Transalkylation of Phosphotriesters Using Cob(I)alamin: Toward Specific Determination of DNA−Phosphate Adducts

Johanna Haglund,*,† Adnan Rafiq,‡ Lars Ehrenberg,† Bernard T. Golding,‡ and Margareta Törnqvist§

Departments of Genetic and Cellular Toxicology and of Environmental Chemistry, Stockholm University, S-106 91 Stockholm, Sweden, and Department of Chemistry, Bedson Building, University of Newcastle upon Tyne, Newcastle upon Tyne NEI 7RU, U.K.

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The supernucleophilic cobalt compound, cob(I)alamin, has been kinetically characterized with respect to its ability to bring about transalkylation of adducts to DNA phosphates (phosphotriesters). The reactivity of cob(I)alamin toward different phosphotriesters (model compounds and methylated DNA), as well as its specificity toward DNA−phosphate adducts, has been investigated. Through nucleophilic displacement on the alkyl by cob(I)alamin, the alkyl groups (methyl and ethyl) were transferred from phosphotriesters within minutes at room temperature. In contrast, methylated nucleosides (base adducts) were stable in the presence of cob(I)alamin.

Introduction

There is a need for a method for the determination of DNA adducts, which permits not only their quantification but also their identification through analysis by mass spectrometry. Existing methods comprise mainly assessment of DNA−base adducts and to some extent DNA−phosphate adducts. In comparison with DNA base adducts, phosphate adducts could, as suggested earlier (*1*), be more useful for the determination of exposures to genotoxic chemicals. This is because they are chemically relatively stable under physiological conditions (*2*-*4*) and are not subject to effective repair.

It has been demonstrated by Haglund et al. (*5*) that it is possible by means of relatively strong nucleophiles to transfer alkyl groups (adducts) from DNA phosphates to give alkyl−nucleophile products that can be analyzed (Scheme 1). This was shown with model phosphotriesters (PTEs)[1] and for DNA methylated by *N*-methyl-*N*-nitrosourea (MNU). With the nucleophiles that were studied, mainly aniline and thiosulfate, a complete (10 half-lives) transfer of alkyl groups was attained. However, the reactions were too slow to be practically useful as a method for the determination of DNA−phosphate adducts.

We have now investigated whether it is possible to apply a more reactive nucleophile, cob(I)alamin (vitamin B_{12s}; Figure 1), to attain a sufficiently fast and, still, specific transfer of DNA−phosphate adducts. The species cob(I)alamin is known to be highly nucleophilic in both chemical (*6*) and enzymatic reactions (*7*). Because of its high reactivity, this cobalt(I) species has been described as a supernucleophile (*8*), with a nucleophilic strengthof

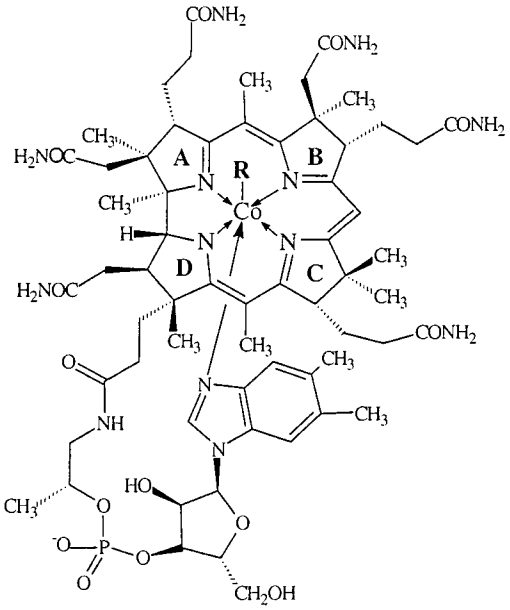
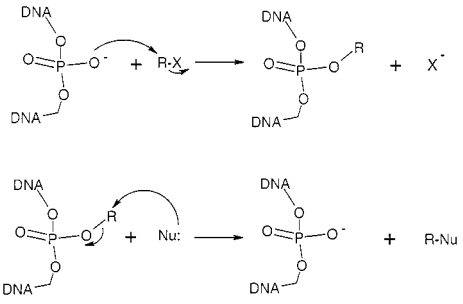


Figure 1. Structure of cobalamins. R is a variable ligand [e.g., OH in hydroxocobalamin; Me in methylcobalamin; an electron pair in cob(I)alamin].

