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Reversibly constraining spliceosome-substrate complexes by engineering disulfide crosslinks

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ABSTRACT

The spliceosome is a highly dynamic mega-Dalton enzyme, formed in part by assembly of U snRNPs onto its pre-mRNA substrate transcripts. Early steps in spliceosome assembly are challenging to study biochemically and structurally due to compositional and conformational dynamics. We detail an approach to covalently and reversibly constrain or trap non-covalent pre-mRNA/protein spliceosome complexes. This approach involves engineering a single disulfide bond between a thiol-bearing cysteine sidechain and a proximal backbone phosphate of the pre-mRNA, site-specifically modified with an *N*-thioalkyl moiety. When distance and angle between reactants is optimal, the sidechain will react with the single *N*-thioalkyl to form a crosslink upon oxidation. We provide protocols detailing how this has been applied successfully to trap an 11-subunit RNA-protein assembly, the human U1 snRNP, in complex with a pre-mRNA.

1. Introduction

Many biological enzymatic reactions are catalyzed by multi-subunit assemblies, such as the complexes that catalyze chromatin modification, DNA replication, transcription, and translation [1–3]. Such large assemblies, and even those with far fewer subunits, often exist in multiple equilibrium states, have interacting partners that bind transiently, and catalyze reactions that pass through various transient intermediates. These aspects make them challenging to study enzymatically and/or structurally. Detailed mechanistic analysis of 'simpler' enzymes as well as multi-subunit assemblies is often greatly aided by availability of an inhibitor(s) that can trap or constrain the enzyme in a discrete intermediate state and/or limit compositional and conformational dynamics. However, identification, characterization, and production of inhibitor(s) that may be useful to trap, or at least decrease compositional and conformational dynamics, and capture an active state, is highly challenging to achieve and often fiscally demanding.

In the mid-1990s, Verdine and colleagues developed a chemical approach utilizing what was termed "convertible nucleosides" to constrain nucleic acid and nucleic acid-protein complexes [4,5]. They initially employed phosphoramidite chemistry to site-specifically introduce into a DNA oligonucleotide a nucleoside analog with a base modification that contained a reactive functionality. The modified base position contained a leaving group that when post-synthesis was treated with a nucleophile could be modified with a thioalkyl, a carbon linker of a desired length and a thiol. Upon oxidation, the thiol can then form a disulfide crosslink with the thiol bearing sidechain of cysteine or a thioalkyl introduced into a complementary DNA oligonucleotide. This approach allows for the constraint of a DNA-protein complex or engineering of a cross-strand disulfide crosslink in duplex DNA, thereby reversibly constraining a helix. Importantly, the engineered modification could be placed on the base edge, e.g., non-base-pairing groups, and the carbon linker engineered to cross the minor or major groove without perturbing structure and function. In addition, by varying the carbon linker length of the modification on one of two strands of a DNA duplex, a bend in the DNA of a desired degree can be introduced. This strategy was ingeniously used to distort double-stranded DNA helical conformation so as to examine the mechanism of transcription factor recruitment [6] (Fig. 1A).

Convertible nucleosides have also been site-specifically incorporated into RNA [7,8]. Pomeranz Krummel et al. (2000) introduced a minor groove spanning crosslink to constrain two

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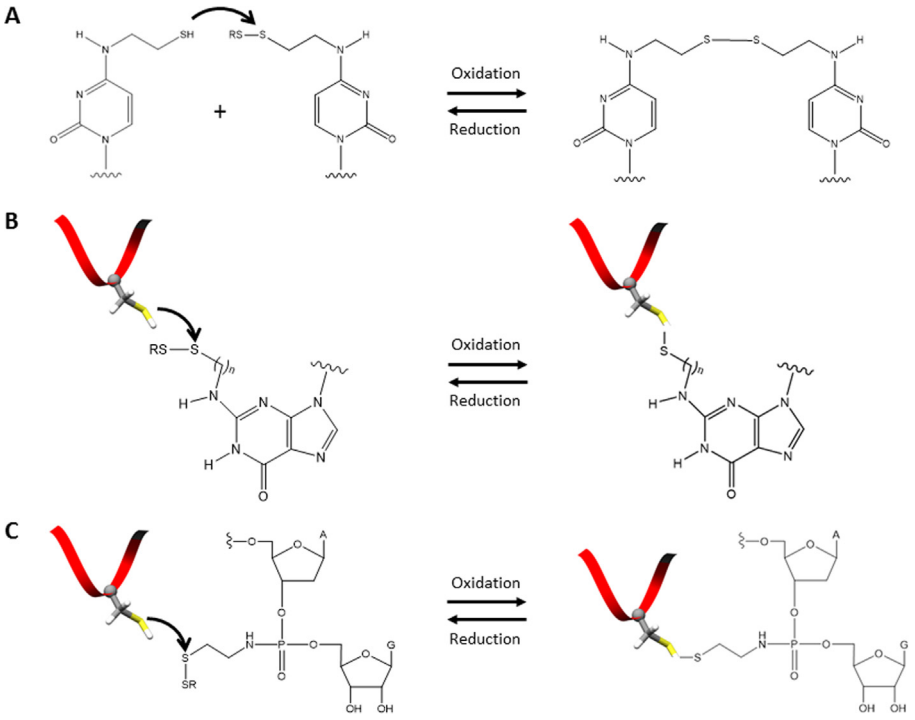

