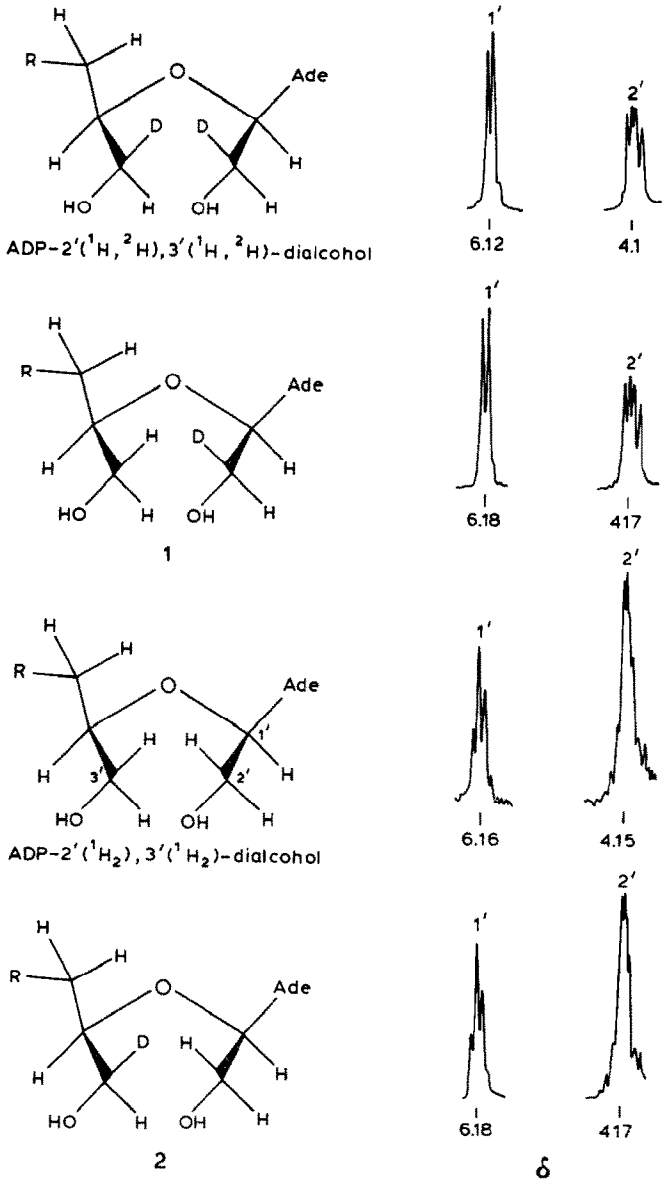

Fig. 1. Structures and ^{1}H-n.m.r. spectra, showing the signals of the 1′- and 2′-protons of the fully reduced compounds. [The structures drawn for the deuterated compounds represent one, arbitrarily chosen, isomer. Ade = adenin-9-yl; R = $P_2O_7H_3$.]

be diagnostic of the isotope incorporation at C-2′, and thereby reflect the step during the reaction in which this carbon atom was reduced.

Four compounds differing in isotope content were synthesized in order to carry out this analysis. The two reference compounds were the fully protonated derivative, the ADP-2′($^1H_2$), 3′($^1H_2$)-dialcohol obtained by reduction of ADP-2′,3′-dialdehyde

with sodium borohydride, and the dideuterated compound, the ADP-2′(^{1}H,^{2}H), 3′(^{1}H,^{2}H)-dialcohol obtained by reduction of the dialdehyde with sodium borodeuteride. The mixed-isotope reduced compounds were synthesized in two steps, using a different isotope for each reduction step. Compound **1** is the dialcohol obtained by effecting the first reduction step with borohydride; the reduction intermediate thus obtained was purified, and subsequently reduced to the dialcohol with sodium borodeuteride. Compound **2** is the dialcohol obtained similarly, but using sodium borodeuteride for the first, and sodium borohydride for the second, reduction step.

The ^{1}H-n.m.r. spectra of these four compounds were compared (see Fig. 1). The ADP-2′($^1H_2$), 3′($^1H_2$)-dialcohol shows a triplet at the anomeric-proton resonance due to the two protons present on the 2′-alcohol. The complex pattern of the 2′-resonance shows vicinal and geminal coupling for both protons. The ADP-2′(^{1}H,^{2}H), 3′(^{1}H,^{2}H)-dialcohol shows a doublet at the anomeric-proton resonance, indicating the presence of only one proton on C-2′, due to the incorporation of a deuterium atom during the borodeuteride reduction. The two overlapping doublets for the 2′-resonance are produced by the two possible isomers obtained during deuterium incorporation. Although there appears to be only one doublet at the anomeric-proton resonance, decoupling at the 2′-resonance collapsed the anomeric resonance to two almost-superimposed singlets (data not shown).

The 1′- and 2′-resonances of compound **1** are identical to those of the deuterated reference compound ADP-2′(^{1}H,^{2}H), 3′(^{1}H,^{2}H)-dialcohol, and the resonances of compound **2** are identical to those of ADP-2′($^1H_2$), 3′($^1H_2$)-dialcohol. In both cases, the ^{1}H-n.m.r. signals indicate that the isotope incorporated at C-2′ of the mixed reduced compounds came only from the reducing agent used during the conversion of the reduction intermediate into the fully reduced compound.