Scheme 1. Illustration of a Nucleophilic Transalkylation of DNA Alkylated at Phosphate O



14.4 on the Pearson scale (*9*). This corresponds to $n \approx 10$ on the Swain−Scott scale (*10*), and therefore, cob(I)-

* To whom correspondence should be addressed.
† Department of Genetic and Cellular Toxicology, Stockholm University.
‡ Department of Chemistry, University of Newcastle upon Tyne.
§ Department of Environmental Chemistry, Stockholm University.
[1] Abbreviations: PTE, phosphotriester; DMS, dimethyl sulfate; DMP, dimethyl phosphate; TMP, trimethyl phosphate; TpMeT, thymidine 3′-[thymidine 5′-(methyl phosphate)]; TpEtT, thymidine 3′-[thymidine 5′-(ethyl phosphate)]; MNU, *N*-methyl-*N*-nitrosourea.

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alamin is expected to react about 5000 times faster than the most reactive standard nucleophile, thiosulfate (n = 6.36), at s values of ≈1. Many alkylating agents react with cob(I)alamin to produce Co-alkylcobalamins (*11*) in reactions that can be formulated as nucleophilic displacement processes. The question arises as to whether this nucleophile will fulfill the criteria necessary for a method for the determination of DNA−phosphate adducts. These criteria are as follows:

The alkyl group at phosphates in DNA has to be a substrate for displacement by the nucleophile.

The alkyl−oxygen bond of the adduct in the PTE should be the only one cleaved.

The rate of the transfer should be sufficiently high for analytical applications.

A sufficient and reproducible yield of the alkyl−nucleophile product should be obtained.

The specificity toward phosphate adducts in DNA should be high.

The alkyl−nucleophile product formed should be separable from the unmodified nucleophile and be identifiable and quantifiable by mass spectrometric methods.

In the study presented here, reactions between cob(I)alamin and different PTEs, including ^{3}H-methylated DNA, have been kinetically characterized with regard to transfer of the alkyl group, with fulfilment of the first five of the criteria listed above.

Experimental Procedures

Caution: *The following chemicals are hazardous and should be handled carefully (12): dimethyl sulfate (DMS) and N-methyl-N-nitrosourea (MNU).*

Materials. The following chemicals have been used: cobalt(II) nitrate, hydroxocobalamin, DNA (calf thymus), methylcobalamin, 7-methylguanine, 3-methyladenosine, O^6-methyl-2-deoxyguanosine, adenosine, and 2-deoxyguanosine from Sigma (St. Louis, MO), thymidine 3′-[thymidine 5′-(methyl phosphate)] (TpMeT) and thymidine 3′-[thymidine 5′-(ethyl phosphate)] (TpEtT) from Campro Scientific (Stockholm, Sweden), sodium borohydride, dimethyl phosphate (DMP), trimethyl phosphate (TMP), and dimethyl sulfate from Acros Organics (Geel, Belgium), and *N*-[^{3}H]methyl-*N*-nitrosourea from Amersham Pharmacia Biotech (Uppsala, Sweden).

Chromatography. All reactions were followed by reverse-phase HPLC, which was carried out on a SERIES 200LC pump (Perkin-Elmer), equipped with a 759A absorbance detector (Applied Biosystems), set at 254 or 267 nm. Reverse-phase chromatography was performed with a Hichrom Kromasil 100-5C-18 column (4.6 mm × 250 mm). The mobile phases used in the HPLC analysis were as follows. For system 1, solution A was 50 mM KH_2PO_4 (pH 5) and solution B 25% acetonitrile (aqueous v/v). The gradient was 5% B for 5 min, from 5 to 100% B within 20 min, and 100% B for 10 min. For system 2, solution A was water and solution B methanol. The gradient was from 5 to 100% B within 20 min. In all analyses, system 1 was used, except for the determination of the level of 7-methylguanine.

