Chapter 2

[4Fe-4S] Cluster Ligand Mutants of *Archaeoglobus Fulgidus* Uracil DNA Glycosylase

UDG protein samples were prepared by L. Engstrom and X. Xu from the laboratory of Professor S. David (University of California, Davis).

Abstract

Archaeoglobus fulgidus is a hyperthermophilic archaeon that expresses a [4Fe-4S] containing uracil DNA glycosylase (UDG) enzyme. UDG proteins excise uracil that has been misincorporated into DNA. This uracil is often formed by deamination of cytosine, a process whose rate is greatly accelerated at high temperatures. In order to cope with this excess DNA damage, thermophilic UDGs may contain unique features that enable them to better detect and repair their substrate. We have proposed that *A. fulgidus* UDG uses its [4Fe-4S] cluster to detect DNA lesions through DNA-mediated CT. To better understand the charge-transfer characteristics of *A. fulgidus* UDG, several variants were created that contain mutations in the cysteine residues that ligate the iron-sulfur cluster. The C17H, C85S, and C101S mutants were examined by cyclic voltammetry on DNA-modified electrodes to determine whether they were CT deficient relative to wild-type UDG. C17H produced a signal that was larger than that of WT UDG, C101S produced a signal within error of that of WT, and C85S produced a weaker signal than that of WT. These results indicate that changes in the iron-sulfur cluster coordination environment of UDG can drastically perturb the DNA-bound CT properties of this enzyme.

Introduction

Extremophilic species have long been of interest to life sciences research

because of their ability to survive in a variety of harsh environments. Among the many extremophiles that currently receive increased research attention is the hyperthermophilic archaeon *Archaeoglobus fulgidus*, which can survive in geothermal springs and high-temperature oil fields [1, 2]. Adapting to such high temperatures probably requires a range of physiological changes relative to organisms that live under less extreme conditions. These changes may include modified DNA repair pathways to combat excess mutagenesis induced by high temperatures. For example, cytosine deamination occurs at greater frequency at high temperatures [3], and this process converts cytosine to uracil in DNA. Although thermophiles likely endure higher rates of cytosine deamination in their natural environment, those whose mutation rates have been examined do not exhibit higher rates of mutation than non-thermophiles [4].

Researchers who study this phenomenon have pointed focused on Uracil DNA glycosylase (UDG), the enzyme that removes misincorporated uracil from DNA. Many thermophilic UDGs, (which belong to Families IV, V, and VI of the UDGs [5–9]) contain a [4Fe-4S] cluster [5–7, 10, 11], (Figure 2.1). The presence of this iron-sulfur cluster in *A. fulgidus* UDG makes it comparable to other [4Fe-4S] cluster base excision repair (BER) enzymes such as MutY and EndoIII, which our laboratory has also studied [12]. The [4Fe-4S] clusters in these enzymes are redox-active at physiologically relevant potentials only when bound to DNA [12]. The data led us to hypothesize that thermophilic UDGs scan the genome electrochemically in search of DNA damage.

In our model for DNA damage detection, a bound enzyme transmits an electron to another protein bound to a distal location on the DNA. Upon reduction, this "distal" protein loses some of its DNA affinity, dissociates, and re-binds to a different region of the genome. However, if a lesion is present in the DNA sequence between the two bound proteins, CT will not proceed efficiently, both proteins remain oxidized and DNA-bound, and then localize to find and repair the damage between them. This model is supported by the fact that DNA-mediated charge-transfer (CT) can occur efficiently over long distances [13, 14], but is attenuated by mismatches and damage sites in the DNA helix [14, 15]. We have also shown that the affinity of certain BER enzymes for DNA is stronger in their oxidized 3^+ form than in their reduced 2^+ form [16]. Because CT would provide a means of DNA damage detection that is much faster than a processive search [17], perhaps the presence of a [4Fe-4S] cluster enables UDGs from thermophilic organisms to detect DNA damage more efficiently and thus cope with excess, temperature-induced DNA damage.

