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The Missing Electrostatic Interactions Between DNA Substrate and *Sulfolobus solfataricus* DNA Photolyase: What is the Role of Charged Amino Acids in Thermophilic DNA Binding Proteins?

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Abstract

DNA photolyase can be used to study how a protein with its required cofactor has adapted over a large temperature range. The enzymatic activity and thermodynamics of substrate binding for protein from *Sulfolobus solfataricus* were directly compared to protein from *Escherichia coli*. Turnover numbers and catalytic activity were virtually identical, but organic cosolvents may be necessary to maintain activity of the thermophilic protein at higher temperatures. UV-damaged DNA binding to the thermophilic protein is less favorable by ~2 kJ/mol. The enthalpy of binding is ~10 kJ/mol less exothermic for the thermophile, but the amount and type of surface area buried upon DNA binding appears to be somewhat similar. The most important finding was observed when ionic strength studies were used to separate binding interactions into electrostatic and nonelectrostatic contributions; DNA binding to the thermophilic protein appears to lack the electrostatic contributions observed with the mesophilic protein.

Graphical Abstract

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INTRODUCTION

DNA photolyase is a structure specific DNA repair enzyme that reverses one of the most common types of UV damage in DNA molecules, the cis-syn cyclobutylpyrimidine dimer (CPD). The protein is found across the family of life with the exception of the placental mammals.^{1,2} The enzyme repairs CPD on UV-irradiated DNA using light-driven electron transfer from a noncovalently bound flavin adenine dinucleotide (FAD) cofactor to the CPD lesion. The active site FAD has three possible oxidation states: the fully reduced FADH^- which is the active state, the one electron oxidized $\text{FADH}^\cdot$ (the neutral semiquinone state), and the fully oxidized FAD state. The repair of the CPD involves several discrete steps including: (a) recognition of and binding to the DNA lesion, (b) absorption of a blue-light photon to drive an electron from the excited active site FADH^- cofactor to the CPD lesion, (c) spontaneous rearrangement of the CPD with electron transfer back to the $\text{FADH}^\cdot$ to reform the active state, and finally (d) release of the repaired DNA from the enzyme. Given the temperature sensitivity of the steps involved in this particular DNA repair cycle, the enzyme will serve as a good model to study how a protein and its required cofactor may be evolutionarily adapted to optimize enzymatic performance over a large temperature range.

The hyperthermophilic photolyase (SsPL) is isolated from *Sulfolobus solfataricus*, an organism commonly found in acidic, hot waters;³ we anticipate SsPL will have a structure similar to that of *Sulfolobus tokodaii* photolyase (StPL, crystal structure 2E0I).⁴ Using a sequence alignment tool with the EBLOSUM62 scoring matrix, the amino acid sequence for StPL is 57.9% identical and 74.2% similar to that of SsPL.⁵ Both SsPL and StPL are unusual photolyases in that they contain two FAD cofactors. One FAD cofactor is part of the active site of the protein and required for both DNA binding and repair. The second cofactor, the putative accessory chromophore, may play a role as a light-harvesting pigment; it is always present in the fully oxidized FAD state.⁶ The active site cycles between FADH^- , the fully reduced form required for activity, and $\text{FADH}^\cdot$, the one-electron oxidized or semiquinone form; SsPL is isolated with the active site mainly in the $\text{FADH}^\cdot$ state. The accessory FAD does not appear to readily undergo any reduction–oxidation chemistry, and it is always found in the fully oxidized state.

In general, thermophilic systems have been found with only subtle changes from the mesophilic system; the main prevailing idea is that thermophilic enzymes need to have decreased flexibility to maintain enzymatic activity at higher operating temperatures. Reported differences include an increase in the number of ion pairs/salt bridges, better packing of hydrophobic amino acids, and increased hydrogen bonding for the thermophilic proteins.^{7–13}

While a number of thermophilic systems have been studied, the DNA photolyase system has some unusual requirements for successful DNA repair, and study of the enzyme will significantly add to our understanding of how such systems are able to adapt to their environmental conditions. First, an unusual oxidation state of the FAD cofactor, the stable neutral semiquinone or $\text{FADH}^\cdot$ state, is part of the enzymatic process. The protein has to stabilize this state from further oxidation to FAD; thus, the reduction potential of the $\text{FADH}^-/\text{FADH}^\cdot$ couple needs to be under tight control for the enzyme to function properly over a

large temperature range. Second, the protein needs to recognize a specific damage site, the CPD, on the DNA molecule. Based upon earlier studies on the mesophilic *E. coli* DNA photolyase (EcPL), we believe the enzyme captures a solvent exposed CPD using a three-dimensional search; it does not appear that the protein uses a facilitated diffusion with sliding or hopping steps.¹⁴ When Fujihashi, Numoto, Kobayashi et al. published the crystal structure for the related archaeal protein StPL and observed changes in the amino acids around the CPD binding site, they speculated that the mechanism by which StPL binds damaged DNA substrate may be different from what has been previously observed.⁴

