Chapter 4

Experimental Approaches to Study DNA Base Flipping

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Abstract

The most dramatic and localized enzyme-induced conformational distortion to the helical structure of DNA is base flipping, in which a nucleobase is unpaired, removed from the stack and further rotated out 180˚ to assume a fully extrahelical position. Since its first demonstration in crystal structures of cytosine methyltransferase-DNA complexes, numerous studies revealed that base flipping is a fundamental mechanism in DNA modification and repair, is involved in initiation of replication, transcription and recombination and lately has been shown to mediate sequence-specific recognition by restriction endonucleases. Here we discuss the variety of experimental approaches that are used to study enzyme-induced base flipping in different systems. X-ray crystallography of protein-DNA complexes is the sole method providing the ultimate proof of base flipping. NMR spectroscopy offers important inroads into dynamic aspects of base flipping, but its potential has not been fully exploited. An attractive method to detect and study base flipping in solution is fluorescent spectroscopy; it uses DNA substrates containing fluorescent base analogs, most often 2-aminopurine. Chemical probing, which exploits enhanced chemical reactivity of flipped out bases in DNA, is a simple method that can be performed in a standard laboratory. Biochemical binding studies often show an enhanced affinity for substrates containing mismatched base pairs, which indirectly points to a disruption of the target base pair upon interaction with enzyme.

The Phenomenon of Base Flipping

Normally, DNA exists as the B-form double-stranded helix in which partner bases on the two complementary strands make Watson-Crick pairs. The base pairs are stacked face-to-face to form the inner core of the double helix with the sugar-phosphate backbone wrapping around the outer edge of the structure. An import inherent feature of the DNA is its conformational plasticity and flexibility. Although the double helix is thermodynamically stable at physiological conditions, it undergoes dynamic conformational fluctuations including spontaneous transient disruptions of base pairing interactions (a phenomenon called DNA breathing). Besides slight sequence-dependent variations, the helical structure is often perturbed by interactions with proteins and other cellular components. The most common distortions of the DNA helix include bending/kinking, unwinding and strand separation, which may occur to a different extent during various stages of DNA metabolism. At the nucleotide level, these changes constitute base unstacking (on one or both faces), base pair twisting and base pair opening events, respectively. The most dramatic and yet highly localized noncovalent distortion to the regular structure is base flipping, in which a nucleobase is unpaired, removed from the stack and further rotated out 180˚ to assume an extreme

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extrahelical conformation. Although such conformations are very unstable in free DNA and can only occur transiently, they can be stabilized upon interaction with other biomolecules. The first demonstration of base flipping appeared in 1994 with a high-resolution crystal structure of the HhaI methyltransferase-DNA complex in which the target cytosine is completely flipped out of the DNA helix and into the catalytic site of the enzyme (see Fig. 1).[1] Although greeted with much surprise, this new phenomenon was subsequently shown to occur in many systems where an enzyme needs to gain access to a DNA base. Numerous studies revealed that base flipping is a fundamental mechanism in DNA modification and repair[2] and is also used by proteins responsible for the opening of the DNA or RNA helix during replication, transcription and recombination.[3,4] More recent and fairly unexpected findings, in which sequence-specific target recognition by restriction endonucleases[5] and hemimethylated CpG-specific UHRF1 proteins[6-8] involves a complete expulsion of nucleotides out of the DNA helix, suggest that many other enzymes or DNA-binding proteins may employ this mechanism in their interactions with DNA. Protein-induced flipping of bases in RNA is also well documented in a variety of systems.[9-11]

Numerous structural and mechanistic studies of DNA base flipping had since been performed in different systems. Examples of the most studied systems are the HhaI DNA methyltransferase and uracil-DNA glycosylase. An important motivation to study base flipping was its wide-spread occurrence among DNA enzymes. As a localized conformational distortion, it offered the promise of an ideal model for new inroads into fundamental mechanisms of protein DNA interactions. On the down side, base flipping presented a significant experimental challenge due its extreme and dynamic nature. Structural features, occurrence in different systems and mechanistic aspects of base flipping have been summarized in a series of review articles.[2,12-15] Computational analysis of base flipping is discussed in the chapter by Priyakumar and MacKerell in this book. Here we attempt to discuss the variety of experimental approaches that were developed to study the occurrence and the mechanisms of base flipping in double helical nucleic acids.