We conclude that the second step of the reaction involves the reduction of the 2′-aldehyde group, and that the first step in the reaction therefore consists in the reduction of the 3′-aldehyde group, to generate the 3′-alcohol as the reduction intermediate. All of the data are consistent with the structures that would result from a 3′ and then 2′ sequential reduction (see Fig. 1).

For the selective reduction of the 3′-aldehyde group in periodate-oxidized nucleosides, a mechanism had been proposed[2] by Khym and Cohn that involves hemiacetal formation between the 5′-hydroxyl group and the 2′-aldehyde group, leaving the 3′-aldehyde group free to be reduced. Although this mechanism could account for the selective reduction of the oxidized nucleosides, it clearly cannot explain the selective reduction of the oxidized nucleotides, because they lack a free 5′-hydroxyl group*. The initial reduction of the 3′-aldehyde group in oxidized nucleotides must, therefore, be attributed to some other property of the molecule.

*The ^{31}P-n.m.r. spectra of all of the dialcohols showed two peaks in the 900–1200-Hz region, confirming that both phosphate groups were still present in the final product.

It would appear that C-3′ is either intrinsically more reactive than C-2′, or is more accessible to the rather bulky borohydride group.

Periodate-oxidized nucleotides are known to form a number of covalent adducts with enzymes[8–14]. Schiff-base formation, which has been well characterized in a number of enzymes, is reversible, but can be stabilized by reduction with borohydride[9–12]. This adduct could involve interaction either of one, or of both, of the 2′- and 3′-aldehyde groups with the amino group of one or more lysine residues. For the enzyme phosphofructokinase (EC 2.4.1.11), there has been proposed a dihydroxymorpholine derivative which would result from the interaction of both the 2′- and the 3′-aldehyde group with a lysine residue[14]. Finally, for ribonucleotide reductase (EC 1.17.4.1), it has been postulated that a covalent complex results from the formation of a Michael adduct between a nucleophile on the enzyme and C-5′, following elimination of the pyrophosphate group from the dialdehyde nucleotide[15]. This mechanism requires that the 3′-aldehyde shall enhance the acidity of H-4′, and thus allow the initial elimination.

The 2′-semialdehyde reduction intermediate described here can only form a single type of Schiff base, and should thus simplify the study of proteins and enzymes that interact with nucleotides.

EXPERIMENTAL

General. — ^{1}H-N.m.r. spectra were recorded at 250 MHz with a Bruker WM250 FT NMR spectrometer, using 16 k transforms without resolution enhancement. Each nucleotide (50 mg) was dissolved in D_2O (0.5 mL). The spectrometric reference standard was external, neat tetramethylsilane.

Materials. — Deuterium oxide having a minimal, isotopic purity of 99.96 atom% ^{2}H was obtained from Aldrich Chemical Co. ADP, AMP, GDP, and GMP were obtained from Sigma, and CDP and UDP from P-L Biochemicals. NaB^2H_4 (from Merck Sharp and Dohme) had a minimal, isotopic purity of 98% of ^{2}H. [β-^{32}P]GDP was a gift from Dr. T. F. Walseth.

Oxidation of nucleotides

ADP-2′,3′-dialdehyde. — Oxidation of nucleotides was accomplished by adding $NaIO_4$ (66 mg) to a solution of the nucleotide (128 mg) in 2 mL of 0.1M ammonium acetate buffer, pH 4.3, and allowing the mixture to stand for 45 min in the dark at room temperature; t.l.c. in 0.3M potassium phosphate buffer, pH 3.4 (*A*; R_F 0).

ADP-2′($^1H_2$),3′($^1H_2$)- and ADP-2′(^1H,^2H),3′(^1H,^2H)-dialcohol. — To the foregoing, oxidized-nucleotide solution (1 mL) was slowly added $NaBH_4$ (20 mg) or NaB^2H_4 (25 mg), and the solution was allowed to stand for 30 min, with occasional mixing, at 0°. ADP-2′($^1H_2$),3′($^1H_2$)-dialcohol, ^{1}H-n.m.r.: δ 8.6 (d, 1 H, H-2), 8.46 (s, 1 H, H-8), 6.16 (t, 1 H, H-1′), 4.14 (m, 2 H, H-2′), and 3.6–4.16 (m, 5 H, H-3′,4′,5′); ADP-2′(^{1}H,^{2}H),3′(^{1}H,^{2}H)-dialcohol, t.l.c. (*A*; R_F 0.68); ^{1}H-n.m.r.:

δ 8.59 (s, 1 H, H-2), 8.43 (s, 1 H, H-8), 6.12 (d, 1 H, H-1′), 4.08 (2 d, 1 H, H-2′), and 3.73–4.0 (m, 4 H, H-3′,4′,5′).

