Caged DNA

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Influence of the Absolute Configuration of NPE-Caged Cytosine on DNA Single Base Pair Stability**

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Abstract: *Photolabile protecting groups are a versatile tool to trigger reactions by light irradiation. In this study, we have investigated the influence of the absolute configuration of the 1-(2-nitrophenyl)ethyl (NPE) cage group on a 15-base-pair duplex DNA. Using UV melting, we determined the global stability of the unmodified and the selectively (S)- and (R)-NPE-modified DNA sequences, respectively. We observe a differently destabilizing effect for the two NPE stereoisomers on the global stability. Analysis of the temperature dependence of imino proton exchange rates measured by NMR spectroscopy reveals that this effect can be attributed to decreased base pair stabilities of the caged and the 3′-neighbouring base pair, respectively. Furthermore, our NMR based structural models of the modified duplexes provide a structural basis for the distinct effect of the (S)- and the (R)-NPE group.*

Over the last years, the concept of protecting a specific chemical function by a photolabile protecting group, originally introduced as "caging" by J. F. Hoffman,[1] has found widespread application in numerous studies ranging from small molecules to peptides, proteins, and nucleic acids.[2] Caged DNA and RNA oligonucleotides have been used to investigate nucleic acid folding, RNA interference, transcription, DNAzymes, and antisense activity.[3–5] The 1-(2-nitrophenyl)ethyl (NPE) cage group investigated here has been used in bistable RNA refolding studies where it was shown to be able to selectively destabilize a single conformation.[5,6] This principle to shift the conformational equilibria between alternative structures of similar stability has been applied to a large number of different RNAs and has been found to be an important mechanism of RNA-based regulation.[7]

The similar fundamental concept of caging has been successfully applied for the destabilization of RNA and DNA duplex structures.[8] However, the exact mechanism how caging groups destabilize DNA and RNA duplexes has thus far remained largely unknown. In a single comparative study, the decrease in DNA melting temperature upon introduction of the NPE group has been reported.[9] As one important aspect, most studies have used mixtures of two caged diastereomers (see Scheme 1) based on the consideration that it was less important to prepare diastereomerically pure nucleobase-caged residues, since the stereogenic center of the cage is removed after photodeprotection. Only in a few studies, an enantiomerically pure caging group has been used,[3,5] mainly because of better analytics in the chemical steps of preparing the caged oligonucleotide.[10]

Here, we investigated the effect of introducing either the (*S*)- or the (*R*)-NPE caging group on both the global and the local stability of a model 15-base-pair DNA duplex by UV melting and NMR-detected hydrogen exchange. In addition, we determined NMR-based structural models of the two NPE-modified duplexes which provide a structural basis of the NPE-induced effect on DNA stability. We found a striking difference in the stability decrease based on the absolute configuration of the NPE stereogenic center. These findings

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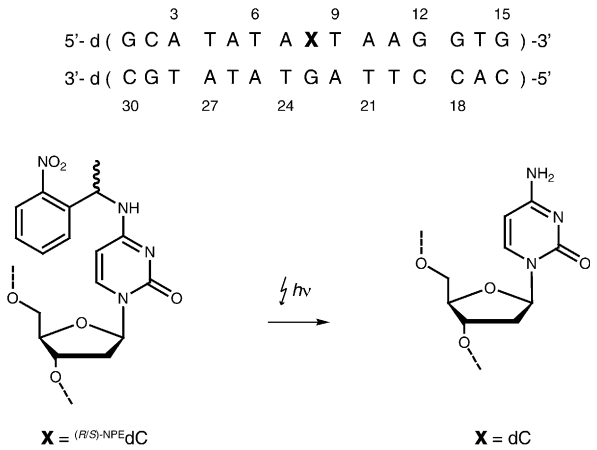

Scheme 1. The investigated DNA sequence contains a photolabile modification at the central cytosine nucleobase. The structure of the NPE-modified nucleotide is depicted below. Upon light irradiation, the unmodified sequence is recovered.

provide key insight for the improvement of caging groups in further chemical and biophysical studies.

The NPE group was introduced at the central cytosine base of the DNA duplex (for sequence see Scheme 1). Cytosine is a particularly interesting nucleobase for the introduction of the photolabile modification since one exocyclic amino hydrogen is retained for base pairing and yet in studies on DNA duplex destabilization by measurements of the melting temperature it was found that a ^{NPE}dC-caged residue destabilizes the duplex surprisingly well.[9]

After synthesis of the single strand, the resulting diastereomeric oligonucleotides were separated by HPLC.[11] To assign the absolute configuration of the NPE group, the (*S*)-NPE containing DNA was synthesized in a stereoselective manner.[12] Imino protons were assigned using ^{1}H,^{1}H NOESY spectra (see Figure S3 in the Supporting Information).[13] The imino proton of the central C8·G23 base pair is observed in all duplexes (Figure 1); from this observation we infer that at temperatures significantly below the melting point, the NPE group does not disrupt base pairing of the central C8·G23 base pair.