In this work, we present the results of our study on the first step in the repair mechanism: substrate recognition and binding as measured by isothermal titration calorimetry (ITC). We will compare DNA photolyase from the hyperthermophilic, acidophilic archaeon *Sulfolobus solfataricus* to protein from the well-studied mesophilic *E. coli*.¹⁵ While the thermodynamic parameters obtained for the SsPL system are surprisingly similar to those obtained for EcPL, there are significant differences between the systems which may serve to help us understand how a thermophilic protein operates at high temperature. In addition, we have some preliminary data that raises questions on how the protein is able to maintain redox control of the active site FAD cofactor.

EXPERIMENTAL METHODS

Cloning and Overexpression of SsPL.

The *S. solfataricus phrB* gene was isolated from the *S. solfataricus* P2 genomic DNA (ATCC) by polymerase chain reaction (PCR) using the following forward and reverse primers (IDT DNA Technology). A *Nco* 1 restriction site was incorporated into the forward primer and a *Xho* 1 restriction site was incorporated into the reverse primer. The restriction sites are underlined.

Forward Primer: 5' – CAC TCC ATG GCG CTC TGC CTA TTT ATA TTT – 3'

Reverse Primer: 5' – CGC CTC GAG CTA TTT TAT TTT AGA TTT – 3'

The *phrB* gene was cloned between the *Nco* 1 and *Xho* 1 restriction sites of the pET 14b vector (Novagen). The gene was under the control of the T7 promoter, inducible by isopropyl β -D-1-thiogalactopyranoside (IPTG). The integrity of the gene and the construct was verified by restriction digestion analysis (data not shown) and by DNA sequencing (Genewiz). The construct was transformed into Novablue competent cells (Novagen) and plated on LB Agar plates with 100 μ g/mL ampicillin to select for transformants. The SsphrB-pET14b plasmid was then transformed into Rosetta 2 (DE3) competent cells (Novagen) which were plated on LB Agar plates with 100 μ g/mL ampicillin.

Isolation of Protein.

DNA photolyase from SsPL was isolated using the procedure described for EcPL¹⁶ with the small modifications as outlined below. Cells were grown at 37 °C with ampicillin, induced with 1 mM IPTG, harvested, and stored at –80 °C in lysis buffer, as described earlier. All steps in the isolation were completed at 4 °C.

The cells were thawed and then broken using a Bio-Neb nebulizer with 100 psi of N₂ gas. The solution was then centrifuged at 38 000g for 30 min, and the supernatant was saved. Ammonium sulfate was added to a concentration of 0.15 g/mL of supernatant. A small amount of yellow-brown solid precipitated with dissolution of the ammonium sulfate. The precipitate was removed with 10 min of centrifugation at 38 000g. Additional ammonium sulfate was added to a total concentration of 0.45 g/mL of supernatant and dissolved with stirring. Proteins were recovered as a white-yellow pellet with 10 min of 38 000g centrifugation. The supernatant was discarded.

The pellet of precipitated protein was carefully resuspended with Buffer A (50 mM Hepes, pH 7.0 with 10% (v/v) glycerol, 50 mM NaCl and 10 mM β -mercaptoethanol (BME)). The resulting yellow green solution was desalted and exchanged into Buffer A using desalting columns (Bio-Rad 10DG, 6 kDa cutoff) and then loaded onto a Cibacron Blue 3GA (Sigma-Aldrich) column of 3 cm by 30 cm, previously equilibrated in Buffer A. The column was rinsed with Buffer A until the eluent was colorless. The protein was then eluted with 1.00 M KCl in 50 mM Hepes pH 7.0 with 10% (v/v) glycerol and 10 mM BME. Green and yellow fractions were collected, combined, and exchanged into Buffer A using desalting columns. The desalted solution was then loaded onto a 1 cm by 30 cm heparin sepharose (GE Biosciences) column and left at 4 °C overnight. The protein was eluted using a salt gradient of 80 mL of Buffer A with 80 mL of 1.00 M KCl in 50 mM Hepes pH 7.0 with 10% (v/v) glycerol and 10 mM BME. The green fractions were collected and combined. The protein was then desalted into 20 mM potassium phosphate buffer, pH 7.0 with 0.400 M K₂SO₄. The protein was concentrated (Millipore, 30 kDa cutoff filter), divided into small aliquots, and stored at -80 °C until needed.

Determination of Oxidation States of SsPL.

