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Converting the Sacrificial DNA Repair Protein N-Ada into a Catalytic Methyl Phosphotriester Repair Enzyme

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The Ada protein is a key component of an inducible pathway in *Escherichia coli* that confers resistance to the genotoxic effects of methylation agents.¹ The N-terminal domain of Ada (N-Ada) repairs *S_p*-configured methyl phosphotriesters in DNA by direct transfer of the methyl group to one of its cysteine residues, Cys38.^{2,3} This methyl transfer results in irreversible loss of repair activity; hence, N-Ada is not an enzyme in the true sense but instead is a sacrificial intracellular reagent for DNA repair.

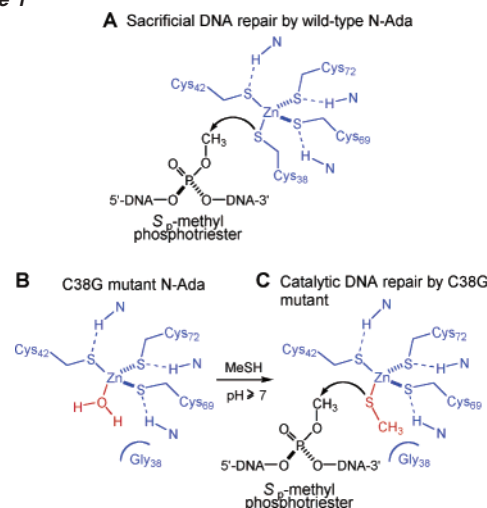
N-Ada contains a zinc ion tightly bound to four cysteine residues, one of which is Cys38, the methyl acceptor identified and reassigned through our recent structural and biochemical studies (Scheme 1).^{2–4} Metal coordination is inextricably linked with the acceptor function of Cys38, thus pointing to a novel mechanism involving metallo-activation of the Cys sulfur atom by its ligated metal. Indeed, N-Ada is the prototype for an emerging group of proteins that use zinc-coordinated thiolates as nucleophiles in alkyl group-transfer reactions. With the exception of N-Ada, all other proteins that use thiol-metalloactivation are bona fide enzymes capable of performing multiple turnovers on nonself-substrates.^{5,6} Here we report the discovery that Ada can be reconfigured to act also as a true enzyme.

Methyl iodide regiospecifically alkylates N-Ada on Cys38,⁷ showing that this particular Cys ligand is electronically activated relative to the other three. Recent structural studies have shed light on the basis for this selectivity. The thiolate sulfur atoms of Cys42, -69, and -72 are hydrogen-bonded to amide protons of the protein main chain, which suppresses their reactivity but stabilizes the protein structure. On the other hand, the Cys38-S[−] is devoid of hydrogen-bonding interactions (Scheme 1A).³ We reasoned that mutation of Cys38 to Gly (C38G) might produce a folded protein having a water molecule occupying the zinc coordination site vacated by Cys38 (Scheme 1B), as the structure shows no residue on the protein that could obviously occupy this site instead. An external thiol source might then displace the coordinated water and be activated by the metal to transfer a methyl group from a methyl phosphotriester (Scheme 1C). Thus, instead of Ada itself acting as the sacrificial methyl acceptor, the external thiol would serve this purpose with the C38G mutant protein, and activity would be retained following turnover so as to constitute a catalytic cycle.

N-Ada16, a truncated version of N-Ada that retains full DNA repair and methylation-dependent DNA binding activity, was used in this study.³ Cys38 of this construct was mutated to a glycine residue (C38G N-Ada16). Colorimetric titration revealed that the mutant protein, like wild-type, contains 0.99 ± 0.02 zinc atom per protein molecule.^{8,9}

The methyl phosphotriester repair activity of C38G N-Ada16 was assayed using T₁₁(OMe), a single-stranded thymidine homopolymer bearing a single, centrally located methyl phosphotriester, as the substrate. As demonstrated previously, removal of a

Scheme 1



methyl group from the phosphodiester backbone of T₁₁(OMe) can be conveniently monitored by anion exchange FPLC.¹⁰ Figure 1A is a trace showing T₁₁(OMe) as obtained directly from synthesis; the major component (retention volume ≈ 12.3 mL) is T₁₁(OMe), and the minor peak (13.1 mL) is T₁₁ that arises via hydrolysis during release from the synthesis resin (Figure 1A). Wild-type N-Ada16 repairs only the *S_p* diastereomer of T₁₁(OMe), which comprises roughly half of the T₁₁(OMe) peak. Repair is therefore complete when half of the T₁₁(OMe) has been converted to T₁₁ (eq 1 and Figure 1E).

Incubation of the C38G mutant protein (10 μ M) with T₁₁(OMe) (10 μ M) at 4 $^{\circ}$ C overnight gave no reaction, suggesting the presumptive metal-bound water is not sufficiently nucleophilic (Figure 1B). Neither was any reaction observed when 50 mM methanethiol was incubated with T₁₁(OMe) in the absence of C38G N-Ada16 (Figure 1C). However, when both the mutant protein (10 μ M) and methanethiol (50 mM) were present, half of the starting T₁₁(OMe) was converted to T₁₁ (Figure 1D). In the presence of excess substrate T₁₁(OMe), we observed up to four turnovers at 4 $^{\circ}$ C overnight, establishing that the system is truly catalytic (Figure S2). It should be noted that phosphotriester repair by wild-type N-Ada16 is 10–100 times faster than that by the C38G mutant protein.