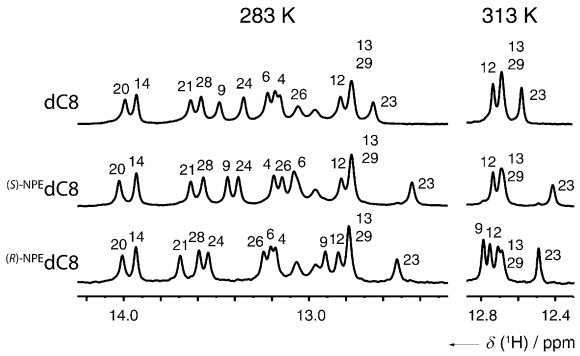

Figure 1. 1D imino proton NMR spectra (600 MHz) of the unmodified DNA duplex (top), the (*S*)-NPE (middle), and (*R*)-NPE (bottom) modified duplexes. Imino proton region at 283 K (left) and imino proton signal of the central GC base pair at 313 K (right). For numbering, see Scheme 1.

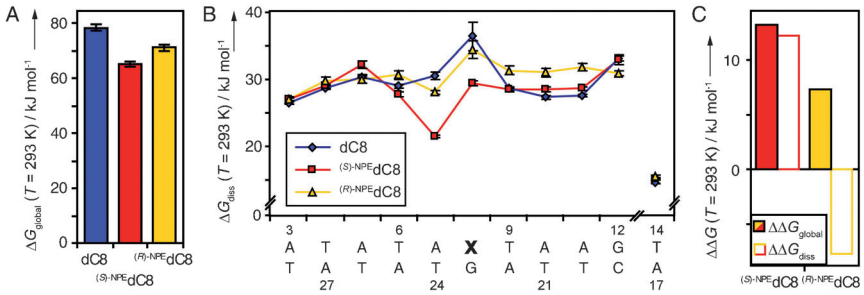

Figure 2. A) Free-energies of global unfolding of the unmodified DNA dC8 (blue) and the photolabile modified DNAs $^{(S)\text{-NPE}}$dC8 (red) and $^{(R)\text{-NPE}}$dC8 (yellow) at 293 K. The duplex concentration is 1 μM. B) Free-energies of base pair opening of the unmodified DNA dC8 (blue) and the photolabile modified DNAs $^{(S)\text{-NPE}}$dC8 (red) and $^{(R)\text{-NPE}}$dC8 (yellow) at 293 K. ΔG_{diss} values were calculated with Gibb's equation with ΔH_{diss} and ΔS_{diss} obtained from imino proton exchange rate analysis. ΔG_{diss} values of G13 and G29 were not determined because of signal overlap. *C*) Free-energy differences $\Delta\Delta G_{global}$ and $\Delta\Delta G_{diss}$ of the native and the modified duplex at 293 K. $\Delta\Delta G_{diss}$ corresponds to the difference of the sum of the ΔG_{diss} values of the native and the modified duplex. All base pair stabilities determined have been taken into account, a positive value indicates a destabilization in comparison to dC8.

The melting temperatures of the three duplexes dC8, $^{(S)\text{-NPE}}$dC8 and $^{(R)\text{-NPE}}$dC8 were determined by UV melting. Our results (see Table S3) do not only support the globally destabilizing character of the NPE group[9] but they furthermore reveal that the DNA destabilization induced by the NPE group depends on the configuration of the stereogenic center. At 1 μM duplex concentration, the (*S*)-NPE group decreases the melting temperature (T_m) by 9.2 K whereas the T_m of the (*R*)-NPE modified duplex is only 4.8 K lower than in the unmodified sequence ($T_m = 326.8$ K). Bearing in mind that a two-state model to theoretically describe global DNA unfolding process can only be applied with caution,[14] we derived free-energies of global dissociation ΔG_{global} as shown in Figure 2 A. At 293 K and 1 μM duplex, ΔG_{global} values exhibit the same trend as the melting temperature: the unmodified duplex is the most stable duplex (78 kJ mol^{-1}) followed by $^{(R)\text{-NPE}}$dC8 (71 kJ mol^{-1}) and $^{(S)\text{-NPE}}$dC8 (65 kJ mol^{-1}).