The protein is isolated with virtually all of the active site FAD in the FADH[•] state but, depending upon the specific preparation, small quantities of the cofactor could also be found in the fully reduced (FADH⁻) or fully oxidized (FAD) form. The oxidation state of the flavin cofactor was determined using UV-vis absorption spectroscopy. The FADH[•] state concentration was determined from the absorbance at 583 nm using a molar absorptivity of 4500 M⁻¹cm⁻¹. The fully oxidized flavin for both active site and antenna was determined from the absorbance at 470 nm after first correcting for the quantity of FADH[•] present: the concentration of fully oxidized FAD = (A₄₇₀ - A₅₈₃)/11 300 M⁻¹cm⁻¹. After acquiring the absorbance spectrum of the native protein to get the semiquinone and oxidized flavin concentrations, the sample was placed in boiling water for 2 min to denature the protein and release all flavin. The resulting solution was spun at 11000 g for 5 min to remove precipitated protein, and the absorption spectrum of the supernatant was obtained. The total flavin content was found from the absorbance at 450 nm using a molar absorptivity of 11 300 M⁻¹cm⁻¹ which then allowed the calculation for the concentration of active site FADH[•] in the original native sample.

Preparation of Substrate Used in Studies.

Undamaged p(dT)₁₀ was purchased from TriLink Biotechnologies. UV-damaged DNA substrate (UV-p(dT)₁₀) was produced as described earlier.¹⁷ The CPD damage site is randomly distributed on the strand with an average of one CPD per DNA strand.

Temperature Dependent Activity Assays.

Protein (~200 nM) and UV-damaged DNA (~6 μ M) were combined in 88 mM K₂SO₄, 20 mM potassium phosphate buffer pH 7.0 (or buffer of choice) to a total volume of 3.00 mL in a quartz cuvette equipped with a septum. The sample, protected from light and on ice, was purged for 10 min with N₂ gas. Fresh dithionite solution (5 μ L of 30 mg/mL concentration) was added via a 10 μ L syringe. The solution was purged on ice for an additional 10 min in the absence of light. The cuvette was transferred to a temperature controlled cuvette holder in a PerkinElmer Lambda 35 UV-vis spectrometer and allowed to equilibrate for 5 min to reach temperature, and the absorption spectrum was obtained. The cuvette was then transferred to a thermostated box equipped with 8 W 365 nm lamp (UVP UVLS-28 EL). The sample was illuminated at 1 min intervals with 365 nm repair light, and the absorption spectrum of the sample was obtained after each interval. The absorbance from repaired DNA at 260 nm was obtained from the spectrum, and the turnover number was obtained as described earlier.¹⁸

Preparation of FADH⁻ Active Site for Binding Studies.

Purified protein was thawed and exchanged into the appropriate buffer using two cycles of microconcentration. The protein was then diluted into the same buffer to a concentration of 15–30 μ M protein along with 5 mM dithiothreitol. Using a quartz cuvette equipped with a septum, the protein solution, on ice, was purged for 10 min with N₂ gas. The solution was then photoreduced via illumination with a small white diode light (Panasonic Dot-It LED) at 4 °C for 10 min; the reduction was checked using absorption spectroscopy. Samples of the reduced protein were removed using a syringe prior to each ITC experiment.

Preparation of Active Site FADH[•] for Binding Studies.

An aliquot of protein was thawed and exchanged into the appropriate buffer using two cycles of microconcentration. The protein was then diluted to a concentration of 15–35 μ M protein in a quartz cuvette equipped with a septum, and the solution was purged on ice for 10 min with N₂ gas. Chemical reduction with sodium dithionite (10 μ L of 20 mg/mL) was used to first fully reduce the active site flavin. After the cofactor was fully reduced, small aliquots (~1 μ L) of 30 mM potassium ferricyanide were added, and the sample absorption was monitored using absorption spectroscopy. The titration was considered complete when ~90% of the protein was in the FADH[•] state with the remaining 10% still fully reduced. Samples for ITC experiments were kept on ice and were removed via a syringe just prior to each experiment.

Isothermal Titration Calorimetry Data.

The temperature dependent binding studies were completed in 20 mM potassium phosphate buffer at pH 7.00 with 88 mM K₂SO₄ (calculated ionic strength of 300 mM). The ionic

strength measurements were done using 50 mM Hepes buffer at pH 7.0 with the appropriate concentration of KCl. Most of the temperature dependent ITC runs for the FADH⁻ state of SsPL were acquired on a GE Microcal ITC 200 while the other work used a TA Instruments NanoITC. Samples were degassed for 5–10 min at the appropriate temperature and ~200 mmHg vacuum using a TA Instruments degasser prior to each ITC experiment. The protein (200 to 300 μ L of 15 to 35 μ M) was loaded in the cell and the syringe was filled with 350–500 μ M UV-p(dT)₁₀ substrate in identical buffer. In a typical run, 20 aliquots of ~2 μ L of substrate were added at 90 s intervals. The integration of the signal and data analysis was carried out using TA Instruments Nanoanalyze or GE Microcal software. In all cases, a one-state independent binding model was used to fit the data. Each binding experiment was replicated at least three times; runs with unusually high ($N > 1.4$) or low ($N < 0.6$) N values (stoichiometry of binding) were discarded.