This model of DNA damage detection assumes that CT proceeds efficiently from the DNA helix to the [4Fe-4S] cluster of a bound enzyme such as UDG. Accordingly, UDG must contain amino acids that ligate the metallocluster, maintain it in a CT-active and stable conformation, and facilitate CT between the DNA and the protein. In order to study which amino acids mediate CT in UDG, several site-directed mutants were prepared and characterized electrochemically.

The *A. fulgidus* UDG mutants examined here, C17H, C85S, and C101S, all

contain mutants at one of the four cysteine residues that ligate the iron-sulfur cluster. In family IV UDG's, the iron-sulfur cluster is ligated by a C-X_2-C-X_n-C-$X_{14\text{-}17}$-C sequence where n=70–100, and the cysteine locations are well conserved [11, 18], (Figure 2.2). These UDG mutants were examined on DNA-modified electrodes, and the signal strength of different mutants' cyclic voltammogram signals were quantified relative to WT protein. An attenuated electrochemical signal indicates a reduced ability to mediate CT. Through these experiments, several mutants of *A. fulgidus* UDG were detected that have CT abilities different than those of WT protein.

Materials and Methods

Oligonucleotide synthesis. The sequences used for quantitative electrochemistry experiments were SH-(C_6H_{12})-(RR)- 5'-GA GAT ATA AAG CAC GCA-3' and complement, SH-(C_6H_{12})- 5'-AGT ACA GTC ATC GCG-3' and complement, and SH-(C_6H_{12})- 5'-AGT ACA GTC ATC GCG-3' paired with RR- 3'- TCA TGT CAG TAG CGC- 5'. "RR" stands for Redmond red, a redox active probe used to quantify the amount of DNA on the electrode surface [19]. All DNA sequences were prepared on an AB 3400 DNA Synthesizer from ABI using standard phosphoramidite chemistry. Phosphoramidites were purchased from Glen Research. Oligonucleotides were purified by HPLC on a C18 reverse phase column and characterized by MALDI-TOF mass spectrometry.

DNA Annealing. For electrochemistry experiments, complementary strands were mixed in a 1:1 ratio in DNA Buffer (10 mM NaPi, 50 mM NaCl, pH = 7.5), heated to 90ºC for several minutes, and then allowed to cool slowly to room temperature over 1–4 hours.

Preparation of DNA-Modified Electrodes. After hybridization to their complement, oligonucleotides duplexes were deposited onto an Au surface in phosphate buffer (50 mM NaCl, 5 mM sodium phosphate, pH 7.0) and incubated for 24–36 hours. Au substrates were purchased from Molecular Imaging. The electrode surface was then washed, and further passivated by incubation with 100 mM mercaptohexanol in phosphate buffer for 30–45 minutes. The electrode surface was washed again in phosphate buffer, and then protein buffer (see below).

Cyclic Voltammetry. Preparation of the DNA-modified Au electrode and subsequent cyclic voltammetry experiments were performed as described above and previously [12, 15, 17, 20–22]. The DNA–modified Au electrode served as the working electrode, a Pt wire served as the auxiliary electrode, and the reference was either an Ag/AgCl electrode modified with an agarose tip or a 66-EE009 Ag/AgCl reference electrode (ESA Biosciences). Unless otherwise noted, all scans were taken at a rate of 50 mV/s on a CH Instruments 760 potentiostat. Each experiment used 50 μL of 50–200 μM of protein in UDG Buffer: (50 mM NaPi pH =7, 50 mM NaCl, 0.5 mM EDTA, 25% glycerol). The protein signal intensity was normalized to DNA concentration using the intensity of the Redmond red signal. For each

comparison, the protein samples were measured consecutively on the same electrode surface.

Protein Purification. UDG was prepared as documented previously [12]. The concentration was quantified using ε_{390}= 20,000 $M^{-1}cm^{-1}$.