To obtain more detailed structural and dynamical insight into the specific effects of NPE incorporation, we then determined the individual base pair stabilities in the three DNA duplexes by NMR-detected hydrogen exchange experiments.[15] Enthalpy ΔH_{diss} and entropy ΔS_{diss} of individual base pair opening were derived from the temperature-dependent exchange rate analysis as described previously.[16] The free-energy of base pair opening ΔG_{diss} is calculated with ΔH_{diss} and ΔS_{diss} according to Gibb's equation.

ΔG_{diss} values of the base pairs in the native duplex range from 14.2 kJ mol^{-1} (T14·A17) to 36.4 kJ mol^{-1} (C8·G23; see Figure 2 B, Table S10). Base pairs in the interior of the DNA duplex exhibit the common feature that GC base pairs are more stable than AT base pairs. Incorporation of the (*S*)-NPE group at the cytosine of the central GC base pair leads to decreased ΔG_{diss} values of C8·G23 (29.5 kJ mol^{-1}) and the neighboring A7·T24 base pair (21.5 kJ mol^{-1}; ΔG_{diss}(dC8) = 30.5 kJ mol^{-1}). Free-energies of the remaining base pairs are comparable to values in the native duplex (Table S11). Unlike $^{(S)\text{-NPE}}$dC8, for $^{(R)\text{-NPE}}$dC8, ΔG_{diss} of C8·G23 (34.4 kJ mol^{-1}) and A7·T24 (28.1 kJ mol^{-1}) are only marginally decreased. Interestingly, ΔG_{diss} of T9·A22, A10·T21, and A11·T20 are higher than in the unmodified sequence (Table S12). Only small ΔG_{diss} changes of $^{(R)\text{-NPE}}$dC8 to dC8 are observed for the other base pairs.

Apparently, introduction of either the (*S*)- or the (*R*)-configured NPE group causes distinct changes to the individual base pair stabilities which depend on the absolute configuration of the NPE group. The (*S*)-NPE significantly destabilizes the base pair at the modification site as well as the neighboring A7·T24 base pair. Interestingly, the destabilizing effect is even more pronounced for the neighbouring base

pair than for C8·G23 at which the (*S*)-NPE group is attached. The destabilizing effect is locally restricted, the free-energy of dissociation of the neighboring base pair on the other side and base pairs further away from the modification are not affected by the (*S*)-NPE group. Likewise, the (*R*)-NPE decreases the stability of the modified base pair and the neighbouring A7·T24 base pair. However, the effect is less pronounced in case of (*R*)-NPE. Additionally, the (*R*) stereoisomer causes further remote changes to the base pair stability in the 3′-direction of the modified strand. Apparently, the effect of the (*R*)-NPE group on the individual base pairs is more complex.

The impact of the altered base pair stabilities in the modified duplexes can be monitored with $\Delta\Delta G_{diss}$ which corresponds to the difference of sums of all base pair stabilities between the native and the modified duplex (see Figure 2C). For $^{(S)\text{-NPE}}$dC8, $\Delta\Delta G_{diss}$ is 12.2 kJ mol^{-1}, the native sequence is more stable than $^{(S)\text{-NPE}}$dC8. Furthermore, $\Delta\Delta G_{diss}$ is in agreement with the difference of global free-energies ($\Delta\Delta G_{global} = 13$ kJ mol^{-1}). The smaller stabilities of pairs A7·T24 (9.0 kJ mol^{-1}) and C8·G23 (7.0 kJ mol^{-1}) result in a decrease in melting temperature of $^{(S)\text{-NPE}}$dC8.

In contrast to $^{(S)\text{-NPE}}$dC8, the values for $\Delta\Delta G_{global}$ (7 kJ mol^{-1}) and $\Delta\Delta G_{diss}$ (−7.7 kJ mol^{-1}) are opposing for $^{(R)\text{-NPE}}$dC8. The individual base pairs of the duplex $^{(R)\text{-NPE}}$dC8 are even more stable than the base pairs of the native sequence at a temperature of 293 K that is significantly below the melting temperature. This increase can be localized at the base pairs T9·A22, A10·T21, and A11·T20, which are significantly more stable. These high ΔG_{diss} values are not compensated by the destabilized base pairs A7·T24, C8·G23, and G12·C19.