Methylation of DNA. DNA was methylated with [^{3}H]MNU (15.3 Ci/mmol) according to the method of Swenson and Lawley (*2*). DNA was also methylated with 88 mM DMS in a 0.38 M Tris buffer (pH 7.55) at 37 °C. Following reaction with either MNU or DMS for 4 h, the DNA was precipitated by adding ice-cold ethanol (2 volumes) to the sample, and after the precipitate had been stored in the freezer for 0.5 h, the DNA was removed by a Pasteur pipet, washed in ethanol (70%), and dissolved in a TE buffer [10 mM Tris and 1 mM EDTA (pH 8)]. The precipitation and washing were repeated twice. The level of 7-methylguanine in MNU- and DMS-treated DNA was determined by depurination, where 0.1 volume of 0.1 M citrate buffer (pH 6) was added to DNA (0.5−1 mg/mL) and the mixture heated at 100 °C for 20 min. The depurination was followed by analyzing samples by HPLC at different time points (5, 10, 15, and 20 min) to ascertain that the process was complete. For quantitation, the measurements were compared with a calibration curve for 7-methylguanine.

Preparation of the Cob(I)alamin. Hydroxocobalamin was dissolved in water at a concentration used in the actual experiment (7−10-fold excess). $Co(NO_3)_2$ (10 mol % of the hydroxocobalamin) was added as a catalyst for the reduction (*13*). The solution was degassed under argon for 20 min. After the reaction vessel had been closed with a septum, a continuous flow of argon was passed through the solution. Sodium borohydride (0.1−0.3 M) was added through the septum in a 7-fold excess to the hydroxocobalamin, to obtain complete reduction of Co(III) to Co(I). The reduced form, cob(I)alamin, should be used immediately after reduction as it is not indefinitely stable either under aerobic or under anaerobic conditions (*14*).

Reactions with Phosphotriester Model Compounds. The PTE model compound (TMP, TpMeT, or TpEtT) was dissolved in water (1−3 mM) and the mixture added to the cob(I)alamin solution (5−20 mM) during argon bubbling. Each reaction was followed by removing samples through the septum at different time points. Immediately after collection, air was bubbled into the sample to oxidize the remaining cob(I)alamin to cob(III)alamin. The methyl- and ethylcobalamin that were produced were analyzed by HPLC.

Reaction between Cob(I)alamin and [^{3}H]MNU-Methylated DNA. The alkylated DNA was added to the reduced cobalamin solution under argon bubbling. The reaction was followed by taking samples at different time points. DNA was precipitated by adding ice-cold ethanol (2 volumes) to the sample. The sample was kept in the freezer for 30 min. The sample was centrifuged, and the supernatant was evaporated under nitrogen prior to HPLC analysis. Fractions were collected at a rate of 1 mL/min, and the formation of [^{3}H]methylcobalamin was assessed by liquid scintillation counting.

Studies of the Specificity of Cob(I)alamin for Alkyl Groups at Phosphates in DNA. (1) DNA. DNA treated with DMS was depurinated, and the 7-methylguanine level was determined before and after incubation with cob(I)alamin. DMS-treated DNA (2 mL, 1.5 mg/mL) was depurinated as a control, and in parallel, 2 mL of DNA was depurinated after incubation with cob(I)alamin (2.23 mM) for three different incubation times (1, 1.5, and 2.5 h). The final time exceeds that needed for transalkylation of PTE to be essentially complete. The 7-methylguanine levels in DNA in the control and after incubation with cob(I)alamin were measured by HPLC.

(2) Methylated Nucleosides. 3-Methyladenosine and O^6-methyl-2′-deoxyguanosine were incubated for up to 1.5 h with an excess of cob(I)alamin in the same way as in the transalkylation experiment with [^{3}H]MNU-methylated DNA and model PTE. The reactions were terminated as described above for PTE. The possible formation of methylcobalamin was monitored by HPLC analysis of samples at different time points.

(3) Dimethyl Phosphate (DMP). A control for the assessment of the transalkylation of TMP was carried out with DMP. The DMP was added to a cob(I)alamin solution, and the reaction was followed by analysis as described above.

Results and Discussion

Rates of Reaction of Cob(I)alamin. The incubation of PTE compounds (TMP, TpMeT, and TpEtT) with cob(I)alamin demonstrated the formation of methyl- and ethylcobalamin. The reaction of cob(I)alamin with TpMeT is illustrated in Scheme 2, and a typical HPLC analysis, showing the formation of ethylcobalamin, is shown in Figure 2. The peak in the HPLC spectrum corresponding to methyl- or ethylcobalamin was not found in control