RESULTS AND DISCUSSION

Characterization of the Isolated Protein.

SsPL was isolated and purified using the procedure developed for the EcPL protein; the only difference between the two proteins during the isolation is that the SsPL appeared to exist mainly in the FADH⁻ state until the protein sat overnight on the heparin sepharose column with exposure to O₂; EcPL is found in the FADH⁻ state during the isolation. The absorption spectrum of isolated SsPL is significantly altered compared to that of the EcPL due to the presence of a second fully oxidized FAD cofactor which appears to replace the nonessential 5,10-methenyltetrahydrofolate cofactor (MTHF, absorption band at 380 nm) found in EcPL, Figure 1. Clearly shown in the SsPL spectrum is the absorption of the putative antenna FAD around 445 and 470 nm which does not disappear with addition of reducing agent. The putative antenna FAD is remarkably resistant to both chemical reduction and photoreduction; we were able to fully reduce this cofactor only in the presence of redox mediators such as benzyl viologen (data not shown).

For SsPL, the presumptive active state FAD appears to be isolated as FADH⁻, the neutral semiquinone form of the cofactor, with the characteristic absorption bands around 635, 590, and 510 nm. The absorption of the FADH⁻ state in SsPL is significantly red-shifted by 6–7 nm compared to that of the EcPL state.

The SsPL FADH⁻ state can be reduced to FADH⁻ with BME, dithiothreitol, or tris (2-carboxyethyl) phosphine hydro-chloride in the presence of blue light or sodium dithionite in the absence of light, in a manner similar to that observed for EcPL; the FADH⁻ state does not absorb strongly in the visible region shown in Figure 1. In the work described below, the antenna FAD is always in the fully oxidized FAD state, while the active site FAD is in either the FADH⁻ or FADH⁻ state.

Temperature Dependence of the Catalytic Activity.

Using a thermostated box equipped with a 365 nm lamp, we were able to measure DNA repair by both SsPL and EcPL as a function of temperature. Turnover numbers (k_{cat}) for the repair are reported in Table 1 for both SsPL and EcPL. To get the FADH⁻ active state from

the FADH⁻ form of the protein, we had to add a reducing agent that was relatively insensitive to temperature. Dithiothreitol and β -mercaptoethanol will reduce the protein in the presence of blue light. At higher temperatures, we found these reducing agents produced a species that also absorbed around 260 nm, the wavelength monitored for repair of the DNA, so fresh sodium dithionite was used as the reducing agent.

Under the specific conditions of the assay, the turnover number for SsPL was measured to be between 2.9 and 3.2 min⁻¹ at lower temperatures. The activity increased slightly to 4 to 5 min⁻¹ from 25 to 45 °C. At 50 °C under the solvent conditions of the assay, the SsPL appeared to denature after repair of the DNA substrate was mostly complete (after roughly 3 min of illumination with blue light); solution turbidity significantly increased as judged by light scatter in the absorption spectrum. Identical measurements on EcPL using the same experimental setup produced turnover numbers of 2.4 to 3.2 min⁻¹ from 10 to 30 °C.

Using the turnover numbers and the binding constants measured with ITC, the catalytic efficiencies of SsPL and EcPL were calculated over the temperature range measured, Table 1. Catalytic efficiency is defined as

$$\text{Catalytic Efficiency} = k_{\text{cat}}/K_{\text{M}} \approx k_{\text{cat}}/K_{\text{D}} \quad (1)$$

where k_{cat} is the rate constant for conversion of product from the enzyme substrate complex (turnover number) and K_{M} is the Michaelis constant for the reaction. Due to the requirement of blue photons for conversion of substrate to product, SsPL does not completely follow the classical Michaelis–Menten mechanism, so it is impossible to measure K_{M} with our experimental set up. Therefore, in eq 1, we replace K_{M} with K_{D} , the dissociation constant measured from our ITC studies. Under the conditions of our activity study, we find that the catalytic efficiency of SsPL is indistinguishable from that of EcPL; neither appears to have a significant temperature dependence over the ranges measured, as shown in Table 1. We did not extend the activity measurement for SsPL above 45 °C since the protein is undergoing denaturation at 50 °C under these particular solvent conditions, as judged by turbidity of the solution; the turbidity interfered with our ability to get the concentration of repaired DNA from the absorption spectrum. Under conditions of low ionic strength and neutral pH, the enzyme does not appear to be especially temperature-resistant; these conditions were determined by our earlier work completed on the EcPL system and were not optimized for SsPL.¹⁵