To elucidate the structural origin of the distinct effects of the (*S*)- and the (*R*)-NPE group on the global duplex stability and the individual base pair stabilities, the NMR structures of the two caged duplexes $^{(S)\text{-NPE}}$dC8 and $^{(R)\text{-NPE}}$dC8 were calculated. Calculations were performed with the software package ARIA/CNS[17] using only homonuclear ^{1}H,^{1}H-NOESY based distance restraints and validated by $^3J_{HH}$ coupling (for detailed information see the Supporting Information). These structures have to be considered as structural models since non-experimental restraints had to be added to obtain a bundle of converging structures. Such constraining for example, of backbone torsion angles is, however, quite common in NMR structure determination of DNA.[18] Constraining the phosphodiester backbone of the DNA duplexes to B-form is further supported by CD spectroscopy that shows a B-form helical structure (Figure S4). Therefore, constraints for dihedral angles for the backbone, the sugar pucker, and the base connection of the B-form DNA helix geometry were applied as well as constraints for Watson–Crick hydrogen bonds and base pair planarity. The middle three base pairs including the modified cytosine were left unrestrained and were thus allowed to adjust freely.

The structure models (see Figure 3A) reveal different orientations of the NPE group in the DNA duplex. The strongly destabilizing (*S*)-NPE group is located between the modified base and the neighboring A7·T24 base pair. In contrast to this, the (*R*)-NPE modification is oriented towards the other side of the strand.

With the structure model at hand, it is tempting to speculate about the origin of the NPE-induced destabilizing effect. In both duplexes, the central C8·G23 base pair exhibits Watson–Crick base pairing. The destabilizing effect of the (*S*)-NPE group occurs on the 5′-side of the modified nucleotide towards which the aromatic ring is oriented. In the right-handed helix, the sterically demanding aromatic ring of the NPE group clashes with the neighbouring base. The base pairs A7·T24 and C8·G23 are tilted and bent in the structure model (Figure 3B). This deviation from regular base pairing possibly affects the stacking interaction of the nucleobases. A decreased stacking interaction would lower the enthalpic contribution to the base pair opening free-energy as is the case for $^{(S)\text{-NPE}}$dC8 (Table S11).

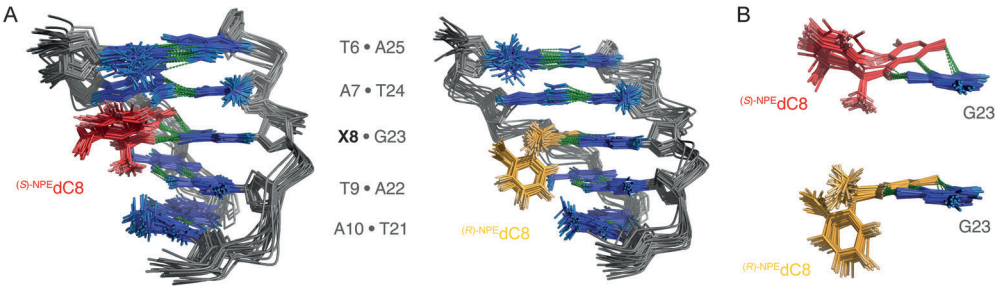

Figure 3. NOE-based structure models of the NPE-modified DNA duplexes. A) Inner five base pairs of $^{(S)\text{-NPE}}$dC8 (left, root-mean-square deviation (rmsd): 0.568 Å) and of $^{(R)\text{-NPE}}$dC8 (right, rmsd: 0.673 Å). The modified nucleotide is color-coded, possible hydrogen bonds are indicated in green. B) Close-up view of the base pair C8·G23 in $^{(S)\text{-NPE}}$dC8 (top) and in $^{(R)\text{-NPE}}$dC8 (bottom). Possible hydrogen bonds are indicated.

In case of $^{(R)\text{-NPE}}$dC8 the inner base pairs are Watson–Crick hydrogen bonded. The NPE group points into the major grove of the helix and the NPE nitro group is oriented towards the 5′-neighboring sugar ring where potential hydrogen bond donors are lacking. However, the individual base pair stabilities revealed remote effects of (*R*)-NPE located towards the 3′-end of the modification site. The increase in ΔG_{diss} of these base pairs in comparison to dC relates to a population difference of the closed conformation smaller than 4×10^{-4}. Detecting these subtle differences in the structure determination remains, however, unfeasible.

In conclusion, we show the remarkably different influence of the two NPE enantiomers. Our results point out that the destabilizing mechanism of the NPE group is not only characterized by decreased hydrogen bonding but has more structural aspects. Our findings offer the possibility to optimize the caging strategy to obtain the maximum desired

effect with a single cage group. Such rational optimization will improve wide spread application of nucleobase cages in chemical biology.

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[12] For information on the synthesis see the Supporting Information. 1D spectra of the stereoselectively synthesized (*S*)-NPE-modified duplex and the HPLC-separated DNAs see Figure S1.
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