DNA Hydrolysis: Mechanism and Reactivity

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1 Introduction

It is essential that the primary, linear structure of DNA is stable to preserve the sequence of bases and hence the genetic code. The bases are assembled as nucleotide units, joined together by phosphate diester links. Given the need to permanently retain genetic information, it is not surprising that the phosphate diester is far more kinetically stable than other common biological functional groups such as amides or esters (Westheimer 1987). However, the backbone must also be cleavable to facilitate the synthesis, manipulation and repair of DNA. This role is naturally carried out by nucleases, which catalyse the cleavage of DNA either by hydrolysis of the phosphate diester bond (with varying degrees of sequence specificity) or by catalysing elimination of phosphate (and destroying one of the nucleotide units). The purpose of this brief review is to summarise the mechanisms relevant to DNA hydrolysis and in particular to estimate the rate of hydrolysis through attack at the phosphate diester under mild, aqueous conditions.

2 The Importance of the Background Reaction

Enzyme-catalysed reaction rates, whether measured through v_{max} or v_{max}/K_m, span a rather small range and so it might appear that enzymes have broadly similar efficiencies. However, the range of rate differences in the corresponding reactions in the absence of catalysis is enormous. If the efficiencies of enzymes are gauged by rate acceleration instead, it becomes apparent that they actually cover a huge range which depends primarily on the corresponding rate of the background reaction and the most efficient enzymes are those that catalyse the slowest reactions (Wolfenden and Snider 2001). Phosphate

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Nucleic Acids and Molecular Biology, Vol. 13
Marina A. Zenkova (Ed.)
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diester hydrolysis falls within this category and quantitative data about this slow reaction is required to reasonably interpret how phosphohydrolases work. In the context of artificial nucleases, it also defines the target that must be achieved to generate a viable artificial catalyst that can function in the same way.

3 Mechanism

Before we can usefully consider the data concerning reactivity, especially with a view to understanding (and directing the design of) catalysts, we need to consider the mechanism(s) of hydrolysis. A considerable body of data has accumulated from physical organic chemistry studies on the substitution reactions of phosphate diesters, and this has been extensively reviewed (Westheimer 1968; Benkovic and Schray 1973; Knowles 1980; Thatcher and Kluger 1989; Gerlt 1992; Gerlt 1993; Anslyn and Perreault 1997; Oivanen et al. 1998). Hence, only the key points will be noted here.

The reaction is an associative process, involving either a pentacoordinate intermediate or transition state. (These are A_N+D_N and A_ND_N mechanisms respectively, according to the IUPAC nomenclature.) For monoesters, a spontaneous unimolecular reaction (D_N+A_N) is a viable pathway (Fig. 1A, X=negative charge); even here, it is thought that the metaphosphate is not sufficiently stable to be a full, diffusable intermediate and the substitution reaction only takes place when a nucleophile is pre-associated with the phosphate (Jencks 1980, 1981). Thus, even for monoesters, the current consensus is of a transition state where there is a significant bonding to both nucleophile and leaving group in the transition state particularly in the enzyme catalysed

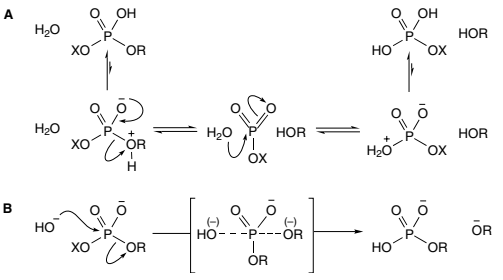

Fig. 1. A Dissociative (D_N+A_N) mechanism for phosphate mono- and diester- hydrolysis. For monoesters, X=negative charge; for diesters, X=R. This pathway accounts for data concerning monoester hydrolysis, but the alkyl metaphosphate intermediate is thought to be too high in energy for this to be a significant contribution to diester hydrolysis (Guthrie 1977). **B** Associative (A_ND_N) mechanism for phosphate diester hydrolysis showing a pentacoordinate transition state with significant bonding to both nucleophile and leaving group

processes. Mildvan (1997) has reviewed this area recently, and evaluates the spectrum of transition states available more precisely in terms of structure. As the metaphosphate ester will be substantially less stable than metaphosphate, this pathway (Fig. 1A, X=R) is not believed to be viable for diesters (Gerlt 1993; Guthrie 1977). Thus, substantial bonding to the nucleophile in the transition state appears to be essential for diester hydrolysis, particularly involving poor leaving groups (Fig. 1B). This can be evaluated by measuring the variation in the reaction rate with the pK_a of the nucleophile, i.e. through a Brønsted plot. If there is substantial bond formation to the nucleophile, then this plot will have a steep slope (which is the β_{nuc} parameter). This is observed for displacement of methoxide by phenoxide in an intramolecular model system (Dalby et al. 1993).

Although phosphoranes may not be stable intermediates when good (aryloxy) leaving groups are involved (Ba-Saif et al. 1990), monoanionic or neutral phosphoranes exist as kinetically significant intermediates when they have alkoxy substituents. This is exemplified by the observations of isomerisation in RNA and related compounds (Westheimer 1968; Thatcher and Kluger 1989; Oivanen et al. 1998). The presence of a phosphorane intermediate provides the most satisfactory explanation for the retention of configuration at phosphorus after isomerisation and in some substitution reactions: intermediate formation followed by pseudorotation and finally leaving group expulsion accounts for this stereochemical observation (Westheimer 1968). However, it is interesting to note that no biological catalysts have been shown to exploit this. Retention of configuration at phosphorus has so far been shown to be the result of a double substitution process, each proceeding with inversion (Knowles 1980; Gerlt 1993).