We investigated the temperature instability of SsPL further using two types of cosolvents in our reaction assay: (a) K₂SO₄ salt and (b) trehalose sugar. In the past, we found the EcPL enzyme to be more resistant to thermal denaturation in the presence of high salt concentrations, such as 0.400 M K₂SO₄; the addition of this concentration of salt appeared to have a negligible effect on the denaturation of SsPL at 50 °C as judged by the activity assay. In contrast, when we added 200 mM trehalose sugar, an organic cosolvent believed to be present in some thermophilic, archaeon organisms,^{19,20} we observed resistance to denaturation at 50 °C as judged from the activity assay and the amount of light scatter measured by absorption spectroscopy. The measured turnover number in the presence of 200 mM trehalose was 4.8 min⁻¹. Since the protein originates from an acidophilic organism, we

also decreased the pH of the assay to 5.0 and 3.0, but the thermal denaturation of the protein was even more severe than at neutral pH. At pH 9, the activity looked similar to that measured for pH 7.0. Further studies are needed to investigate what cosolvents and solvent conditions may be necessary to stabilize both the protein structure and the cofactor redox chemistry at higher temperatures.

ITC Binding Studies.

The substrate used in our binding experiments, UV-p(dT)₁₀ (denoted as ssDNA), is a single strand oligothymidylate with an average of a single CPD lesion randomly arranged on the 10-mer; the substrate was prepared the same way as the ssDNA used in our earlier study of the EcPL system, and the temperature dependence of the binding was measured under identical solvent conditions.¹⁵ Temperature dependent binding studies of the FADH[•] and FADH[−] states of the active site flavin were completed from 10 to 50 °C with a typical ITC run and binding curve shown in Figure 2. The heat of binding was consistently exothermic over the entire temperature range examined, as seen in Figure 2. The integrated area of the peaks allows for the calculation of the enthalpy of binding while the steepness of the binding curve denotes the binding constant (K_A , G) along with the stoichiometry (N) of the substrate required.²¹

Using absorption spectroscopy, the oxidation state of the protein before and after the ITC experiment was measured to determine the extent of oxidation during an experiment. We did not observe a significant decrease in native SsPL during our binding studies, in contrast to our earlier observations with the activity assay. We were able to obtain binding data up to temperatures of 50 °C, but at higher temperatures we had trouble maintaining the oxidation state of the active site FAD cofactor during the experiment. Below 50 °C, we typically lost less than 20% of the reduced state to semiquinone over the ~1 h long ITC run. The standard deviation for the enthalpy of binding increases with temperature due in part to our inability to control the redox state fully.

The thermodynamic parameters (ΔH° , ΔG° , ΔS°) of ssDNA binding to SsPL obtained from replicate ITC measurements are shown in Table 1. In general, binding of ssDNA to both the FADH[•] and FADH[−] states of SsPL gets significantly more exothermic as the temperature increases but the Gibbs free energy of binding is virtually unchanged over the same temperature range, as shown in Figure 3. Substrate binding to SsPL is ~2 kJ/mol less favorable than binding to EcPL.

Plotted in Figure 4 is the enthalpy of binding for both SsPL and EcPL under identical conditions with identical substrate. Heat capacity is defined as the change in enthalpy with temperature, holding the pressure constant:²¹

$$C_p = \left(\frac{\partial H}{\partial T} \right)_p \quad (2)$$

The heat capacity of the system changes as substrate binds to the protein, thus we can obtain the change in the heat capacity upon binding (ΔC_p) using the slope of the change in enthalpy plotted versus temperature in Figure 4. As published earlier, the plots for the EcPL states

appear to be linear over a range of temperature which may mean that there are not significant structural changes occurring in EcPL upon substrate binding.^{22,23} The data for SsPL is more ambiguous, especially for that of the SsPL FADH⁻ state, since the SsPL data is noisier. The data in Figure 4 were fit to lines and, within the error of the fit, the change in heat capacity upon substrate binding for the two oxidation states of SsPL are indistinguishable from each other with a C_p of $-820 (\pm 100) \text{ J K}^{-1} \text{ mol}^{-1}$ and $-960 (\pm 100) \text{ J K}^{-1} \text{ mol}^{-1}$ for the FADH⁻ and FADH[·] states, respectively. The values for SsPL do appear to be more positive by 200 to 300 $\text{J K}^{-1} \text{ mol}^{-1}$ than what had been obtained previously for the EcPL system.¹⁵