Fig. 1. Examples where nucleoside chemistry can be utilized to engineer site-specific disulfide crosslinks. (A) A DNA or RNA inter- or intra-disulfide crosslink can be generated between nucleoside analogs across strands. In this example, a pyrimidine modified with a thiol (N^4-thioethyl-C) forms a disulfide bond crosslink upon oxidation with another N^4-thioethyl-C. Such a crosslink can span the minor groove of a DNA or RNA double-stranded helix without perturbing structure-function [4,7]. (B) A DNA-protein crosslink can be engineered by introducing a thiol-bearing cysteine residue proximal to a modified base, as shown here. When the distance and angle between reactants is satisfactory, the sidechain will react with the thiol group in the DNA. In this example, a purine modified with a thiol (N^2-thioethyl-dG) forms a disulfide bond or crosslink upon oxidation. The thiol in this case is not at a canonical Watson-Crick base-pairing position, but on the minor groove edge of the base [15,16]. (C) An RNA-protein crosslink can be engineered by introducing a thiol-bearing cysteine residue proximal to a modified base, as shown here. When the distance and angle between reactants is satisfactory, the sidechain will react with the thiol group in the RNA. In this example, a single backbone phosphate modified with an *N*-thioalkyl forms a crosslink upon oxidation [17].

partially complementary oligonucleotides [8]. By constraining the helix, it was shown that the tRNA processing endonuclease RNase P, which recognized and cleaved the reduced form of this modified substrate, locally denatures the helix proximal to the scissile bond for efficient phosphodiester bond breakage. Convertible nucleosides have also been used to constrain nucleic acid/protein complexes [9–16] (Fig. 1B). Guided by structure, Huang et al. (2000) set about constraining HIV reverse transcriptase in complex with its template DNA and RNA primer [15,16]. In this case, a cysteine was introduced in the reverse transcriptase proximal to a thioethyl on a template base. Schellenberg et al. (2011) applied a similar chemical approach to constrain an RNA-protein bimolecular complex, but rather than have the modification on a base it was engineered site-specifically in the backbone [17] (Fig. 1C).

We set out to employ the convertible nucleoside approach to constrain a large spliceosome complex. The spliceosome is not a pre-formed enzyme and undergoes a series of discrete and reversible assembly steps to form a catalytic unit [18] (Fig. 2). During these assembly steps, one or more U snRNPs recognize primary sequence in a pre-mRNA and/or protein(s) bound to the pre-mRNA in preceding steps of the assembly cycle. The first critical state, which serves to nucleate spliceosome assembly, is recognition of a 5′ splice site sequence in the pre-mRNA by U1 snRNP. The U1 snRNP, however, is displaced from the 5′ splice site prior to formation of a catalytic assembly. Once the catalytic spliceosome is assembled, changes to the enzyme are primarily structural. Due to the significant challenges posed by the spliceosome's large size and highly dynamic properties, there is no clear molecular understanding of the spliceosome's pre-catalytic structure in general and the role that U1 snRNP plays in its formation in particular. Thus far, the largest complex constrained by engineering a disulfide bond between a site-specifically modified nucleic acid and a protein has been bimolecular. We reasoned that the U1 snRNP, an 11-subunit ribonucleoprotein particle, could be reversibly constrained to a pre-mRNA by engineering a disulfide crosslink between a pre-mRNA site-specifically modified with a thioalkyl and a proximal cysteine of a protein (U1-C) that forms part of the U1 snRNP (Fig. 3). Based on crystal structures of the human U1 snRNP at 5.5 Å [19] and a higher resolution crystal structure of a compositionally and structurally engineered minimized U1 snRNP [20], we chose the sites to introduce the modifications in an RNA substrate and protein. We detail how a specific crosslink was generated and highlight steps taken as well as trialed to achieve this goal.

2. Material and methods

2.1. Preparation of site-specifically modified thioalkyl oligonucleotides

2.1.1. Reagents and equipment for synthesis

- ABI 394 synthesizer or equivalent.
- 2′-TBDMS RNA phosphoramidites are available from a variety of commercial sources.
- For the purposes of phosphonate oxidation, acetonitrile and pyridine are either freshly distilled after drying over CaH_2 or obtained in anhydrous form (Aldrich Sure/Seal).

2.1.2. Synthesis

RNAs were synthesized on an ABI 394 synthesizer using standard protocols with 2′-*tert*-butyldimethylsilyl ether (TBDMS) pro-