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Acid binding and detritylation during oligonucleotide synthesis

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ABSTRACT

Under the conditions normally used for detritylation in oligonucleotide synthesis, the haloacetic acid binds strongly to the oligonucleotide. Acetonitrile also forms a complex with the deblocking acid, in competition with the oligonucleotide, and drastically slows detritylation. Incomplete removal of acetonitrile during the deblock step may slow the kinetics enough to result in incomplete detritylation of the oligonucleotide. Acid binding to the growing oligonucleotide causes striking chromatographic effects in the presence of high oligonucleotide mass densities. In packed-bed column reactors, at low linear velocities, the acid binding almost completely depletes free acid from the deblocking solution. This results in an advancing zone within which the oligonucleotide is saturated with acid. Detritylation occurs mostly in a narrow band at the front of the advancing saturated zone. Increasing the DCA concentration in order to achieve quick saturation can give faster and more complete detritylation while minimizing the exposure time of the oligonucleotide to acid.

INTRODUCTION

High product yields from oligonucleotide synthesis require quantitative removal of the dimethoxytrityl (DMT) group during the deblocking step. Incomplete deblocking causes sequence deletions that are not terminated in the capping step, the resulting failures of which continue to propagate during subsequent coupling cycles. There is increasing evidence (1,2) that the deblocking step is sometimes incomplete.

Most oligonucleotide synthesis protocols use a low concentration of the deblocking acid (3,4) to minimize the risk of depurinating the oligonucleotide. As a result, the deblocking step is usually the bottleneck of the synthesis cycle. As syntheses at much larger scales have become common, the strategy of using dilute acid has resulted in the generation of large volumes of chlorinated waste with the accompanying high costs of disposal.

This report demonstrates that the acid used in the detritylation reaction binds strongly to the oligonucleotide during the deblocking step. Massive acid binding to the oligonucleotide results in large transients in the acid concentration. An accompanying paper (5) measures the kinetics of depurination by haloacetic acids to assess the probable damage resulting from higher (and uncontrollable) acid concentrations occurring during oligonucleotide synthesis. Accounting for the consequences of acid binding appears to be important for achieving high product yields in synthesis.

MATERIALS AND METHODS

DNA synthesis reagents

Acid binding experiments used DNA synthesized on high load CPG (100 μmol/g). The DNA sequences were based on the following standard oligonucleotide:

5′-TCAATTGAGCCTACGGT-3′ (A)

or on an oligonucleotide containing half of this sequence:

5′-GCCTACGGT-3′ (B)

Oligonucleotides were prepared on CPG on an 8800 synthesizer from PerSeptive Biosystems (Framingham, MA). The β-cyanoethyl phosphoramidites and high load CPGs used for DNA synthesis were from PerSeptive Biosystems. Dichloroacetic acid (DCA) and trichloroacetic acid (TCA) were purchased from Aldrich (Milwaukee, WI). Methylene chloride and dry acetonitrile were from EM.

Analyzing binding isotherms

Binding isotherms are all illustrations of the law of mass action. Binding curves can take several forms depending on the complexity of the binding reaction under study and how it is being measured (6). The following experiments determine binding isotherms describing the amount of acid retained by the oligonucleotide and isotherms describing the competition for the deblocking acid between the oligonucleotide and acetonitrile. This competition produces significant chromatographic and kinetic effects observed during the detritylation step.

If we denote the ligand (i.e., the haloacetic acid) by X and a binding site on the oligonucleotide by M, then the reaction equation is

$$M + X \rightarrow MX \quad \mathbf{1}$$

At saturation, the number of binding sites M can be estimated from the amount of ligand X removed from solution. The mass action binding constant is

$$K_1 = \frac{[MX]}{[M]_x} \quad \mathbf{2}$$

where x is the activity of the acid. The degree of binding for the simple binding isotherm can be written:

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$$\overline{X} = \frac{[MX]}{[M] + [MX]}$$

$$= \frac{K_1 x}{1 + K_1 x} \quad \textbf{3}$$

The amount of DCA that binds to acetonitrile is deduced from its influence on acid binding to the oligonucleotide. This requires a more specific binding model, such as 1:1 or 2:1 complex between acid and acetonitrile. The reaction equation describing a 1:1 complex can be written:

$$X + Y \rightarrow XY \quad \textbf{4}$$

where Y is acetonitrile, and the XY complex cannot bind to the oligonucleotide or participate in detritylation. The mass action binding constant for this competing reaction is

$$K_2 = \frac{[XY]}{y \cdot x} \quad \textbf{5}$$

where y is the acetonitrile activity. The acid concentration in solution now consists of two components:

$$x' = x + [XY]$$
$$= x \cdot (1 + K_2 \cdot y) \quad \textbf{6}$$

The activity of the DCA entering into formula **3** is therefore:

$$x = \frac{x'}{(1 + K_2 \cdot y)} \quad \textbf{7}$$

which shows that acetonitrile lowers the apparent binding constant of the acid for the oligonucleotide. The total acetonitrile concentration is

$$y' = y + [XY] \quad \textbf{8}$$

In the presence of acetonitrile, the activity of DCA (i.e., can be determined from expression **2** by measuring the DCA 'lost' from solution through binding to the oligonucleotide, which directly gives [*MX*]. Then

$$x = \frac{[MX]}{K_1 \cdot ([M_0] - [MX])} \quad \textbf{9}$$

where $[M_0]$ is the known number of binding sites on the oligonucleotide.

As we know both x and x′, we also know [*XY*] and can obtain

$$K_2 = \frac{[XY]}{x \cdot (y' - [XY])} \quad \textbf{10}$$

A 2:1 complex formed by binding a second DCA molecule to the 1:1 complex may best describe the binding behavior, but this cannot be developed into a testable model in the absence of a way to measure the concentrations of both complexes. We can look at a model containing the single 2:1 complex:

$$2X + Y \rightarrow X_2Y \quad \textbf{11}$$

The mass action binding constant for this reaction can be found in terms of measurable variables in the same way as was shown with expression **10**:

$$K_3 = \frac{[X_2Y]}{x^2 \cdot (y' - [X_2Y]} \quad \textbf{12}$$

where

$$x' = x + 2[X_2Y] \quad \textbf{13}$$

$$y' = y + [X_2Y] \quad \textbf{14}$$

The binding isotherms for this model can be calculated by solving equations **12**, **13** and **14** for x, which results in a cubic equation with coefficients expressed in x′ y′ and K_3.

Measuring binding isotherms

Deblock solutions were prepared by weighing dichloroacetic acid or trichloroacetic acid into volumetric flasks and adding methylene chloride (or an appropriate mixed solvent) to make the volume up to 10.0 ml. The deblocking acid concentrations ranged from 0.025 to 2.0 N, and were always checked by titration.

For acid binding measurements, 4.0 ml of a deblock solution was added to each 1.000 ± 0.003 g sample of CPG-x and allowed to reach equilibrium for a period of 30 min. The free acid concentration was measured by titrating it with a suitable concentration of aqueous NaOH.

Measurements of competition binding isotherms required measurements of both acetonitrile and DCA concentrations in equilibrium with the oligonucleotide (**8**). The low volatility of TCA precluded its use in these experiments, which were made on a Hewlett-Packard 5890 Series II Gas Chromatograph, using an HP-17 (50% Phenyl–50% Methylpolysiloxane; 10 m, intermediate polarity) capillary column and the thermal conductivity detector of the instrument. The carrier gas was Helium at a flow rate of 4.0 ml/h. The elution program began at 30°C for 2.0 min to resolve methylene chloride and acetonitrile, followed by a ramp to 180°C over an interval of 5.0 min to elute the DCA.

Measuring detritylation kinetics

The detritylation kinetics were measured on a Hewlett-Packard 8452A Diode Array Spectrophotometer, collecting absorbance data at 498 nm and at a frequency of 1 Hz. The experiments used 10 µM phosphoramidite (dissolved in methylene chloride) in DCA concentrations high enough to ensure pseudo first order detritylation kinetics. Experiments were performed by mixing 20 µl of the phosphoramidite in 1.0 ml of the acid solution. All experiments were analyzed as single exponential decays according to the formula:

$$\frac{d}{dt}[DMT] = k_1\{1 - exp(-k_2[DCA]t)\} \quad \textbf{15}$$

where k_2 is a rate constant for detritylation in a given solvent, and k_1 is proportional to the amidite concentration.

Spectroscopic identification of the DCA/acetonitrile complex

An effort to identify the DCA/acetonitrile complex proposed to explain the behavior of the binding isotherms was carried out on a Nicolet 205 FTIR spectrometer. Solutions containing methylene chloride, acetonitrile and dichloroacetic acid in different proportions were sandwiched between KBr plates, and 8 scans per spectrum were taken in the region from 4000 to 400 cm^{-1}, at a resolution of 2 cm^{-1}, with particular attention to the range between 2400 and 1600 cm^{-1}.