The change in heat capacity upon substrate binding is linked to the change in accessible surface area as the substrate binds. Using the empirical relationship described by Spolar and Record,²⁴

$$\Delta C_p (\text{in J mol}^{-1}) = 1.34(\Delta A_{\text{nonpolar}}) - 0.586(\Delta A_{\text{polar}}) \quad (3)$$

where A_{nonpolar} and A_{polar} are changes in the nonpolar and polar surface area (in Å²) upon substrate binding to the protein. When Fujihashi, Numoto, Kobayashi et al. reported the crystal structure for the StPL,⁴ they observed changes in the amino acids around the CPD binding site compared to that of PL from *Anacystis nidulans* (AnPL).²⁵ The changes included the replacement of Asn349 (AnPL) with Gly309 (StPL), Gly38 (AnPL) with Asn349 (StPL), and two water molecules connecting to Tyr290 (AnPL) with the polar amine group of Asn349 (StPL). These changes would result in minor differences in the amount of polar and nonpolar surface area buried with substrate binding. The difference we are detecting is more likely due to the composition of amino acids at the surface of the protein; the hydrophobicity does appear to be significantly different between EcPL and SsPL. Using the ExPASy calculator,²⁶ the grand average of hydropathicity was -0.64 for SsPL and -0.395 for EcPL; the more negative value denotes more hydrophobic residues. The less negative C_p observed for SsPL may simply reflect a slightly more hydrophobic amino acid composition buried by substrate in SsPL. Given the similarities in turnover numbers, catalytic activities, and thermodynamic parameters, it seems unlikely that the mode of CPD binding in the thermophilic enzyme is significantly altered from that of the mesophilic system. Based upon our earlier analysis of crystal structures and given the size of the C_p found experimentally, it appears SsPL also captures the solvent exposed CPD as the main step in the damage recognition mechanism.¹⁵

Role of Electrostatic Interactions in Formation of Complex.

The ionic strength dependence of the binding can be used to quantify the electrostatic interactions between the protein and the substrate.^{27,28} An electrostatic interaction is specifically defined as an interaction between charged species while hydrogen bonding and van der Waals interactions are defined as nonelectrostatic or nonelectrical interactions. We will use the counterion condensation (CC) model tested by Privalov, Dragon, and Crane-Robinson²⁸ to quantify the extent of the nonelectrical interaction where

$$\log K_A = \log K_{\text{nonelectrical}} - Z\psi \log[\text{salt}] \quad (4)$$

where K_A is the measured binding constant at a specific salt concentration and $K_{\text{nonelectrical}}$ is the contribution from $K_{\text{nonelectrostatic}}$ interactions. The parameter Z is the number of DNA phosphate groups that interact with the protein, and ψ is the number of cations released by the phosphate charge upon substrate binding (assumed to be 0.7 in this study since we have a short oligonucleotide²⁸). While eq 4 may be overly simplified for DNA binding to a protein, there is a theoretical framework that underlies the linear relationship between $\log K_A$ and $\log[\text{Salt}]$.²⁹ In the CC model, the salt-dependent component of the Gibbs energy of binding is entirely entropic ($G_{\text{electrical}} = -T S_{\text{electrical}}$) with the enthalpic component of binding dependent upon the nonelectrostatic interactions ($H_{\text{binding}} = H_{\text{nonelectrical}}$).²⁸

We measured binding at a number of different ionic strengths (from 200 to 1000 mM) to generate a plot using the equation shown, Figure 5. The ionic dependence of substrate binding to SsPL is significantly different from that of the mesophilic EcPL under identical experimental conditions; at 25 and 45 °C DNA binding to SsPL is virtually independent of ionic strength.

In Table 2, we show the calculated nonelectrostatic contribution to binding for the different oxidation states of SsPL; we used the y-intercept of the plots in Figure 5 to get the $K_{\text{nonelectrical}}$ and $G_{\text{nonelectrical}}$. We assumed that there was no net electrical contribution to the enthalpy of binding so the enthalpy reported in Table 1 is nonelectrical,^{30–32} and from that we were able to calculate the $S_{\text{nonelectrical}}$. Our data appears to be consistent with the CC model: the enthalpy of binding in the 200–1000 mM ionic strength buffers were indistinguishable from the enthalpy of binding measured with 88 mM K_2SO_4 , 20 mM potassium phosphate pH 7.0 (300 mM ionic strength) buffer at the same temperature (Table 1). In addition, the overall entropy of binding in Table 1 is indistinguishable from the nonelectrostatic entropy of binding reported in Table 2 for the same temperature.

Measurements of substrate binding to EcPL at 300 mM ionic strength, the ionic strength of the temperature dependent measurements, found approximately 25% of the Gibbs energy for binding is due to the favorable electrostatic interaction between the DNA molecule and the protein, with approximately 3 electrostatic interactions formed between EcPL FADH^- and ssDNA substrate.¹⁵ From our experimental data on SsPL, we conclude that the electrostatic interactions do not make any appreciable contribution to the overall Gibbs energy of binding and the number of electrostatic interactions have decreased to one contact for the fully reduced state, Table 2.