Results

Quantitative electrochemistry of BER Enzymes on DNA-modified electrodes. WT UDG, C17H, C85S, and C101S were examined on electrodes prepared with Redmond-red modified DNA. Redmond red is a redox-active probe whose cyclic voltammetry signal intensity can be used to quantify the amount of DNA present on the electrode surface [19]. The Redmond red was positioned so that it could contact the Au surface directly without requiring a DNA-mediated signal. In cyclic voltammograms taken on these electrodes, two peaks are visible, one from Redmond red and one from the protein sample (Figure 2.3). In these experiments, the signal from C17H is consistently larger than that of WT UDG, the signal strength from C101S is within error of that of WT UDG, and the signal from C85S is consistently weaker than that of WT UDG. The signal strength ratios of these variants were 2.2, 0.5, and 1.0, respectively (Table 2.1).

Discussion

All the mutants examined contain substitutions in one of the cysteine residues that ligates the iron-sulfur cluster. Histidine-substituted mutants were

prepared because this residue is a naturally occurring iron-sulfur cluster ligand in some proteins [23]. Substitution of histidine for cysteine did not severely reduce the strength of the mutants' cyclic voltammetry signals relative to wild-type enzyme. As a matter of fact, C17H produced a stronger signal on DNA-modified electrodes than WT UDG, possibly because histidine's aromatic character may render it particularly effective at mediating CT [24]. Serine is not known to occur as a cluster ligand in nature, but perhaps its hydroxyl side chain could substitute for the thiol found in cysteine. C101S, for example, produced a signal strength within error of that of WT UDG. C85S produced an incredibly weak signal relative to wild-type enzyme, although the discrepancy in signal attenuation between the two serine mutants could result from their position within the UDG structure. In the crystal structure of *T. thermophilus* UDG, another thermophilic organism, the residue equivalent to C101 is oriented towards the interior of the protein, making it the least solvent-exposed of the cysteines [11]. Consequently, perturbations at this residue may only minimally affect the stability of the [4Fe-4S] cluster. It is also possible that solvent could ligate the [4Fe-4S] cluster in some of the mutants and/or that minor structural rearrangements could occur that allow other residues to ligate the cluster instead of the native cysteines [25].

These data have identified types of amino acids that can effectively substitute for cysteine in ligating the [4Fe-4S] cluster of UDG and still permit efficient CT. Of the three mutants examined here, the histidine mutant was most likely to be functional and the serine mutants were somewhat functional.

Importantly, though, all of these mutants exhibited a midpoint potential within error of that of WT UDG, which was reported as 95 mV versus NHE [11]. These measurements demonstrate that the [4Fe-4S] cluster is intact in these mutants, and that they are in the range characteristic of high potential iron proteins (HiPIPs) that transition between the 2^+ and 3^+ oxidation states [26]. This potential is also physiologically relevant, suggesting that UDG could participate in electron transfer reactions *in vivo.* These experiments also show that changes in the coordination sphere of the iron-sulfur cluster of UDG can dramatically impact its electrochemical activity. Further work will be necessary to determine whether such mutations undermine the ability of UDG to detect and repair DNA damage inside a cell.

Figure 2.1: Structure of DNA-bound Family V UDG from *T. thermophilus* HB8. A uracil substrate is shown in purple. Adapted from reference [7] and formatted in PyMol [27] PDB File: 2DEM.

