Towards the Generation of Artificial O^6-Alkylguanine-DNA Alkyltransferases: In Vitro Selection of Antibodies with Reactive Cysteine Residues

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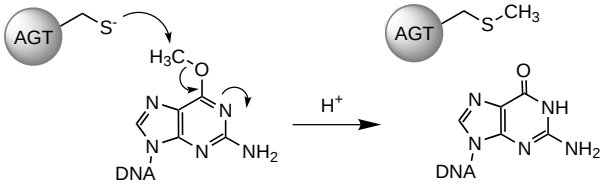
catalytic antibodies · DNA · molecular evolution · phage display · transferases

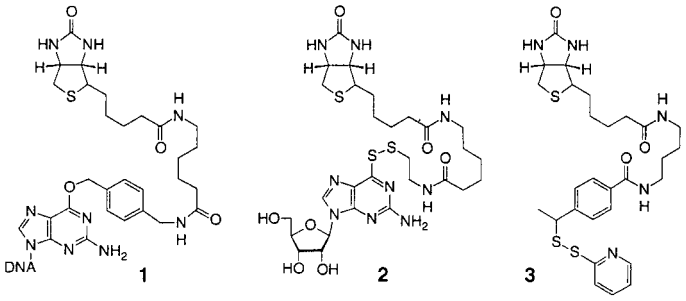
O^6-alkylguanine-DNA alkyltransferases (AGT) are DNA repair proteins that reverse O^6-alkylations of guanine in DNA.[1] The underlying repair mechanism is unusual as the AGT transfers the alkyl group in an S_N2 reaction to one of its own cysteine residues to yield an irreversibly alkylated protein (Scheme 1). In order to



Scheme 1. *Repair of O^6-methylguanine-DNA by AGT.*

increase our understanding of this DNA repair mechanism, we became interested in the generation of artificial AGTs based on an antibody scaffold. It has been shown that the generation of catalytic antibodies, their detailed characterization, and comparison to their natural counterparts can yield important insights into enzyme catalysis.[2, 3] In the case of an artificial AGT, it would be particularly interesting to see how a reactive cysteine residue is generated, how the guanine leaving group in the S_N2 reaction is activated, and if a base-flipping mechanism is used. Here, we introduce a stepwise approach to the development of artificial AGTs and its application to the in vitro selection of antibodies with highly reactive cysteine residues. These experiments open the way for the generation of artificial AGTs based on antibody scaffolds.

Recently, we showed that the incorporation of O^6-alkylguanine derivatives of the type **1** into oligonucleotides leads to covalent



biotinylation of human AGT (hAGT).[4] Together with the display of hAGT on the surface of phage λ, **1** can be used to link the DNA repair activity of hAGT with its genotype.[4] A similar approach appears feasible for the direct selection of the desired DNA repair activity from antigen-binding fragment (Fab) phage antibody libraries. However, as a result of the low reactivity of O^6-alkylated guanine as a substrate in S_N2 reactions, we envisioned a stepwise selection of artificial AGTs in which sulfhydryl-specific reagents of the types **2** and **3** are first used to generate antibodies with reactive cysteine residues. These antibodies are then the basis for selections against O^6-alkylguanine derivatives of the type **1**. We reasoned that **2** might allow for the selection of a reactive cysteine residue as well as a binding site for guanine. Sterically hindered disulfides such as **3** have been used previously for the in vitro selection of reactive cysteine residues that possess Fab from phage antibody libraries, which leads to the generation of antibodies with esterase activity.[5] Details of the synthesis of **2** and **3** and the kinetic characterization of the antibodies is available in the Supporting Information.

As a source of diversity, the previously described antibody Fab phage library k10E was used.[5] This library is based on a human antitetanus toxoid antibody in which the complementarity-determining region 3 of the heavy and light chains (HCDR3 and LCDR3, respectively) were randomized. Selections were carried out by using either **2** or **3** at concentrations of 10 – 100 pM to form biotinylated phage, which were captured on streptavidin-coated magnetic beads. Bound phage were eluted with dithiothreitol, which reduces the disulfide bond between the antibody and biotin. The phage were then amplified in *Escherischia coli* XL-1 Blue for the next round of selections. No enrichment was observed in the selections with **2**, in contrast to the selections with **3**. Control experiments with helper phage VCSM13 indicated that **2** leads to unspecific biotinylation of the phage. To compare the reactivities of **2** and **3** towards a sulfhydryl group within a protein environment, their rate constants for the reaction with the cysteine residue (Cys34) of bovine serum albumin (BSA) were determined. Compound **2** possesses a 6000-fold higher reactivity than **3**, as indicated by

