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Thionucleoside Disulfide Cross-Linked Duplex DNA

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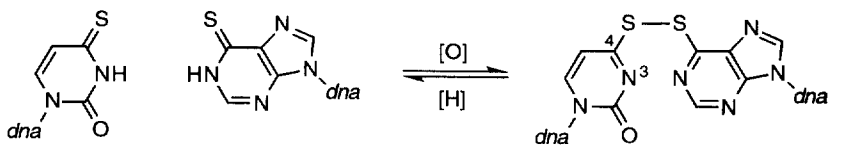
Abstract: A strategy for covalent cross-linking of duplex oligonucleotides is described, and is based on a direct base-to-base disulfide bond formed by iodine oxidation of two appropriately positioned thionucleoside bases. Cross-link formation between 4-thio-2′-deoxyuridine and 6-thio-2′-deoxyinosine is demonstrated in three sequence contexts (3′ to 3′, 5′ to 5′, or opposed).

Elucidation of the relationship of biological function to oligonucleotide structure is an important goal in nucleic acids chemistry.[1] Methods to constrain oligonucleotide conformation have been important as adjuvants in many studies of oligonucleotide structure and function.[2] Control of oligonucleotide structure can affect conformational immobilization, reduction of dynamic motion, and stabilization of disfavored conformations. We describe a fundamentally novel and highly effective strategy for cross-linking duplex DNA that is based on a direct, base-to-base disulfide bond between two appropriately positioned thionucleoside bases. This provides the desired covalent cross-link with the absolute minimum number of atoms, which may be useful when trying to mimic naturally occurring structures, since there is no sterically bulky tether. Because of the unconventional manner in which the interstrand disulfide is formed, the potential utility of our strategy is complementary to existing protocols that use thiols tethered to the deoxyribose or heterocyclic bases. Work on disulfides as covalent constraints of oligonucleotide structure comes most prominently from the groups of Glick[3] and Verdine,[4] among several others.[5] These strategies typically use extraneous tethers for supplying the conformational lock (*e.g.*, N^3-(2-thioethyl)thymidine[3] or N^6-(3-thiopropyl)deoxyadenosine[4]).



$d^{S4}U$ $d^{S6}I$

We demonstrate herein quantitative and reversible formation of disulfide cross-links within duplex DNA using the thionucleoside bases 4-thio-2′-deoxyuridine[6,7] ($d^{S4}U$) and 6-thio-2′-deoxyinosine[8] ($d^{S6}I$) in three permutations: (1) $d^{S4}U$–$d^{S4}U$; (2) $d^{S6}I$–$d^{S6}I$; (3) $d^{S4}U$–$d^{S6}I$, and in three sequence contexts: (1) 3′ to 3′; (2) 5′ to 5′, or; (3) directly opposed. The synthetic

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incorporation of thionucleosides into oligodeoxynucleotides was easily achieved using *S*-cyanoethyl phosphoramidite reagents.[7]

Using a modified AMBER* molecular mechanics force-field[9] that we had specifically parameterized for the thioimidate disulfide,[10] we examined the topology of the thiocarbonyl groups in the starting duplex structures and evaluated the distortion of the B-DNA double helix induced upon disulfide bond formation. The energetic contributors to helix distortion are the N–C–S–S and C–S–S–C torsions, which have energy minima at 0° and 80-90°, respectively. Within duplex DNA where the bases are roughly co-planar, a compromise between these two dihedral angles must be reached if the thionucleoside disulfide does not unwind the DNA. Typically, the C–S–S–C torsion was found to fall in the range 55-75°, with the N–C–S–S torsion in the range 20-90°.[11] The major distortion of the thionucleosides and adjacent bases was propeller and buckle motions.[12]

We found that 5′-disposed $d^{S4}U$ residues would be more effectively positioned to form interstrand disulfide bonds compared to the less favorably 3′-disposed bases. Disulfide formation between two $d^{S4}U$ bases induced less helix distortion when another pyrimidine base was positioned opposite to the $d^{S4}U$ bases by creating the conformational flexibility necessary to attain bonding distance.

opposed

5′d(T A A T A C G A C **N** C A C T A T A)3′
3′d(A T T A T G C T G **N** G T G A T A T)5′

5′-disposed

5′d(T A A T A C G A C **N** C A C T A T A)3′
3′d(A T T A T G C T G C **N** T G A T A T)5′

3′-disposed

5′d(T A A T A C G A C **N** C A C T A T A)3′
3′d(A T T A T G C T **N** C G T G A T A T)5′

The –CNC– containing oligomer was ^{32}P end-labeled and purified using a Sephadex G25 spun column prior to use. Oligonucleotides were denatured at 65-95 °C, cooled to 25 °C over 30 min, and cross-linked by addition of oxidant. Rapid formation of interstrand disulfide cross-links was achieved in high yield by treatment of the annealed duplex (10 μM) in 10 mM phosphate buffer (pH 8) containing 5 mM NaCl with 1 mM I_2/satd KI to a final $[I_2]$ = 200 μM. Reactions were complete in ≤10 min at 25 °C. Other oxidants were much less effective with respect to yield or rate. Cross-linking was analyzed by 20% denaturing polyacrylamide gel electrophoresis (PAGE)[13] and quantitated using a phosphorimager.