However, there is still debate concerning the stability of the dianionic phosphorane, and whether it is a true intermediate. It should be noted that experiments to demonstrate the presence of an intermediate unambiguously rely on secondary processes such as pseudorotation and/or protonation, so a lack of racemisation or isotope exchange into the diester does not indicate that the dianion cannot be an intermediate, but that breakdown to starting material or product is more rapid than pseudorotation and/or protonation. Theory indicates that in the gas phase, the dianion is not stable (Lim and Karplus 1990), but including solvent suggests that it is (Uchimaru et al. 1991; Dejaegere et al. 1991; Yliniemela et al. 1993). Considering the greater intrinsic barriers involved in formation of pentacoordinate phosphoranes in solution as deduced by Guthrie (1977), it would appear likely that such an intermediate will have a finite lifetime, and hence be a viable intermediate. Whether this lifetime is long enough to allow the anionic oxygen atoms to be protonated is yet to be clarified and requires data on the pK_a values of the putative intermediate. Current estimates suggest values for the first and second pK_as of 6.5–11 and 11.3–15, respectively (Davies et al. 2002; Lopez et al. 2002; and as summarised in Anslyn and Perreault 1997).

Studies on the pH dependence of RNA isomerisation (Fig. 2) show that acid catalysed or pH independent regions exist, which can be rationalised in terms of a neutral or monoanionic phosphorane intermediate that forms and pseudorotates. Isomerisation does not appear to have a base catalysed component, which would provide evidence for a dianionic intermediate (which is stable enough to pseudo roatate). If the dianionic intermediate is forming, it breaks down at least 10^4-fold faster than it can pseudorotate (Oivanen et al. 1993) and presumably requires a kinetically important protonation (i.e. before the rate limiting step) to lead to isomerisation. It should be noted that hydrolysis dominates rapidly above pH 7 and obscures the observation of potential base catalysed isomerisation. However, even a phosphonate analogue of RNA where cleavage is prevented still does not show base catyalysed isomerisation up to pH 10 (where the substrate starts to react through base degradation). Thus, although the presence of a metastable phosphorane intermediate is likely for all the substitution reactions of phosphate diesters, it may not be kinetically significant for the attack of hydroxide on the diester anion.

Figure 3 shows the ionic species involved in acid, spontaneous (pH independent) and base catalysed dialkyl diester hydrolysis, along with the likely key intermediates arising from attack at phosphorus. Under strongly acidic conditions, acid catalysed hydrolysis of the neutral diester can also be observed (Bunton et al. 1960), but at higher than pH 1 this is insignificant relative to the spontaneous reaction of the neutral diester. This scheme does not cover all the plausible mechanisms that can be proposed, but illustrates that a complex series of tautomers are involved in many cases and to provide a framework for the reactivity estimates that follow below.

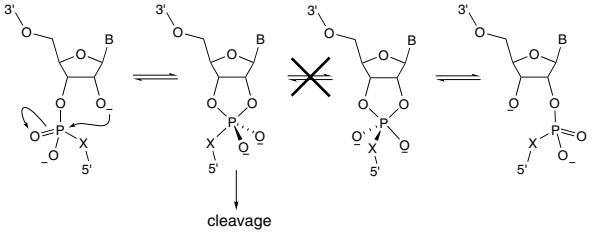

Fig. 2. Potential pathway for base catalysed isomerisation in RNA. When X=O, cleavage dominates and no isomerisation is observed. When X=CH_2, cleavage is prevented, but isomerisation is still not accelerated by base. Thus, either the dianionic phosphorane intermediate does not form, or it has too short a lifetime for pseudorotation to occur

4 Spontaneous Hydrolysis

The most appealing method of determining the background reactivity is to simply take DNA and monitor its cleavage. Unfortunately, the stability of the phosphate diester bonds in DNA exceeds that of the nucleotides (Lindahl 1993) under neutral conditions. Reaction can occur at the base position, with deamination of C being particularly important as it results in formation of U and consequently modifies the genetic code. However, the key process when considering strand cleavage is depurination/depyrimidination at the anomeric centre to give an abasic site, which can subsequently lead to strand cleavage via C–O bond cleavage. This effectively rules out the use of the complete DNA structure to evaluate the reactivity of the diester group to spontaneous hydrolysis.

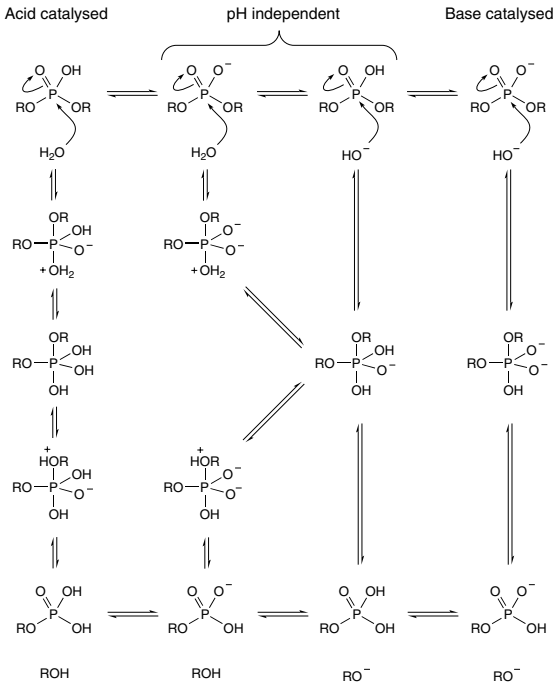

Fig. 3. A_N+D_N mechanisms for phosphate diester hydrolysis at different pHs. The dianionic phosphorane intermediate may not be kinetically significant (i.e. able to undergo reactions other than decomposition to starting materials or product)