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rate constants of $1.8 \times 10^5\,min^{-1}\,M^{-1}$ and $30\,min^{-1}\,M^{-1}$, respectively. We conclude that the reactivity of **2** was too high for the envisioned phage selections, which stresses the importance of a careful choice of the reactivity of the substrate in a given selection system. Clones obtained after four rounds of selection with **3** were further analyzed. Approximately 20% of the phagemids (phage that contain a plasmid) isolated after the fourth round had a Fab gene of the correct size. Thirteen of the phagemids that possessed a Fab insert were characterized by sequencing. Of these, seven possessed at least one cysteine residue in either the LCDR3 or HCDR3 chain (Table 1). It is interesting to note that one of the cysteine residues of the RD1 antibody is part of the sequence PCTR. The motif PCHR is conserved in the active site of O^6-alkylguanine-DNA alkyltransferases, which suggests that it might be a general motif for the generation of reactive cysteine residues.[1]

Table 1. *Sequence of CDR3s of cysteine-containing Fab fragments.*[a]

Clone	HCDR3	LCDR3
	GXXXXXXXXXXXXXDXW	YXXXXXXT
RD1	G***C***KP***C***TRPISVIQ***C***DGW	YGLLKRGT
RD2	GAVFMZMRVGSGPPD***C***W	YVWGLVRT
RD3	G***C***MMPGSSRMSFL***C***DIW	YGARQSGT
RD4	GATVTSEG***C***VSRHGDNW	YGRAWRLT
RD5	GS***C***QRFMWMPLRGVDAW	YGGGRMRT
RD6	GQTPGRPQMWQYSLDVW	YGFP***C***GGT
RD7	GZAGZVTNYVFSGRDGW	YTLQRR***C***T

[a] X represents a randomized position; Z is a TAG stop codon, which is suppressed as Q in XL-1 Blue.

To examine whether the selected antibodies indeed possess reactive cysteine residues, the *gp3* gene, which anchors the Fab on the phage, was deleted from the phagemids and crude extracts of *E. coli* XL1-Blue that expressed Fab were analyzed.[6] These extracts were incubated with **3**, biotinylated Fab was immobilized on streptavidin-coated microplates, and this immobilized Fab was detected by using an antihuman $F(ab')_2$-alkaline phosphatase conjugate. A clone picked at random from the initial library, designated as RP1, was also examined. Extracts from bacteria that express clones RD1 – 5 and RD7 produced signals at least twofold above the corresponding values of the RP1 antibody, with the strongest signal produced by extracts from bacteria that express clone RD3. These data suggest that the selected antibodies indeed possess reactive cysteine residues.

To simplify expression and purification for a detailed characterization of one of the antibodies and, in particular, to avoid the deletion of antibody genes in future selections, the clone RD3 was transformed into a single-chain format (scFv) by using the vector pAK300.[7] This vector possesses a higher stability than pComb3 and the expression of the antibody is tightly controlled. For the construction of the scFv RD3 (scRD3) antibody from the two variable domains, the orientation V_H-linker-V_L was chosen, with a nonrepetitive peptide of 20 amino acids as a linker (V_L = light variable domain, V_H = heavy variable domain).[8] scRD3 was expressed and purified following published procedures and was isolated as a dimer in a yield of 0.8 mg per liter shake-flask culture.[9] The formation of dimers or oligomers is not uncommon for scFv proteins and does not necessarily affect binding properties.[10] To ensure that the transformation of the Fab RD3 clone into an scFv did not significantly affect its activity, we also expressed and purified the corresponding Fab RD3 antibody.[6] About 0.6 mg of Fab was isolated per liter shake-flask culture.

The reactions of the scRD3 and RD3 antibodies with **3** were then analyzed in detail (Figure 1). scRD3 reacts with about 1.5 equivalents of **3**. The reaction can be divided into a fast and a

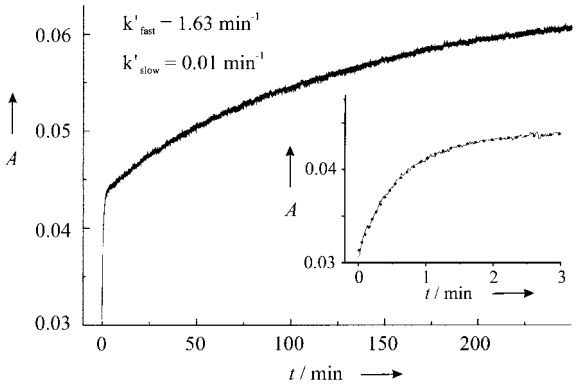

Figure 1. *Reaction of scRD3 (4 μM) with* ***3*** *(100 μM) in 3-(N-morpholino)propanesulfonic acid (30 mM), acetonitrile (4% v/v), and ethylenediaminetetraacetate (0.5 mM) at pH 7.4, 25 °C. The reaction was followed by measurement of the increase in absorbance at 343 nm and can be best fit to a two-exponential function to yield the pseudo first-order rate constants k'_{fast} and k'_{slow}. Inset: First three minutes of the reaction. The dotted line represents the best fit to a two-exponential function.*