A crystal structure of a CPD photolyase with a double stranded CPD analog bound (1TEZ, ref 25) was analyzed for the specific electrostatic interactions between the CPD containing strand of DNA and AnPL protein. From the AnPL structure, there appears to be three positively charged (R232, R350, and K414) side chains along with one negative charge (E283) that may interact with the CPD strand; Figure 6 shows the location of the charged amino acid residues. An alignment of the amino acid sequences of AnPL, EcPL, and SsPL was completed,³³ and results for the amino acids that appear to specifically interact with substrate are reported in Table 3. EcPL appears to use the same charged amino acids as AnPL to bind substrate though it may have one more positive charge since Q461 in AnPL is

replaced with R458 in EcPL. SsPL appears to have two differences compared to AnPL. First, positively charged K414 in AnPL is replaced with neutral T372 and neutral N407 in AnPL is replaced with negatively charged D365. N407/D365 amino acid may not have any significant interaction with the DNA substrate since the residue may be pointing away from the substrate. Some caution needs to be applied with this analysis since the crystal structure was obtained with a double stranded DNA substrate, and we may not be identifying the exact interactions for single stranded substrate.

The electrostatic contacts we measure in our ionic strength experiments are qualitatively in agreement with the amino acid analysis above; we see a larger electrostatic interaction with EcPL, as predicted. Due to irradiation with X-ray, the FAD in the AnPL crystal structure is most likely in the fully reduced FADH^- state. As noted above, we concluded that the FADH^- state of EcPL had three favorable electrostatic interactions while the FADH^- state of SsPL had one electrostatic interaction.

Thermophilic enzymes are commonly observed to have a greater number of salt bridges than their mesophilic cousins;^{7,10,13} based upon our analysis of the crystal structures of EcPL and StPL, this observation also holds for our systems. As shown in Table 4, we find 37 salt bridges in the EcPL structure (1DNP)³⁴ compared to 47 in StPL (2E0I).⁴ The number of charged amino acids found for each protein can also be quantified: EcPL appears to have 51 and 53 negative and positive charges, respectively, and StPL has 62 negative and positive charges. SsPL appears to be different from both EcPL and StPL: it has 71 positively charged amino acids and 63 negative amino acids. It seems counterintuitive that a DNA binding protein with an excess of positively charged amino acids is not using significant electrostatic interactions to bind the negatively charged DNA substrate.

We can propose several explanations for the observed behavior. First, the electrostatic interactions contribute 20 to 25% to the substrate binding in EcPL at 300 mM ionic strength;¹⁵ is it more important for SsPL to use the charged groups in salt bridges to maintain protein structure rather than to use the charges to bind DNA? The answer to that question may lie in work published earlier; Privalov, Dragan, and Crane-Robinson tested the CC model using a number of DNA binding proteins.²⁸ They concluded that the electrostatic component to the Gibbs energy of binding was independent of DNA sequence and was a nonspecific, albeit large component for formation of the DNA protein complex. They observed that specificity of the interaction was determined solely by the nonelectrostatic (dehydration effects, van der Waals interactions, and hydrogen bonding) interactions. Therefore, in the absence of electrostatic interactions, SsPL will not have lost its specificity for the CPD lesion. It may be more beneficial for the organism to use the salt bridges to maintain structural integrity at the cost of losing some thermodynamic favorability in binding to the CPD lesion.

Second, the lack of electrostatic interactions in formation of the DNA protein complex may be a result of the specific environment of the SsPL protein; if the protein has to function in an unusually high ionic strength environment, electrostatic interactions would be useless due to the ionic atmosphere that would occur around any of the charged groups on the DNA or protein surface. *Sulfolobus solfataricus* does not require high salt growth conditions, but it is not clear how much salt the species will tolerate.³

Third, we may be detecting a change in the nature of the electrostatic interactions formed in the complex. It is possible that EcPL uses contact ion pairs (ions are in direct contact) in binding the DNA, but that SsPL uses a solvent separated ion pair where the ions are separated by intervening water molecules.³⁵ In this case, the effect of the higher salt content would be attenuated by the nature of the ion pair; this hypothesis would predict that the fewer waters would be released upon formation of the protein–substrate complex in SsPL compared to EcPL, and thus, the entropy of binding would be greater with EcPL than SsPL. As shown in Table 1, we observe that SsPL has a marginally larger entropy of binding, so our data do not support replacement of contact ion pairs in EcPL with solvent separated ion pairs in SsPL.