X-Ray Crystallography

X-ray crystallography of protein-DNA complexes holds the crown among experimental methods for providing the ultimate proof of base flipping. Indeed, a high resolution cocrystal structure of a reaction complex can reveal the position of a target base relative to the rest of the helix, show the conformation of the nucleotide and its neighbors on both strands of the DNA. Examples of crystallographically proven base-flipping systems include DNA methyltransferases, DNA glycosylases, apurinic/apyrimidinic endonucleases, glucosyltransferases, restriction endonucleases and

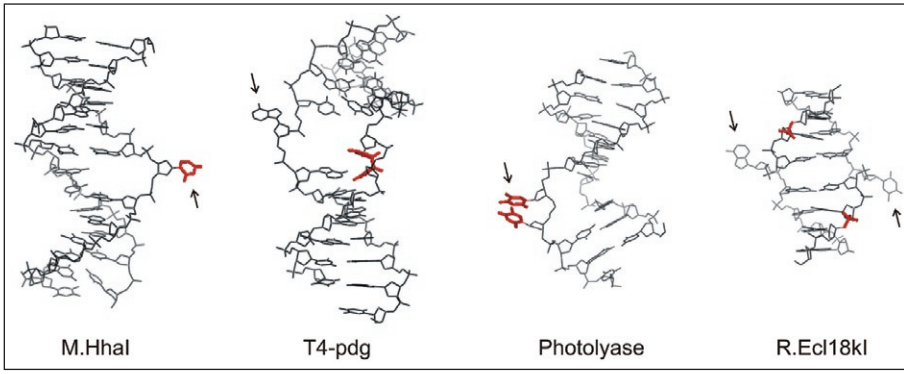


Figure 1. Types of enzymatic DNA base flipping observed in crystal structures of protein-DNA complexes (left to right): target nucleotide flipping (HhaI DNA methyltransferase, PDB entry 1mht), opposite nucleotide flipping (T4-pdg, formerly known as T4 endonuclease V, 1vas), damaged dinucleotide flipping (DNA photolyase, 1tez) and flipping of both nucleotides in a central base pair (restriction endonuclease Ecl18kI, 2fqz). Highlighted are DNA sites targeted by the enzymes, arrows point at flipped out bases. Protein residues are omitted for clarity.

Table 1. Base-flipping systems proven by crystal structures of protein-DNA complexes

Specific Protein	Catalytic Reaction	Primary Reference	PDB Entry
DNA methyltransferases			
M.HhaI	Forms 5-methylC on both strands of a DNA recognition site	1	1mht
M.HaeIII	Forms 5-methylC on both strands of a DNA recognition site	23	1dct
M.TaqI	Forms *N6*-methylA on both strands of a DNA recognition site	101	1g38
M.T4Dam	Forms *N6*-methylA on both strands of a DNA recognition site	102	1q0t
M.EcoDam	Forms *N6*-methylA on both strands of a DNA recognition site	19	2g1p
DNA glycosylases			
T4-Pdg (formerly known as T4 endonuclease V)	Removes pyrimidine dimers from DNA	18	1vas
Human UDG	Removes uracil from DNA	103	4skn
E. coli MUG	Removes uracil or thymine from DNA containing G:T or G:U	104	1mwi
Human AAG	Removes 3-methylA from DNA	105	1bnk
E. coli AlkA	Removes 3-methylA from DNA	106	1diz
hOGG1	Removes 8-oxoG from DNA	107	1ebm
B. stearothermophilius EndoIII	Removes oxidized pyrimidine from DNA	108	1p59
E. coli MutY	Removes adenines from mismatch base pair	109	1rrq
Apurinic/apyrimidinic endonucleases			
E. coli endonuclease IV	Cleaves the DNA backbone at apurinic/apyrimidinic sites	20	1qum
Human apurinic/apyrimidinic endonuclease (HAP1 or APE1)	Cleaves the DNA backbone at apurinic/apyrimidinic sites	110	1dew
Other DNA repair proteins			
S. cerevisiae Rad4	Binds to the lesion and recruits the multi-subunit transcription factor TFIIH	17	2qsh
E. coli AlkB	Oxidizes N-alkylated base lesions to restore standard bases in single-stranded DNA and RNA	111	3bkz
Human ABH2	Oxidizes 1-methylA damage to restore A in double-stranded DNA	111	3btx