RESULTS AND DISCUSSION

DCA and TCA binding to oligonucleotides in methylene chloride

The binding capacity of DCA to the oligonucleotide in methylene chloride, determined from the saturation asymptote of the binding isotherm, is proportional to the length of oligonucleotide. Table 1 shows that binding roughly corresponds to one molecule of acid per monomer unit. The binding affinity does not change with the length of the oligonucleotide. The amount of acid bound to the oligonucleotide during each successive cycle of a synthesis tracks the overall crude product yield.

Table 1. Parameters of the isotherms describing binding of haloacetic acids to oligonucleotides

Conditions	Binding constant (N^{-1})	Binding capacity (μmol)
DCA, 17mer[a]	17.8	2.4
DCA, 9mer[b]	18.0	1.3
TCA, 17mer[a]	44.0	2.4

[a]Sequence is 5′-TCAATTGAGCCTACGGT-3′.
[b]Sequence is 5′-GCCTACGGT-3′.

The binding capacity for the oligonucleotide is the same for both DCA and TCA, although TCA has a much higher affinity for the oligonucleotide than DCA.

Binding isotherms modulated by acetonitrile

The competition binding experiments used deblocking solutions with acetonitrile concentrations from 0.50 through 5.0 M and DCA concentrations x′ of ~0.28 N (in equilibrium with the CPG). GC analysis of the deblocking solutions before and after exposure to the CPG shows that the concentration of acetonitrile in the deblocking solution is unchanged, whereas DCA concentration is significantly reduced by binding to the oligonucleotide. Table 2 shows initial and final deblock concentrations, where x′(0) is the concentration of DCA prior to adding the deblocking solution to CPG, and x′ is the concentration of DCA in equilibrium with the CPG. The corresponding values of K_2 for a 1:1 complex decrease monotonically with increasing [ACN], indicating a failure of this model to describe the binding behavior. The values of K_3 for a 2:1 complex are roughly constant over a 10-fold range of acetonitrile concentrations, indicating that this model is an acceptable description of the binding.

Table 2. Competition between acetonitrile and the oligonucleotide for DCA

[ACN]	x′(0)[a]	x′[b]	K_2[c]	K_3[d]
0.50	0.631	0.297	6.9	70
1.00	0.576	0.286	4.1	60
2.00	0.511	0.289	3.5	86
5.00	0.420	0.266	2.2	96

[a]Initial [DCA] before adding CPG-oligo.
[b]Final [DCA] depleted by acid binding.
[c]Binding constant for a 1:1 complex.
[d]Binding constant for a 2:1 complex.

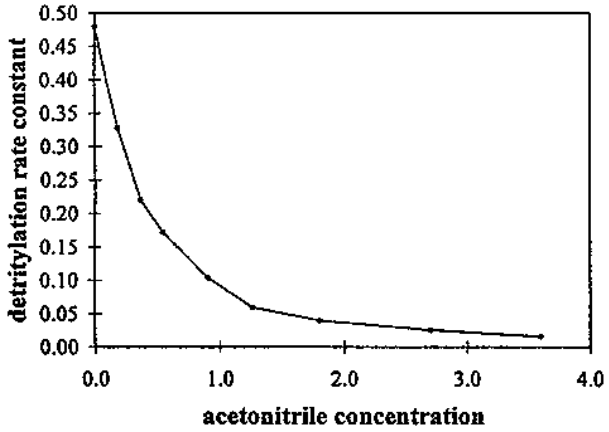

Figure 1. The detritylation rate declines rapidly in the presence of acetonitrile. A 2% solution of acetonitrile is 0.36 M.

Spectroscopic identification of the DCA/acetonitrile complex

At 2292 cm^{-1}, acetonitrile has a strong vibrational band when it is neat or in dichloromethane solution. Upon addition of DCA to the solution, this band shifts to 2269 cm^{-1}. As more DCA is added, the peak at 2269 cm^{-1} grows at the expense of the 2292 cm^{-1} peak. This 23 cm^{-1} shift is characteristic of hydrogen bonding interactions, indicating a hydrogen bonded complex between acetonitrile and DCA.