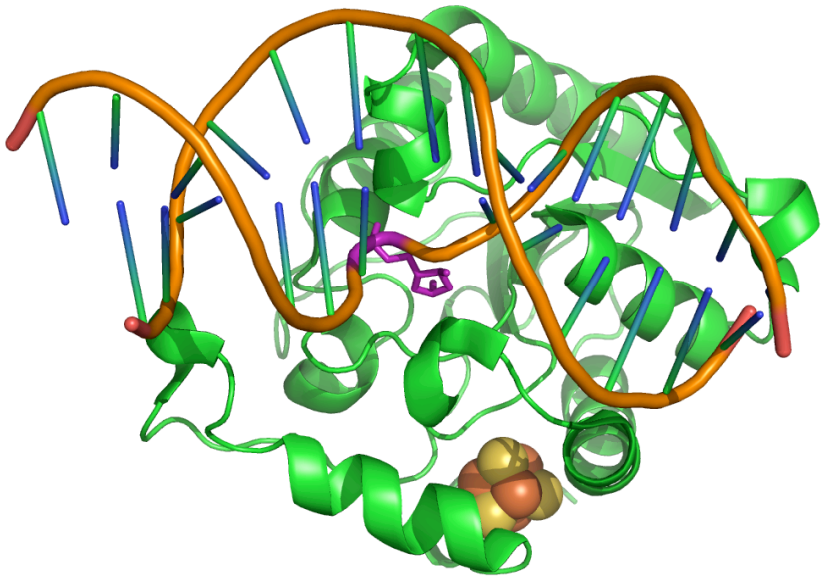

Figure 2.2: Sequence alignment of Family IV UDG proteins. All sequences are from thermophilic organisms. Alignment was made using ClustalW. Conserved cysteine residues are emphasized in orange, other highly conserved residues are highlighted in blue.

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TThermophilus   -----MTLELLQAQAQNCTACRLMEGRTRVVFGEGNPDAKLMIVGEGPGEEEDKTGRPFV 55
Tmaritima       MYTREELMEIVSERVKKCTACPLHLNRTNVVVGEGNLDTRIVFVGEGPGEEEDKTGRPFV 60
Afulgidus       ----MESLDDIVREIMSCRKCDLHKTKTNYVPGVGNEKAEIVFVGEAPGRDEDLKGEPFV 56
                              *  *

TThermophilus   GKAGQLLNRILEAAGIPREEVYITNIVKCRPPQNRAPLPDEAKICTDKWLLKQIELIAPQ 115
Tmaritima       GRAGMLLTELLRESGIRREDVYICNVVKCRPPNNRTPTPEEQAACG-HFLLAQIEIINPD 119
Afulgidus       GAAGKLLTEMLASIGLRREDVYITNVLKCRPPNNRDPTPEEVEKCG-DYLVRQLEAIRPN 115
                                        *               *

TThermophilus   IIVPLGAVAAEFFL----GEKVSITKVRGKWYE---WHG-IKVFPMFHPAYLLRNPSRAP 167
Tmaritima       VIVALGATALSFFVD---GKKVSITKVRGNPID---WLGGKKVIPTFHPSYLLRNRS--- 170
Afulgidus       VIVCLGRFAAQFIFNLFDLEFTTISRVKGKVYEVERWGKKVKVIAIYHPAAVLYRPQ--- 172

TThermophilus   GSPKHLTWLDIQEVKRALDALPPKERRPVKAVSQEPLF 205
Tmaritima       ---NELRRIVLEDIEKAKSFIK-KEG------------ 192
Afulgidus       -----LREEYESDFKKIGELCGKKQPTLFDYL------ 199
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Figure 2.3: Cyclic voltammograms of UDG mutants on DNA-modified electrodes. Representative cyclic voltammograms are shown for C17H (orange), C85S (green), and C101S (red). In each image, the scan of WT UDG is also shown (blue), along with a buffer-only control scan (gray).