continued on next page

Table 1. Continued

Specific Protein	Catalytic Reaction	Primary Reference	PDB Entry
Anacystis nidulans DNA photolyase	Repairs pyrimidine dimers via photo-induced cleavage of the cyclobutane ring	71	1tez
Glucosyltransferases			
T4 bacteriophage BGT	Transfers the glucose moiety of UDP-glucose to the 5-hydroxymethylC bases making β-glucosidic bond	16	1m5r
T4 bacteriophage AGT	Transfers the glucose moiety of UDP-glucose to the 5-hydroxymethylC bases making α-glucosidic bond	112	1y8z
Sequence-specific endonucleases			
R.HinP1I	Cleaves phosphodiester bonds on both strands of a recognition site	113	2flc
R.Ecl18kI	Cleaves phosphodiester bonds on both strands of a recognition site	5	2fqz
R.PspGI	Cleaves phosphodiester bonds on both strands of a recognition site	96	3bm3
Tn5 transposase	Excises and integrates a transposon	114	1muh
Other DNA binding proteins			
SRA domain of UHRF1 (also known as ICBP90, Np95)	Directs Dnmt1 methylation to hemi-methylated CpG sites	6-8	2zkf 3clz 2zo1

some other systems (see Table 1). Crystallographic studies showed that DNA base flipping comes in a variety of flavors (see Fig. 1) such as sole flipping of the target base itself,[1,16] flipping of a base located on the opposite DNA strand to the target base (repair enzymes)[17,18] or flipping of both nucleosides of a target base pair (repair enzymes, M.EcoDam, restriction endonucleases).[5,19,20] In many cases, a concerted bending of the DNA helix is also observed.[16,20]

Although crystal structures reveal many structural details at atomic resolution, they provide only static snapshots, usually at the end of a flipping pathway; many dynamic and mechanistic aspects can only be discerned using other methods (see below). Thus, crystallography lays down a structural basis for further solution studies. An important extension of the method is the use of DNA substrates containing conformationally restricted nucleotide analogs, or mutant proteins to trap base-flipping intermediates.[21,22] However, interpretation of such experiments requires utmost caution since chemical alterations to a system may cause unnatural conformations in the target nucleotide.

A major limitation of the method is that cocrystallization of proteins with their DNA substrates is often tedious or even impossible. Covalent cross-linking with catalysis-based analogs[1,23,24] or alkyldisulfide tethers[25] can be used to obtain stable protein-DNA complexes amenable to crystallization. In lack of cocrystals, base flipping can be predicted on the basis of topological considerations. This is valid in cases when catalytic residues are located in a concave pocket of a protein and thus cannot come to close proximity with the target base in B-DNA without a substantial conformational rearrangement of the protein-DNA complex. Many examples show that the rod-shaped helical DNA molecule is more flexible than a globular protein and thus the former often undergoes the

required conformational changes, although cases when conformational changes in the protein accompany binding of the flipped out base are not uncommon.[13]

NMR Spectroscopy and Imino Proton Exchange

NMR spectroscopy is a powerful technique that is well established to tackle various aspects of nucleic acids structure.[26] In contrast to crystal structures, NMR can potentially give insights into dynamic aspects of base flipping. Smaller molecules are amenable to structure determination using heteronuclear labeling and 2D or 3D sampling techniques. However, dealing with larger protein-DNA complexes may be a challenge due to slow molecular tumbling or insufficient solubility.

The first attempt to study enzyme-induced base flipping by NMR in solution was performed for the M.HhaI DNA methyltransferase.[27] Two 5-fluorocytosine residues were incorporated into the target and a reference position within a cognate DNA substrate. ^{19}F chemical shift analysis of the free DNA duplex and the M.HhaI-DNA complexes revealed the existence of multiple conformers of the target 5-fluorocytosine along the base flipping pathway that were not seen in the previous crystal structures. To assess the exchange dynamics between stacked and flipped-out states, the T_1, T_2 and $T_{1\rho}$ spin relaxation times of ^{19}F for the free duplex and the enzyme-DNA binary complex were determined. The observed relaxation parameters indicated that base pair lifetimes of the target and the reference residue are longer than 1 ms and are most likely similar; hence no dramatic acceleration of the internal motional processes in the DNA duplex upon binding of M.HhaI could be detected in these experiments.