DCA displays a strong C=O stretching vibration at 1746 cm^{-1}, both neat and in dichloromethane solution. This has been assigned to the C=O stretching vibration of the hydrogen bonded DCA dimer. Upon addition of acetonitrile to the solution, this band shifts to 1763 cm^{-1}, a higher frequency characteristic of the DCA monomer. This 17 cm^{-1} shift is consistent with the breakup of the carboxylic acid dimer concomitant with the formation of a DCA/acetonitrile complex. The exact structure of the DCA/acetonitrile complex cannot be deduced from the FTIR spectra.

Kinetics of detritylation in methylene chloride mixed with acetonitrile

The rate of detritylation is reduced drastically in the presence of acetonitrile. The curve of Figure 1 was measured at a fixed DCA concentration of 0.25 N while increasing the concentration of acetonitrile from 0.0 up to 3.6 M.

The practical consequence of this behavior can be seen at the start of the deblocking step, when the reactor bed is still steeped in acetonitrile. The acetonitrile complexes with the DCA, resulting in a slow start of the deblocking reaction because the concentration of free DCA remains very low. These binding results confirm observations that washing the reactor with methylene chloride prior to the deblocking step improves the initial rate of detritylation (especially in batch reactors). Very thorough removal of acetonitrile is essential for repeatable performance of the detritylation step.

The binding models of DCA to the oligonucleotide and to acetonitrile permit a much closer scrutiny of the mechanism of the detritylation reaction. The extent of binding of DCA to the oligonucleotide was calculated (using the 2:1 complex model) in Figure 2a. The total DCA concentration was fixed at 0.25 N, and

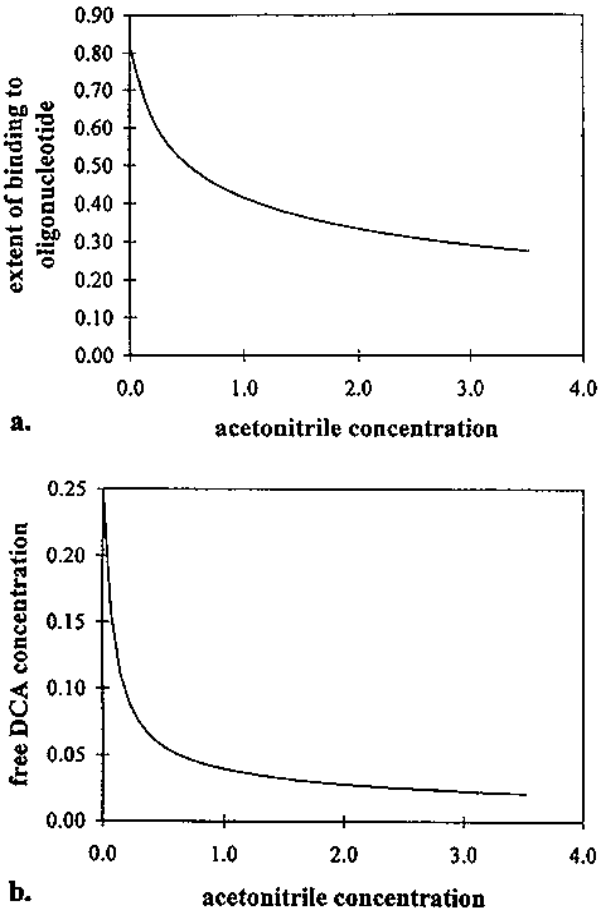

Figure 2. Detritylation kinetics caused by DCA bound to the oligonucleotide would be different from detritylation kinetics caused by free DCA in the presence of acetonitrile. (**a**) The extent of bound DCA does not track observed detritylation kinetics. (**b**) The calculated free DCA concentration does track observed detritylation kinetics in the presence of acetonitrile.

the acetonitrile concentration was allowed to vary from 0.0 to 3.5 M, covering deblocking conditions similar to the detritylation kinetics of Figure 1. The extent of binding is not asymptotic to zero at these concentrations, suggesting that DCA bound to the oligonucleotide is not involved in the detritylation reaction.

The free DCA concentration is plotted against the acetonitrile concentration in Figure 2b. In contrast to the behavior of the DCA binding, this curve is rapidly asymptotic to zero and tracks the detritylation kinetics more closely. It suggests that the detritylation rate is directly proportional to the DCA activity in solution, and that DCA bound up with either the oligonucleotide or acetonitrile is effectively removed from participation in the detritylation reaction.