slow process with an amplitude ratio fast/slow of about 0.9. These data indicate that the scRD3 antibody possesses two unpaired cysteine residues with significantly different reactivity. The fact that each cysteine residue undergoes only 75% reaction with **3** is most likely a result of oxidation of the unpaired cysteine residues. The biotinylation of purified scRD3 by **3** was verified by Western blotting by using a streptavidin – peroxidase conjugate for the detection of biotinylated antibody. Incubation of the antibody prior to the reaction with the sulfhydryl-specific reagent *S*-methyl methanethiosulfonate blocked the reaction between scRD3 and **3**.[11] Measurement of the reaction at different concentrations of **3** and plots of the observed pseudo-first-order rate constants as a function of the concentration of **3** gave second-order rate constants (k_{fast}, $(1.9 \pm 0.1) \times 10^4\,min^{-1}\,M^{-1}$; k_{slow}, $(9 \pm 1) \times 10^1\,min^{-1}\,M^{-1}$). Analysis of the reaction of the Fab RD3 antibody with **3** revealed that the RD3 clone also possesses two cysteine residues with differing reactivity. The rate constant for the fast process is $8 \times 10^3\,min^{-1}\,M^{-1}$. This value is within a factor of 2.5 of the corresponding rate constant for the scRD3 antibody and thus shows that the properties of clones RD3 and scRD3 are indeed very similar.

The second-order rate constant for the fast reaction of scRD3 with **3** is 80 times higher than that for reaction of **3** with the corresponding amino acid ($k = 250\,min^{-1}\,M^{-1}$). Considering that an unpaired cysteine residue of an antibody displayed on phage

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has to be protected against oxidation, this rate enhancement is remarkable. Compared to the cysteine residue in BSA ($k = 30\ min^{-1}\,M^{-1}$), the reactivity of the scRD3 cysteine residue is 670-fold higher. Two scenarios appear plausible for the mechanism of the reaction of the antibody with **3**. In the first, the two different cysteine residues react in an ordered fashion. In the second, the first reaction is followed by intramolecular disulfide exchange between the two cysteine residues before the antibody reacts with another molecule of **3**. To investigate which of the two cysteine residues Cys^{96} and Cys^{100k} in the RD3 antibody is the more reactive one and whether their reactivity is mutually dependent, each was mutated to alanine to give the mutants ^{Ala96}RD3 and ^{Ala100k}RD3. The expression level for both mutants as a Fab were significantly below that for wild-type RD3 antibody. However, 0.16 mg of the ^{Ala100k}RD3 mutant could still be isolated from one liter of shake-flask culture whereas we were not able to isolate sufficient amounts of the ^{Ala96}RD3 mutant. The ^{Ala100k}RD3 mutant possesses 0.9 equivalents of free thiol per Fab and its second-order rate constant for the reaction with **3** is $2.5 \times 10^3\ min^{-1}\,M^{-1}$. These data demonstrate that the presence of both cysteines is important for the folding of the antibody and that their reactivity is mutually dependent. The fact that the mutant ^{Ala100k}RD3 possesses a higher reactivity than the less reactive cysteine residue of wild-type RD3 antibody suggests that Cys96 is the reactive residue.

In summary, this work represents an important step in our efforts towards the generation of artificial DNA repair proteins. The established in vitro selection system and the isolated clones with reactive cysteine residues, in particular scRD3, open the way for selections of antibody-based AGTs by using derivatives of the type **1**.

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Synthesis and NMR Structure of Peptidomimetic α4β7-Integrin Antagonists

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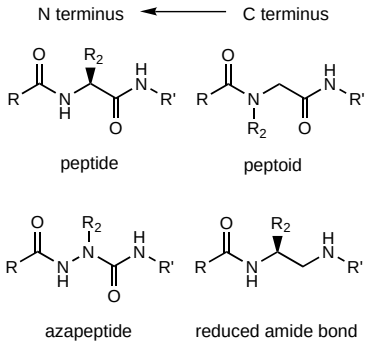
The development of new anti-inflammatory drugs is currently one of the great challenges in medicinal chemistry.[1] Recently, α4β1- and α4β7 integrins were shown to be promising targets for treating inflammatory and autoimmune diseases.[2–4] α4β7 integrins bind to the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) through their Leu-Asp-Thr sequence (LDT sequence) specifically and with high affinity.[5, 6] An overexpression of MAdCAM-1 occurs in mouse models of colitis, in human inflammatory bowel disease (IBD), and in chronic inflammatory liver disease.[7, 8] Recently, we and others were able to derive peptidic α4β7 integrin antagonists from the natural LDT-binding sequence.[9–11] For the further development of nonpeptidic and highly selective drugs, we identified the structural and functional requirements of the LDT recognition sequence within α4β7 integrin antagonists by using several peptidomimetic variations such as peptoids, azapeptides, and reduced amide bonds (Scheme 1). An aromatic residue *N*-terminal to the LDT sequence



Scheme 1. *Comparison of different peptidomimetics.*

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