More recent NMR analysis of interactions between cyclobutane pyrimidine dimer photolyase and its single and double-stranded DNA substrates was performed employing ^{13}C or ^{15}N segmentally labeled DNA substrates.[28] Chemical shift differences of ^{1}H-^{13}C HSQC resonances from the cyclobutane pyrimidine moiety upon binding of the deuterated protein and its mutant indicated intimate contacts between the DNA lesion and a Trp residue in a cavity in the enzyme. In light of largely preserved base pairing in the rest of the DNA duplex (derived from analysis of the imino region of a ^{1}H-^{15}N HSQC spectrum), a very localized but dramatic conformational change at the damaged dinucleotide (i.e., base-flipping) was proposed.

A series of NMR experiments have been devoted to study the dynamics of base pairing in DNA in solution[29,30] and in solid state.[31] As mentioned above, double helical nucleic acids undergo spontaneous conformational fluctuations at physiological conditions which include transient disruptions of base pairing interactions. The imino protons, which reside on N1 of guanine and N3 of thymine/uracil, are not accessible to bulk solvent in a closed base pair, but can be exchanged with those of water in an open state. Based on a two-state model, the lifetimes of the closed and open state for individual base pairs can be derived from the analysis of spin inversion recovery or spin saturation transfer from water. In general, the base pair lifetimes (in the closed state) have been found to be in he range of 1-5 ms for A:T base pairs and 10-50 ms for G:C pairs at 15˚C, but can vary by a large margin in different sequence contexts.[30]

Analogous comparative experiments have also been performed using DNA-protein complexes and corresponding free DNA duplexes in order to establish the roles of enzymes in the base flipping mechanism.[27,32] A lack of or a small acceleration of the breathing rate upon binding of an enzyme was typically observed and interpreted as a passive mechanism by which the enzyme merely catches the spontaneously flipped out base.[13,22,32] It should be noted that, due to their dynamic nature, the NMR-detectable open base pairs have not been structurally characterized by other experimental means. Computational estimates of the minimum rotation of a base that is required to allow hydrogen exchange with solvent are in the range of 30-40˚, which is only 20-25% of the full 180˚ rotation observed in most flipped out complexes.[32] An estimated free energy barrier for the open state derived by Arrhenius treatment of an average equilibrium constant of 10^{-7} [30] is around 9 kcal/mol, which accounts for roughly a half of the total 15-20 kcal/mol required for a complete rotational expulsion of the nucleotide.[33,34] The majority of stacking interactions may still be preserved in such open intermediates especially in cases when the complementary bases move asymmetrically towards opposite DNA grooves. In all likelihood, the nucleobases remain largely obscured within the DNA stack in such open

base pairs and therefore, they cannot be regarded as extrahelical or flipped-out. However, in many reports dealing with mechanistic issues of passive and active role of enzymes, hardly any distinction is made between the terms "base pair opening", "base flipping", "extrahelical base", which are indiscriminately used as synonyms.[22,32] Most importantly, the conformational motions that are observed in such NMR experiments largely reflect early events along the pathway to a fully flipped out state and such bases are insufficiently exposed to be simply captured in a concave catalytic site of an enzyme in a passive manner.