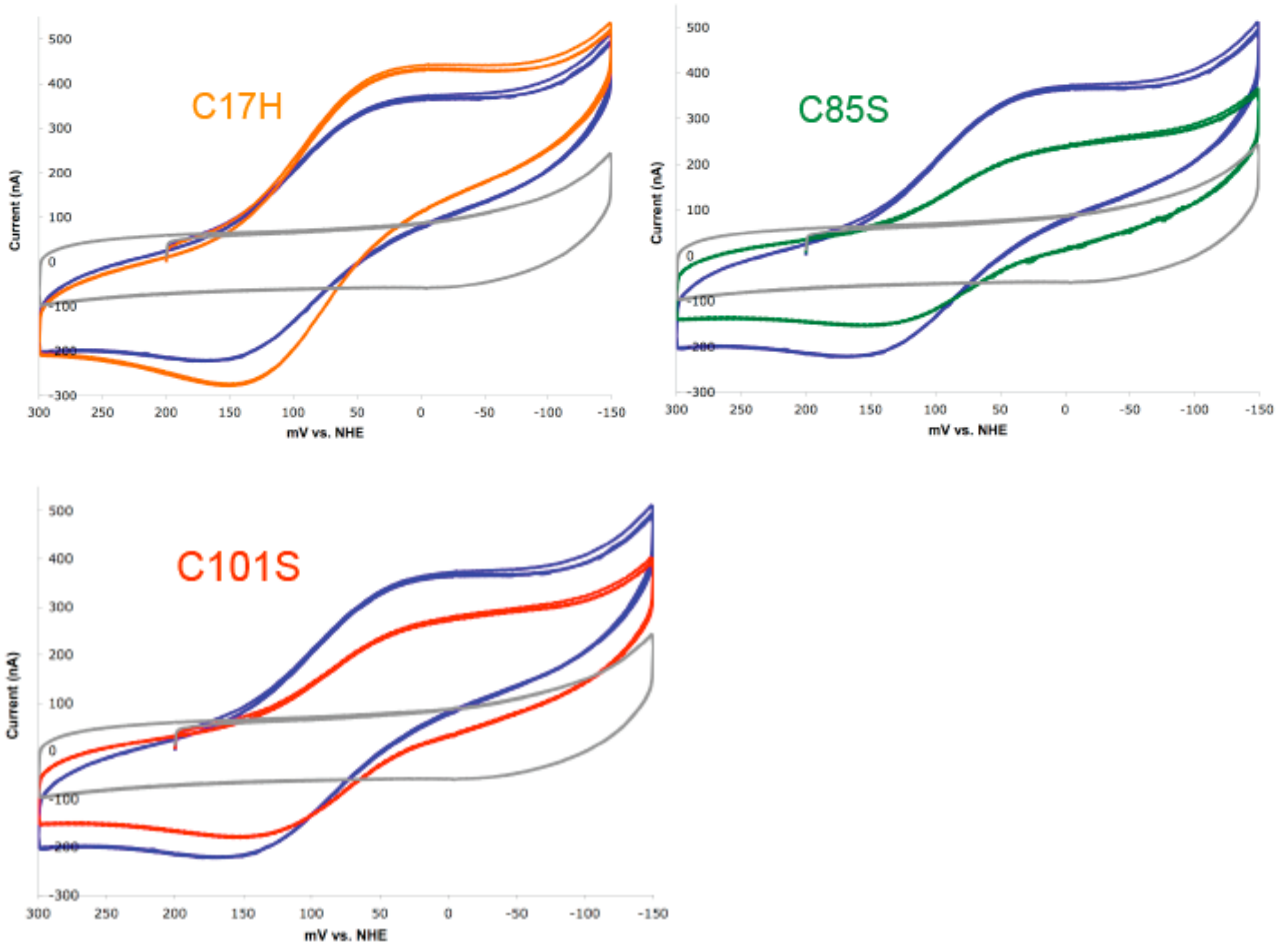

Table 2.1: Quantitative summary of electrochemical data for UDG mutants. Three scans of each mutant were taken. For each sample, WT UDG was measured on the same electrode. The signal strength ratio (area under the cyclic voltammogram curve) was tabulated for each trial.

Trial	Mutant to WT Signal Strength Ratio (RR Corrected)		
	C17H	C85S	C101S
1	3.2	0.5	1.0
2	1.1	0.4	0.9
Average	**2.2**	**0.5**	**1.0**

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