For systems with opposed and 5′-disposed bis-$d^{S4}U$ bases, cross-linking was effectively quantitative (≥ 90%). Increasing the ratio of unlabeled to labeled oligomer from 1.5:1 to 10:1 increased the cross-linking yield from 60% to greater than 90%. The 3′-disposed system

underwent cross-linking in poor yield (30%) as a consequence of the energetically disfavored geometry that must be attained for disulfide formation to occur.

The sequence dependence of cross-link formation partly disappeared for bis-d^{S6}I systems, reflective of the decreased duplex distortion that occurs upon disulfide formation. Cross-linking yields ranged from 82-83% for the opposed and 3′-disposed thionucleosides to quantitative (≥ 95%) for the 5′-disposed system. These results are in accord with molecular modeling predictions, which demonstrated S–S bond formation could occur without major distortion of the B-DNA double helix and with close to equilibrium torsional angles about the thionucleoside disulfide.

Near sequence independence was observed in the mixed d^{S6}I–d^{S4}U sequences (X = ^{S6}I; Y = ^{S4}U), again reflecting modeling predictions. In the case of the 3′-disposed system, cross-linking occurred in 85% yield, whereas with the opposed and 5′-disposed cases cross-linking was quantitative (≥ 95% respectively). Modeling predicted that the opposed d^{S6}I–d^{S4}U cross-link would have the best combination of low duplex distortion and optimal disulfide dihedrals.

opposed

5′ d (A A T A C G A **X** A C A C T A T A T) 3′
3′ d (T T A T G C T **Y** T G T G A T A T A) 5′

5′-disposed

5′ d (A A T A C G A **X** A C A C T A T A T) 3′
3′ d (T T A T G C T C **Y** G T G A T A T A) 5′

3′-disposed

5′ d (A A T A C G A **X** A C A C T A T A T) 3′
3′ d (T T A T G C **Y** C T G T G A T A T A) 5′

Melting studies in 0.1 M NaCl showed the expected large increase in melting temperature (T_m) and poorly defined helix-coil transition upon interstrand disulfide formation (*e.g.*, ΔT_m = 24.9 °C for the 5′-disposed d^{S4}U·d^{S4}U system). We found no correlation between the expectedly unrelated parameters of cross-linking yield and T_m of non-cross-linked duplex. Characterization of the cross-linked duplex by enzymatic digestion was not possible because the parent *bis*-thionucleoside disulfides underwent oxidative hydrolysis and the disulfide bond is highly sensitive to reducing agents present in commercial enzyme preparations.[11] Stability of the disulfide bond within duplex DNA in pH 8 buffer at 25 °C, measured by denaturing PAGE, ranged from 20% degradation at 48 h for the *bis*-d^{S4}U systems to 0% degradation at 7 d for the *bis*-d^{S6}I systems.

Circular dichroism studies showed that the disulfide cross-link did not significantly distort the B-DNA helix. Besides the disappearance of the weak absorption at 320-330 nm attributable to loss of the thiocarbonyl group, there were no major differences between the CD spectra of

the oxidized and reduced systems. The positive ellipticity at 282 nm characteristic of B-DNA underwent a slight red-shift (1-3 nm) upon disulfide formation in all cases, although the interpretation of this shift is not unambiguous.[14]

This methodology for cross-linking oligonucleotides has potential applicability to problems in oligonucleotide structure, for example as a constraint in sequence dependent DNA bending studies. Because of the high reduction potential of the thioimidate disulfide and low oxidation potential of the corresponding thioamide, this methodology may prove useful as a rapidly activated trigger or latch for kinetic studies on RNA folding. Incorporation of thionucleosides into DNA is straightforward, and the protocol for disulfide bond formation is rapid, quantitative, and reversible. Our protocol complements existing work on constrained DNA duplexes[3-5] because the cross-link is introduced without an extraneous tethers. In optimal sequence contexts, the thionucleoside disulfide induces only a minimal distortion of the B-DNA helix.

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