A more realistic model for a passive base flipping comes from observing the capture of extrahelical guanine bases by macrocyclic glycans such as β-cyclodextrin. The β-cyclodextrin macrocycle traps a guanine base in a high affinity guest-host complex. Due to nearly irreversible capture of extrahelical guanines at saturating concentrations of this compound, DNA undergoes a first-order denaturation reaction (low temperature melting) with a rate of 0.003 s^{-1} at 51°C.[35] Remarkably, the latter number matches the apparent rate of target cytosine flipping ($k_{flip} \sim k_{chem} = 0.2$ $min^{-1} = 0.003$ s^{-1} at 37°C) in a mutant (Q237G) of the HhaI methyltransferase that is deficient in promoting active base flipping.[36] Although such a close match of the rates observed in a chemical and enzymatic systems may appear fortuitous, it clearly illustrates that the events of spontaneous flipping of nucleobases into extended extrahelical positions in DNA occur at frequencies several orders of magnitude lower than the NMR-detectable imino proton exchange. This means that the NMR derived exchange rates are less predictive than were generally thought (and were often overexploited) for assigning an active or passive role for an enzyme in base flipping and at best can provide an upper estimate for the rate of spontaneous appearance of unpaired bases in DNA. Since most DNA modification and repair enzymes operate at turnover rates (k_{cat} or k_{chem}) faster than 1 min^{-1} they cannot fully rely on DNA breathing for their base flipping needs. For example, extensive NMR and kinetic studies of DNA uracil glycosylase conclude that partial capture (with <10% efficiency) of spontaneously (pre)flipped bases occurs at an early intermediate point (which comprises 17% of the full flipping trajectory) and that further rotation of the uracil into the catalytic site occurs with active participation of the enzyme.[37]

Biochemical Studies

Following the discovery of the base flipping, biochemical studies of DNA cytosine-5 methyltransferases revealed that binding affinity of DNA duplexes containing mismatched base pairs at the target site is increased as compared to cognate duplexes containing the C:G base pair.[38,39] This unexpected feature turned out to be quite general for base flipping enzymes and was observed for DNA methyltransferases (reviewed in ref. 2) as well as DNA repair enzymes.[40] Improved binding is even more prominent if the target base or entire nucleotide is removed (abasic sugar moiety, gapped strands) and in some cases of strand discontinuity (missing phosphodiester linkages).[41] Such an inverse correlation between the DNA binding affinity and the target base pair stability implies that the energy needed to disrupt the target base pair is offset from the total energy gained upon formation of multiple protein-DNA contacts, i.e., the binding energy is partially used to destabilize the target base pair and advance the flipping (substrate strain). DNA straining by pushing with a protruding residue[36] or backbone compression with a serine pinch[15,42] are examples of strategies used by enzymes to promote the base flipping process. On the other hand, substitutions in the target base pair may also have energetic costs, as the result of lost or altered interactions in the complex. Therefore, qualitative studies undoubtedly have an indirect predictive value for base flipping, whereas quantitative analyses require a thorough examination of various contributions. Higher catalytic rates with mismatched substrates have been observed for the human Dnmt1 methyltransferase,[43] however the latter trend is not as general as the enhanced mismatch binding under noncatalytic conditions.

Another important mechanistic outcome of these studies was that the identity of the target base is not scrutinized by most enzymes during initial flipping and thus the target base can be substituted with various analogs such as fluorescent probes etc. To this end, fluorescent bases (see below) and structural nucleotide analogs such as steric probes[44,45] or conformationally locked sugars[46] had been synthetically introduced into DNA to permit mechanistic studies of the base flipping.

Optical Spectroscopy

Natural DNA nucleobases are relatively good chromophores ($\varepsilon_{260} \sim 10^4\ M^{-1}\ cm^{-1}$), however, the absorbance is very weakly dependent on their conformational state. Circular dichroism spectroscopy is more responsive to conformational changes in the helical structure. Because of their low sensitivity, these methods have not been of much utility to the study of base flipping. Selective conformational examination of particular positions in DNA duplexes is possible using synthetically incorporated modified nucleotide probes such as 6-thioguanine,[47] although this approach has not yet been directly applied for probing DNA base flipping.

A much higher sensitivity is generally achieved using fluorescence spectroscopy. Unfortunately, the DNA nucleobases are poor fluorophores themselves and thus their chemical analogs need to be employed to achieve the required sensitivity and enhanced selectivity. The most widely used fluorescent probe for detecting base flipping is 2-aminopurine (2AP), a close structural analog of adenine. 2AP has a high quantum yield in aqueous solution but the fluorescence is highly quenched when incorporated into DNA.[48] Furthermore, the excitation (305-320 nm) and emission (370 nm) maxima are well separated from those of proteins and DNA, increasing its usefulness for studying protein-DNA interactions.