Acetonitrile is the washing solvent that both precedes and follows the deblocking step. Although complexing of DCA by acetonitrile interferes with the detritylation reaction, it also makes acetonitrile highly effective as a wash solvent after deblocking by actively removing the bound haloacetic acid from the oligonucleotide. Exhaustive acetonitrile washing to remove acid after the deblocking step appears to be superfluous. Most other common solvents (e.g., alcohols) also appear to complex with DCA, which may explain why methylene chloride has remained the preferred solvent for deblocking oligonucleotides.

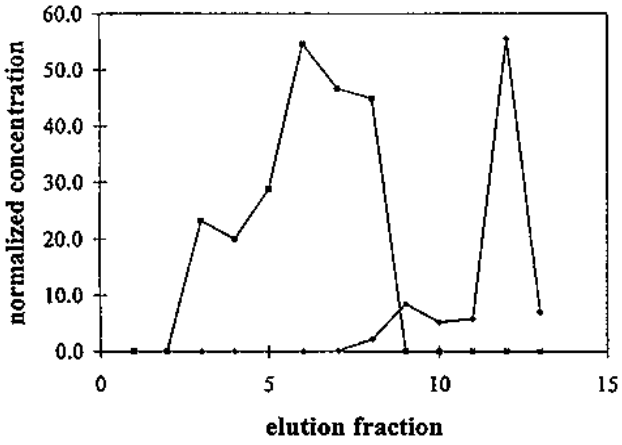

Figure 3. Elution profile of DCA and dimethoxytrityl emerging from a reactor during the deblocking step. The lefthand trityl peak emerges while the bed is becoming saturated with DCA. The DCA front emerges with fraction 8.

Chromatographic behavior of the deblocking step

Efficient and rapid exchange of reagents during oligonucleotide synthesis is best achieved by a reactor structured like a chromatography column. If the linear velocity of the deblocking solution is slow enough to permit diffusional equilibrium to the center of the CPG particles, the acid binding to the oligonucleotide is strong enough to deplete the methylene chloride of acid. This creates a zone in the reactor saturated with acid, preceded by a zone of methylene chloride containing too little acid to give perceptible detritylation. The detritylation reaction takes place mostly at the frontal boundary of the saturation zone, where the free DCA concentration also rises abruptly. The amount of acid bound to the support increases in each successive coupling cycle in proportion to the mass of DNA, causing the frontal boundary to move more slowly in each successive cycle. Acid binding to the oligonucleotide is exothermic, and the amount of heat released in this reaction is also proportional to the amount of DNA. The hot zone coincides with the saturation front, and sometimes reaches temperatures high enough to boil the methylene chloride (b.p. 39.8°C) unless the system is under pressure.

Pronounced chromatographic behavior is observed most easily at large synthesis scales, where the mass density of the oligonucleotide is large and the linear velocities of reagents through the reactor are small. Figure 3 shows a typical elution profile from an 8 mmol synthesis in which the concentrations of DCA (diamond) and DMT (square) were estimated by NMR. The first appearance of DCA at fraction 8 coincides with the emergence of the saturation zone from the reactor. The DCA concentration rises to the inlet DCA concentration (2.5%) for several fractions. In this case, the acetonitrile wash commences at fraction 9, and the emergence of the first acetonitrile in fraction 12 is accompanied by a pulse of DCA released by acetonitrile that reaches a concentration of almost 25%!

Oligonucleotide synthesis

Numerous oligonucleotide syntheses at scales between 1 and 8 mmol, using flow-through mechanics for the deblocking reaction, have shown equal or higher crude product purities (measured by ion exchange HPLC) when deblocked with higher DCA concentrations.

For example, a comparison of the cycle efficiencies for two syntheses of the oligonucleotide standard (A), each at a scale of 5 mmol, gave a cycle efficiency of 97.7% for 2.5% DCA and a cycle efficiency of 98.3% at 15% DCA.

The amount of deblock solution required to complete the detritylation reaction is determined by the mass of the oligonucleotide in the reactor. Both increase throughout the course of the synthesis. Use of acid concentrations higher than those traditionally employed allows the reactor bed to be saturated more quickly, promoting faster detritylation. The acid can then be promptly removed with acetonitrile, minimizing depurination. This observation is supported (5) by measurements of the depurination rates of various CPG-bound oligonucleotide intermediates. DCA is preferred to TCA as the deblock acid because the molar exposure required to reach saturation is similar for both acids, whereas the stronger acidity of TCA makes depurination a greater concern.

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