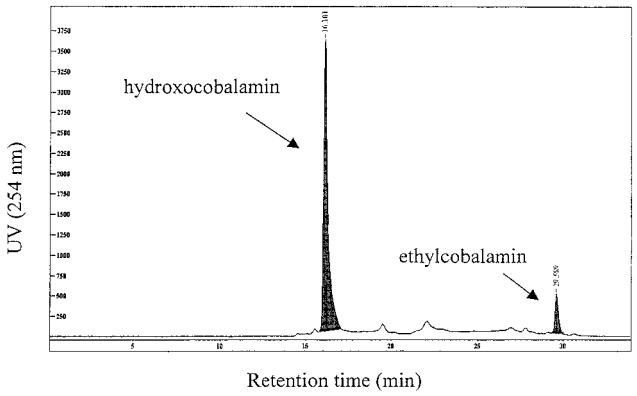
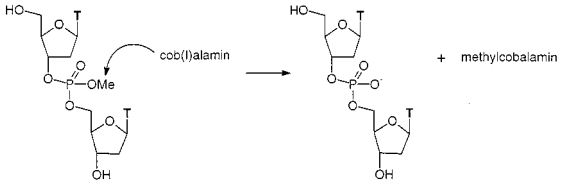


Figure 2. Typical HPLC analysis on the formation of ethylcobalamin.

Scheme 2. Illustration of the Transalkylation by Cob(I)alamin of TpMeT, Forming TpT and Methylcobalamin



samples. The corresponding decrease in the level of the PTE was plotted as the natural logarithm against time (Figure 3). The rate constants of the reactions were calculated from the slopes and are summarized in Table 1.

Cob(I)alamin was found to demethylate [^{3}H]MNU-treated DNA and the TpMeT at rate constants that were 4400−4700 times higher than that of thiosulfate.

According to the Swain−Scott relation (*10*), the ratio of the rates should be

$$\log(k_1/k_2) = s(n_1 - n_2) \qquad (1)$$

The substrate constant *s*, for methylated DNA and methyl PTE model compounds, was judged to be 0.84 (*5*), i.e., somewhat lower than the *s* value of 0.91 determined for TMP (*15*). In the Swain−Scott system, the nucleophilic strength of thiosulfate $n_2 = 6.36$ (*16*). Solving eq 1 after insertion of $k_1/k_2 = 4700$ for TpMeT and this value of n_2 for thiosulfate gives an *n* value for cob(I)alamin (n_1) of 10.7. This value is in agreement with that estimated by the rule of thumb division of the Pearson value (n = 14.4) by a factor 1.4 (*17*). It should be noted that there is an uncertainty about the range of the validity of this factor.

The two nucleophiles that are compared [cob(I)alamin, Figure 1, and thiosulfate, $S_2O_3^{2-}$] are both negatively charged (−1 and −2, respectively). Their rates of reaction with TpMeT, which is electroneutral, are not influenced by this charge. However, the rate of transalkylation of methylated DNA, a polyanion, is reduced with both thiosulfate and cob(I)alamin. The influence of the charges is expected to lead to a repulsion due to the Coulomb forces, with lower rate constants as a consequence (*18*, *19*).

It should be noted that the rate constants are still subject to considerable uncertainty. For instance, the rates of reaction of thiosulfate at 22 °C were extrapolated from measurements at 37 °C, on the basis of a rough estimation of E_a. However, for the purpose of using the fast transalkylation of alkyl groups by cob(I)alamin for specific determination of DNA−phosphate adducts, this uncertainty has no practical importance.

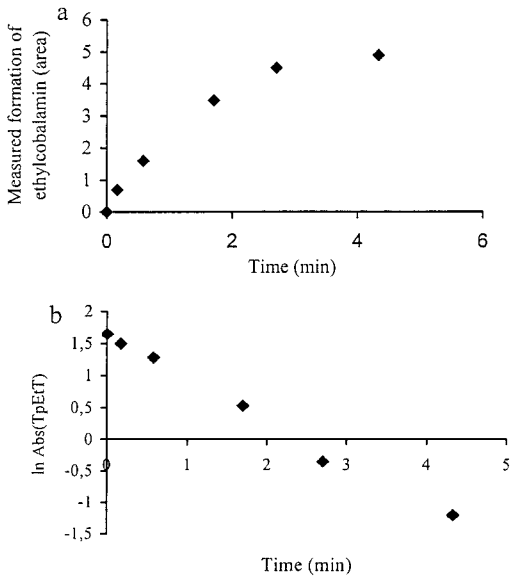


Figure 3. Measurement of reaction rates, exemplified by (a) the reaction between cob(I)alamin and TpEtT showing the formation of ethylcobalamin plotted against time and (b) the natural logarithm of the inferred amount of remaining TpEtT plotted against time.