ADP-2′,3′-semialdehyde. — Treatment of the oxidized-nucleotide solution (2 mL) with $NaBH_4$ (24 mg), or NaB^2H_4 (28 mg), added slowly during 10 min at room temperature, gave a mixture of the semialdehyde and dialcohol forms. The course of the reaction was monitored by thin-layer chromatography, to assure that all of the ADP-dialdehyde had disappeared, as this nucleotide was found to co-chromatograph with ADP-semialdehyde in the anion-exchange system used to purify the nucleotides. The nucleotides were then separated by anion-exchange chromatography as described in the next paragraph. Fractions containing the semialdehyde (as determined by t.l.c.) were combined, and lyophilized.

Dialcohols **1** *and* **2.** — The lyophilized pool of ADP- semialdehyde was dissolved in 1.5 mL of 100mM ammonium acetate buffer, pH 7, bringing the final pH to 5. The further reduction of the semialdehyde was then accomplished by adding $NaBH_4$ (12 mg), or NaB^2H_4 (15 mg), and allowing the reaction to proceed for 10 min at 0°. Compound **1**, t.l.c. (*A*; R_F 0.66); ^{1}H-n.m.r.: δ 8.7 (d, 1 H, H-2), 8.53 (s, 1 H, H-8), 6.18 (d, 1 H, H-1′), 4.17 (2 d, 1 H, H-2′), and 3.76–4.08 (m, 5 H, H-3′,4′,5′); compound **2**, t.l.c. (*A*; R_F 0.68); ^{1}H-n.m.r.: δ 8.63 (d, 1 H, H-2), 8.47 (s, 1 H, H-8), 6.16 (t, 1 H, H-1′), 4.14 (m, 2 H, H-2′), and 3.55–5.0 (m, 4 H, H-3′,4′,5′).

Purification of nucleotides by anion-exchange chromatography. — Nucleotides were purified by a gravity-flow adaptation of a liquid chromatography system developed by Axelson *et al.*[3] for the separation of nucleotides. Nucleotide samples were applied to a column (1.5 × 12 cm) of Bio-Rad AG MP-1 (trifluoroacetate) anion-exchange resin, equilibrated in 15mM trifluoroacetic acid and eluted isocratically. Fractions were monitored for absorbance at 254 nm, and aliquots were analyzed by thin-layer chromatography to identify the nucleotides. This system allowed separation of the semialdehyde from the completely reduced dialcohol forms. It also removed the nucleoside monophosphate contaminants and borate and iodide (salts), which passed directly through the column.

Thin-layer chromatography. — Nucleotides were analyzed by chromatography on 10-cm plates of poly(ethyleneimine)cellulose, as previously described[1]. Chromatograms were developed in 0.3M potassium phosphate buffer, pH 3.4 (buffer *A*), or 125mM potassium phosphate buffer, pH 3.4 (for CDP and UDP).

Reduction kinetics. — To a 10mM solution of [β-^{32}P]GDP-2′,3′-dialdehyde (1 mL) at 0° was added freshly prepared M $NaBH_4$ (120 μL); aliquots (50 μL) were removed at various times, and the reaction was terminated by the addition of acetone (10 μL). Portions (10 μL) of the solutions were chromatographed, and the radioactive spots were "counted" as previously described[1].

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REFERENCES

1 J. W. Bodley and J. Gordon, *Biochemistry*, 13 (1974) 3401–3405.
2 J. X. Khym and W. E. Cohn, *J. Am. Chem. Soc.*, 82 (1960) 6380–6386.
3 J. Axelson, J. W. Bodley, and T. F. Walseth, *Anal. Biochem.*, 116 (1981) 357–360.
4 B. G. Van Ness, J. B. Howard, and J. W. Bodley, *J. Biol. Chem.*, 225 (1980) 10,717–10,720.
5 N. K. Kotchetkov and E. I. Budovskii (Eds.), *Organic Chemistry of Nucleic Acids*, Plenum Press, London, 1972, pp. 467–468.
6 F. Hansske and F. Cramer, *Carbohydr. Res.*, 54 (1977) 75–84.
7 C. D. Hurd, P. J. Baker, Jr., R. T. Holysz, and W. H. Saunders, Jr., *J. Org. Chem.*, 18 (1953) 186–191.
8 D. H. Rammler, *Biochemistry*, 10 (1971) 4699–4705.
9 J. T. Powell and K. Brew, *Biochemistry*, 15 (1976) 3499–3505.
10 C. T. Evans, N. H. Goss, and H. G. Wood, *Biochemistry*, 19 (1980) 5809–5914.
11 S. B. Eastbrook-Smith, J. C. Wallace, and B. D. Keech, *Eur. J. Biochem.*, 62 (1976) 125–130.
12 S. N. Kotchetkov, T. V. Bulargina, L. P. Sashchenka, and E. S. Severin, *Eur. J. Biochem.*, 81 (1977) 111–118.
13 R. T. Borchardt, S. E. Wu, and C. S. Schasteen, *Biochem. Biophys. Res. Commun.*, 81 (1978) 841–849.
14 M. R. Gregory and E. T. Kaiser, *Arch. Biochem. Biophys.*, 196 (1979) 199–208.
15 P. K. Tsai, *Fed. Proc.*, 40 (1981) 1867.