The major decay pathway of the excited fluorophore involves static quenching via charge transfer to nearby guanine bases, with less contribution from hydrogen bonding and collisional quenching.[49-52] Since the fluorescence intensity of 2AP in the DNA helix is mostly dependent on base stacking, removal of both stacking partners during base flipping process should be easily detectable by monitoring emission intensity. Indeed, in most cases, substitution of the flippable base with 2AP gives a strong increase in fluorescence upon addition of a base-flipping enzyme.[53,54] The largest increase (several ten-fold) is observed when the 2AP is flipped out into a polar environment,[54,55] whilst a smaller enhancement (several-fold) is observed upon its interaction with nonpolar or aromatic residues in the flipped out state.[53,56,57] A moderate increase can not serve as a reliable signature of base flipping, since similar changes in fluorescence intensity can arise from other types of helical distortions such as kinking/bending, flipping of an adjacent base,[58] etc. In other words, complete flipping of 2AP from the DNA into a quenched environment cannot be unequivocally distinguished from partial unstacking of the 2AP base in the helix based on the fluorescence intensity changes alone.[59-61]

A higher level of sophistication is achieved with time-resolved fluorescence spectroscopy, which can provide a more detailed information on the environment of the fluorophore. Based on time-resolved fluorescence measurements of MTase-DNA complexes in single crystals and in solution, it was established that the fluorescence decay function of 2AP shows a pronounced, characteristic response to base flipping: the loss of the very short (~100 ps) decay component and the large increase in the amplitude of the long (~10 ns)[55] or an intermediate decay component.[57] Comparison of the amplitudes and rates of the four decay components offers a more precise characterization of the chemical environment of the fluorophore in the helical and flipped out states. However, collection of time-resolved data and analysis of multi-exponential decay functions is mostly accessible to specialized laboratories and thus are likely to be resorted to if conventional fluorescence intensity studies yield no conclusive results.

The utility of 2AP for studying base flipping by adenine modifying enzymes in part derives from the fact the stability of the synthetically introduced 2AP:T base pair is similar to the canonical A:T base pair.[62,63] This is further reinforced by a recent cocrystal structure of the TaqI adenine methyltransferase, which shows that the flipped out target 2AP residue perfectly fits into in the active site of the enzyme.[57] However, an important caveat of the 2AP:T base pair is the lack of the exocyclic 6-amino group in the major groove of the DNA as compared with the A:T pair, which may perturb or even abolish important interactions with proteins.[36] Examples of the failure to open up the 2AP:T pair are the EcoRV DNA methyltransferase[59] and RNA polymerase[64] for which no fluorescence enhancement was observed in complexes when the target base was replaced with 2AP. Moreover, 2AP is not nearly as good a structural mimic of cytosine or thymine, since not only the geometry[65] but also the strength of the base pair (2AP-G vs C:G) is dramatically altered.[63] With that in mind, stopped-flow kinetic and steady state fluorescence measurements still can provide

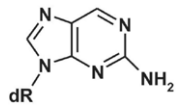
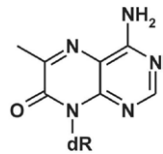
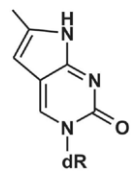
useful information on particular aspects of the base flipping mechanisms of cytosine MTases[36,66] and pyrimidine-glycosylases.[58,67]

A series of new fluorescent analogues of adenine and cytosine have recently been developed and are now available as building blocks for oligonucleotide synthesis. Most of these compounds are capable of Watson-Crick pairing to natural complementary bases and thus do not introduce considerable structural alterations into DNA. However, due to substantial increase in steric bulk these analogs may potentially perturb interactions with enzymes in the major groove. Two such analogs (Table 2) were used to study base flipping by DNA photolyase. 6MAP, a derivative of pteridone, has a high quantum yield (excitation and emission maxima are at 330 nm and 430 nm, respectively) and the fluorescence decreases when the base is incorporated into DNA.[68] Similarly, pyrrolo-dC, a derivative of C, is a useful probe of base stacking interactions in DNA. DNA duplexes containing the fluorescent analogs were used to probe how far base flipping propagates along the duplex. It was found that base destacking around the lesion decays rapidly with distance, which was consistent with the protein-DNA crystal structure.[69-71] The most recent addition is 1,3-diaza-2-oxophenoxazine, tC^{O}, a fluorescent derivative of cytosine.[72,73]