A hallmark of wild-type Ada is its stereochemical selectivity for *S_p* methyl phosphotriester.¹ To evaluate the stereoselectivity of the C38G protein, we utilized an HPLC-based nucleoside composition assay.^{10,11} Following the repair reaction, the DNA product mixture was digested down to its constituent nucleosides using snake venom phosphodiesterase, Benzonase, and alkaline phosphatase. Under these conditions, all phosphate mono- and diester bonds are hydrolyzed to completion, but the methyl phosphotriester linkage is completely resistant to cleavage. The *S_p* and *R_p*

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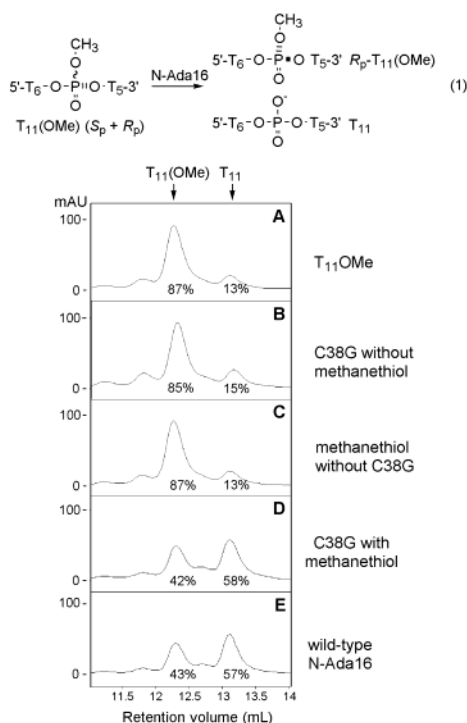


Figure 1. DNA methyl phosphotriester repair by N-Ada16 and C38G N-Ada16. (Top) The substrate T₁₁(OMe) is a diastereomeric mixture at the phosphotriester center. Wild-type N-Ada16 repairs only the S_p diastereomer. (Bottom) Results of repair reactions with wild-type and C38G N-Ada16 under various conditions.

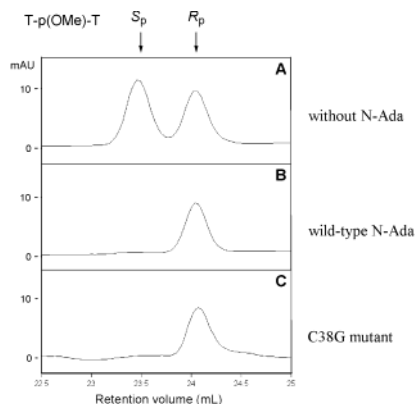


Figure 2. Stereochemistry of phosphotriester repair by wild-type and C38G N-Ada16. Arrows denote the retention volumes of the S_p and R_p diastereomers of T-p(OMe)-T.

diastereomers of the unhydrolyzed dinucleoside phosphotriesters, T-p(OMe)-T, are separable by reversed-phase HPLC (Figure 2). The digest of T₁₁(OMe) clearly reveals the presence of the S_p and R_p diastereomers in roughly equal amounts (Figure 2A). Upon treatment of T₁₁(OMe) with wild-type N-Ada16 at 4 °C overnight, the peak corresponding to the S_p diastereomer disappeared completely, while the amount of the R_p diastereomer remained the same (Figure 2B), consistent with the known stereochemical preference of N-Ada. When the C38G mutant N-Ada16 was treated with T₁₁(OMe) in the presence of excess methanethiol under the same conditions, complete repair of only the S_p diastereomer was again

observed (Figure 2C). Thus, C38G N-Ada16 exhibits the same stereochemical preference as wild-type N-Ada, despite the fact that the mutant enzyme is utilizing an exogenous sacrificial acceptor.

The catalytic methyl transfer reaction was observed to proceed at pH 7–11 but did not occur to an appreciable extent at pH 6, consistent with the expectation that methanethiolate is the nucleophile in the reaction.^{12,13} The pK_a of methanethiol in aqueous solution is at the upper end of this range, 10.33,¹⁴ lending credence to the notion that coordination of the methanethiol sulfhydryl group to the Lewis acidic Zn²⁺ ion in C38G N-Ada16 lowers its pK_a by several units.

Whereas methanethiol supported phosphotriester repair in this system, ethanethiol, 2-mercaptoethanol and dithiothreitol (DTT) were all ineffective (data not shown). Mutation of Cys38 to glycine creates a small pocket in the active site. Inspection of the N-Ada16/DNA complex structure shows that nothing larger than a methyl group can be accommodated in this pocket, if the protein fold were to remain the same after the mutation. Methanethiol (50 mM) alone does not show noticeable repair activity of T₁₁(OMe) at pH 11, indicating that besides deprotonating the coordinated thiol group, the protein also plays an important role by bringing the substrates together and positioning them for the methyl-transfer reaction.

Here we have shown that the sacrificial DNA repair protein can be converted into a true enzyme by removal of its internal nucleophile and supplying an external nucleophile as a methyl group acceptor. Why Nature chose not to use the latter strategy is a matter of some curiosity. Irreversible methylation of the protein may help N-Ada to perform its second function: sensing methylation challenge and activating transcription of methylation resistance genes.

Acknowledgment. This research was supported by NIH Grant GM 44853. C.H. was supported as a Merck Fellow of the Damon Runyon Cancer Research Foundation.

Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA028046A