Table 1. Summary of Second-Order Rate Constants for Transalkylation of a Phosphotriester by Cob(I)alamin and, for Comparison, by Thiosulfate

phosphotriester	rate constant for reaction with cob(I)alamin (22 °C) (dm^3 mol^{-1} s^{-1})	rate constant for reaction with thiosulfate (5) (22 °C) (dm^3 mol^{-1} s^{-1})	$k_{cob(I)alamin}/k_{thiosulfate}$
TMP	0.22[a]	1.1×10^{-4} [b]	2000
DMP	0[c]		
TpMeT	0.61[a]	1.3×10^{-4} [b]	4700
TpEtT	0.022[a]		
methylated DNA	0.11[d]	0.25×10^{-4} [b]	4400

[a] The yield was 100% with a range of ±2%. [b] The rate constants for thiosulfate at 37 °C were extrapolated to room temperature (22 °C) using an activation enthalpy E_a of 70 kJ mol^{-1} determined for the reaction of TpMeT. [c] A control reaction for that between TMP and cob(I)alamin, confirming only one methyl group being transferred from TMP. [d] The radioactivity released as [^{3}H]methylcobalamin corresponded to that expected (12−15%) at phosphate in DNA after [^{3}H]MNU treatment.

Specificity and Efficiency. The different methylated nucleosides studied in the presence of cob(I)alamin showed no formation of methylcobalamin (according to the detection limit, the extent of transfer of methyl groups is less than 0.2%). 3-Methyladenosine and O^6-methyl-2-deoxyguanosine were stable for 1.3−1.5 h, and 7-methylguanine was stable for 2.5 h, i.e., times exceeding the time required for complete transalkylation of alkyl groups from studied PTE. The inability of cob(I)alamin to demethylate O^6-methylguanosine contrasts with the DNA repair protein O^6-methylguanine-DNA methyltransferase (*20*).

In all the experiments whose results are presented herein, where alkyl groups have been transferred from PTE to cob(I)alamin, the amount of Co-alkylcobalamin formed after completion of the reaction corresponds within a range of ±2% to the initial amount of the PTE.

This shows the high efficiency of cob(I)alamin in transalkylation of an alkyl group from a PTE. The agreement of the amount of [^{3}H]methylcobalamin that was formed, after transalkylation of ^{3}H-methylated DNA to cob(I)alamin, with published data (*21*) for the level of methyl groups bound to phosphates in DNA after MNU treatment shows not only the high efficiency but also a specificity for phosphate alkylations. If methyls were transferred from other sites in DNA, this value would have been higher, and in addition, the kinetics would have deviated from the observed pseudo-first-order type. The stability of methylated nucleosides in the presence of cob(I)alamin during time further confirms the specificity of cob(I)alamin.

In the reactions between cob(I)alamin and the DNA model compound PTE (methyl- and ethylthymidyl phosphates), TpT was the only detectable leaving group. This shows that displacement on the deoxyribose carbons was negligible as expected.

Analytical Applications, and Sensitivity. In its present state, procedures for assessing DNA-P adducts by transalkylation to cob(I)alamin can be applied to animal experiments for analysis by HPLC and detection with radioactivity or UV detection (detection limit of 0.01−0.05 nmol; cf. ref *22*). Cobalamins have been determined by MS techniques with detection levels in the femtomole range (*22*), indicating the applicability to certain occupational exposures. For studies of background adduct levels in individuals without known environmental exposure, a further development of MS techniques toward higher sensitivity is required. From the experience of protein adduct analysis, it is concluded, however, that detection levels around 100 amol may be reached with MS techniques.

Conclusion

Five of the six criteria set out in the Introduction for a nucleophile being useful for PTE transalkylation have been fulfilled. The alkyl group in a PTE was shown to be the substrate for nucleophilic displacement by cob(I)alamin. The rates of transalkylation as well as the obtained yield of the alkyl−nucleophile product show the suitability of cob(I)alamin as a nucleophile for transferring an alkyl group from a PTE. Specificity toward PTE has been demonstrated, and it has been shown to transfer methyl adducts from PTE in MNU-treated DNA. With regard to the sixth criterion, quantification at higher sensitivity and for identification of adducts from PTE in DNA, mass spectrometric methods are being developed, with good prognosis.

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