Chemical Probing

Many chemicals have been developed to probe structural variations in DNA or RNA.[74-76] Detection of nonstandard conformations in DNA relies on differential chemical reactivity of individual residues (nucleobase, sugar or phosphodiester moiety). Hyper-reactivity of a base typically reports its enhanced accessibility, which may come from (partial) base unpairing or unstacking events. Conversely, protection against modification (footprint) is likely to be observed upon ligand and

Table 2. Fluorescent nucleobase analogs used to study DNA base flipping

Name	Chemical Structure	Systems Used	References
2-Aminopurine		DNA methyltransferases, DNA repair enzymes, restriction endonucleases	Reviewed in 2 56
6MAP (4-amino-6-methyl-7(8*H*)-pteridone)		DNA photolyase	69
Pyrrolo-C		DNA photolyase	70

*(dR denotes a 2′-deoxyribose moiety within DNA)

protein binding, or upon formation of compact tertiary structures within a nucleic acids molecule. Protein-induced base flipping is expected to show either or both of the two—hyper-reactivity at or around the extrahelical residue itself and protection of residues obscured by protein contacts outside the lesion. Subsequent visualization of the chemically modified sites is most often achieved by piperidine-induced strand cleavage, followed by electrophoresis on denaturing gels. In cases when the modifications block base pairing, enzymatic strand cleavage with single-strand-specific nuclease S1[77] or polymerase extension-based approaches can be used.[78,79] Chemical probing is thus cost-effective, requires no specialized equipment and, in certain cases, can also be applied in vivo.[78,79] On the other hand, in many cases the probing chemicals attack not only the DNA but also the interacting proteins, so the stability of protein-DNA complexes can be affected during the reaction. Since flipping of DNA bases is observed upon interaction with proteins, only those chemical reactions that proceed at near physiological conditions can be employed (Table 3). Three such examples are discussed below.

$KMnO_4$ treatment leads to conversion of thymine to *cis*-thymine glycol (5,6-dihydroxy-5, 6-dihydrothymine);[78] the oxidized base undergoes further degradation leading to cleavage of the DNA strand upon piperidine treatment. Since the attack on the C6=C5 bond takes place from a face of the pyrimidine ring, permanganate reactivity depends upon solvent accessibility of a thymine residue; thus double-stranded DNA is relatively resistant to permanganate oxidation compared to single-stranded DNA. The suitability of potassium permanganate for "positive display" of flipped-out thymine residues in mismatched DNA substrates was demonstrated for cytosine[80] and adenine[60] DNA methyltransferases and recently for a sequence-specific transposase.[81] This assay was also used to demonstrate thymine nucleotide flipping in mismatched DNA duplexes upon interaction with a small macrocyclic bis-intercalator.[82]

Chloro- and bromoacetaldehyde are known to primarily react with unpaired adenine and cytosine bases in DNA yielding 1,*N*6-ethenoadenine and 3,*N*4-ethenocytosine derivatives, respectively.[83] Such haloacetaldehyde-modified residues can be detected by piperidine-induced strand cleavage. An intermediate reaction with formic acid, hydrazine or dimethylsulfate can be performed following the chloroacetaldehyde modification and prior to piperidine cleavage to achieve a better signal-to-noise ratio.[84] Recently, the suitability of the chloroacetaldehyde modification for mapping flipped-out cytosine bases has been demonstrated for two restriction endonucleases and also for a series of DNA cytosine-5 methyltransferases.[85]

Hydroxyl radicals generated in the Fenton reaction attack DNA by abstracting a deoxyribose hydrogen in the backbone which eventually leads to strand breaks at the modified residues. The hydroxyl radical is a proven probe for high-resolution foot-printing of protein-DNA complexes that shows no base or sequence selectivity.[86] Consistent with the known crystal structures of the closed ternary complexes, footprints of Type II bacterial cytosine-5 MTases showed no regions of enhanced deoxyribose accessibility.[87,88] However, enhanced susceptibility to hydroxyl radicals at and around the target nucleotides was observed with the Type I DNA methyltransferase M.EcoR124I[89] suggesting that a different base flipping strategy is employed by these large heteromultimeric enzymes.

Even water can be exploited as a probe to track base-flipping events in DNA under physiological conditions in vivo. Hydrolytic deamination of C to U is a spontaneous damage to DNA that occurs

Table 3. Chemical probes used to study base flipping in protein-DNA complexes

Reagent	Residues Probed	Systems Used	References
Potassium permanganate	Thymine	M.HhaI, M.TaqI, M.EcoP15I, Tn5 transposase	80 60 81
Chloroacetaldehyde	Cytosine	M.HhaI, M.AluI, M.SssI, R.Eco18kI, R.PspGI,	85
Hydroxyl radical	Deoxyribose	M.EcoR124I	89

at a relatively slow rate compared to the other chemical reactions described above. However, the rate of C to U mutations can be measured at a particular locus in the genome using a genetic reversion assay.[90] It is known that in single-strand DNA cytosines deaminate at a 140-fold higher rate than those in double-stranded form[90] and therefore elevated mutation rates can serve as indirect evidence for base flipping events in vivo. Indeed, such genetic reversion studies indicated that target sites of DNA cytosine-5 MTases are mutational hot spots in vivo.[91] However, in the case of cytosine-5 methyltransferases, it is not clear whether the higher deamination rates are due to (i) the enhanced solvent exposure of the flipped out cytosines, (ii) transient covalent activation of the target base by the enzymes,[92,93] or (iii) higher intrinsic deamination rate of methylated cytosine residues as compared to cytosines in DNA.[91] Recently, this assay was used to predict that the PspGI restriction endonuclease, which was known to bind and cleave DNA at the target CC(A/T)GG sites,[94] opens up the central base pair upon binding to the near-cognate CC(C/G)GG sites. Subsequent in vitro studies using 2AP fluorescence[56,95] and chloroacetaldehyde probing[85] and X-ray crystallography[96] confirmed this assumption. A major limitation of this approach is that a specific DNA locus is examined by a particular genetic system, which needs to be redesigned for probing other DNA sequences.

Photochemical Approaches

Light-activated nucleobase analogs can be incorporated into DNA to probe protein contacts via photo-induced covalent cross-linking reactions. When incorporated at a target position, 5-iodouracil or 2-thiouracil formed covalent cross-links with certain DNA methyltransferases[97-99] and Tn5 transposase[81] upon exposure to UV light. Such experiments can identify the region of a protein proximal to the target base, but do not per se indicate base flipping. However, the topological argument can be applied when the modified residues map to a distal location in the protein that requires flipping of the target base.[81,98] A more informative approach is to examine the nucleobase-mediated charge transfer along the DNA helix. Such long-range charge effects can be studied by analyzing the oxidation of guanine residues near one end of the DNA duplex that is induced by a light-harvesting intercalating rhodium photo-oxidant tethered to the other end of the DNA. It was observed that yields of the long-range guanine damage correlate with protein-induced changes in the DNA base stacking.[100] For example, wild-type M.HhaI effectively turns off the charge transfer by inserting the protruding Gln237 residue to replace the target cytosine in the DNA, whereas the Q237W mutant, in which the aromatic Trp residue enters the DNA restoring the helical stack, leads to an efficient long-range charge transfer.[100]

Concluding Remarks and Future Prospects

X-ray crystallography of protein-DNA complexes holds the crown among experimental methods for providing the ultimate proof of base flipping. Although crystal structures reveal many structural details at atomic resolution, they provide static snapshots, typically at the end of the base-flipping pathway. A major limitation of the method is that cocrystallization of proteins with their DNA substrates is often tedious or even impossible. NMR spectroscopy can give further insight into dynamic aspects of base flipping, but its potential has not yet been fully exploited. An attractive "low resolution" method to study base flipping in solution is fluorescent spectroscopy. However, it requires modified bases such as 2-aminopurine to be introduced in DNA and simple steady state emission analyses do not always yield unequivocal results. Chemical probing exploits enhanced chemical reactivity of flipped out bases; it requires no specialized equipment and can be performed in a standard laboratory. Biochemical and kinetic studies involving protein mutants and DNA substrates containing nucleotide analogs can provide valuable insights into protein-DNA interactions leading to base flipping. However, many mechanistic aspects of base flipping still remain obscure in the majority of systems studied and further efforts combining various experimental and computational approaches are required to tackle this exciting enigma of macromolecular interactions.

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