Complicating the picture, work published by Kernchen and Lipps on single-stranded binding protein (SSB) from *Sulfolobus solfataricus* found that both the dissociation constant and the number of ions released when oligonucleotide bound to the protein were strongly dependent upon the identity of the salt anion present.³⁶ They examined four salts: choline chloride, potassium glutamate, potassium chloride, and potassium fluoride. They reported the order of binding affinity of DNA to SSB from most favorable to least favorable salt present as potassium fluoride, potassium glutamate, potassium chloride, and choline chloride. In addition, not only did the number of ions released change with the anion present, but ion release was also temperature dependent for potassium fluoride and potassium glutamate. We may be seeing this lack of electrostatics in binding solely due to our anion.

CONCLUSIONS

From activity measurements, it appears that the thermophilic SsPL may require specific solvent conditions or organic cosolvents to control the redox chemistry of the active state FAD and to function more efficiently at temperatures higher than 50 °C. At temperatures below 50 °C and low ionic strength, the thermophilic SsPL is similar to the mesophilic EcPL with similar turnover numbers and catalytic efficiencies. The surface area buried upon complex formation with SsPL may be slightly less polar than what has been observed with EcPL. DNA binds to SsPL with slightly less favorable Gibbs energy, and the complex formed appears to lack 2 to 3 electrical contacts that are formed in the EcPL system even though SsPL has a large number of positively charged amino acid side chains. The electrostatic effect warrants further study to understand if this is due to the type of ion pair formed or anion used in the study. Corresponding studies on a psychrophilic photolyase will be completed to understand the importance of this effect.

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Figure 1.

Absorption spectra of the FADH⁻ and FADH[·] states of PL. Spectra were normalized for FADH[·] absorbance. Solutions at 10 °C in 20 mM phosphate buffer pH 7.0 with 88 mM K₂SO₄.

Figure 2.

Typical ITC data and binding curve. Cell contained 240 μL of 36 μM FADH⁻ SsPL at 25 °C in 1000 mM ionic strength buffer, pH 7.0 and 280 rpm stirring. Syringe contained 350 μM UV-p(dT)₁₀ in same buffer. Substrate was added in 2.37 μL aliquots with 90 s per addition.

Figure 3.

Comparison of the Gibbs energy of binding from ITC. The values for EcPL as published earlier.¹⁵ Data obtained as described in the text. The FADH^- state is “red” and the FADH^+ state is “SQ”. Errors for all points are reported in Table 1.

Figure 4.

Comparison of the enthalpy of binding. The values for EcPL as published earlier.¹⁵ Data obtained as described in the text. Dotted lines are the least-squares analysis of the data. Slopes (in $\text{kJ K}^{-1} \text{mol}^{-1}$) for each: The FADH^- state is “red” and the $\text{FADH}^\bullet$ state is “SQ”. SsPL red = $-820 (\pm 100)$, SsPL SQ = $-960 (\pm 100)$, EcPL red = $-1170 (\pm 20)$, EcPL SQ = $-1120 (\pm 80)$. Individual errors are reported in Table 1.

Figure 5.

Comparison of ionic strength dependence of binding of ssDNA. ITC experiments were run in 50 mM Hepes, pH 7.0 with appropriate concentration of KCl. The FADH^- state is “red” and the FADH^+ state is “SQ”. The dashed lines reflect the least-squares analysis of the data. The EcPL data as published previously.¹⁵ Uncertainty in data reported in Table 2.

Figure 6.
Interaction between CPD containing DNA and AnPL (1TEZ, ref 25).

Table 1.

Comparison of Thermodynamic and Kinetic Properties of SsPL with EcPL^a

	<i>T</i> , K	<i>H</i> ^o , kJ mol ⁻¹	<i>G</i> ^o , kJ mol ⁻¹	<i>S</i> ^o , J K ⁻¹ mol ⁻¹	<i>k</i> _{cat} , min ⁻¹	cat eff, min ⁻¹
SsPL FADH ⁻	283	-29 (1) ^b	-32.9 (0.6) ^b	13 (5) ^c	3.2 (0.3) ^b	4 × 10 ⁶
	288	-36 (8)	-32.5 (0.7)	-10 (30)	3.2 (0.1)	2 × 10 ⁶
	293	-35 (7)	-32 (1)	-10 (30)	2.9 (0.3)	2 × 10 ⁶
	298	-39 (10)	-33 (2)	-20 (40)	4.8 (0.1)	3 × 10 ⁶
	303	-47 (2)	-32 (2)	-50 (10)	3.9 (0.3)	1 × 10 ⁶
	308	-43 (8)	-33.3 (0.9)	-30 (20)	3.9 (0.1)	3 × 10 ⁶
	313	-45 (10)	-36.6 (2)	-30 (40)	4.4 (0.7)	6 × 10 ⁶
	318	-58 (10)	-34.4 (0.8)	-70 (40)	5.0 (0.2)	3 × 10 ⁶
SsPL FADH ⁻	323	-68 (20)	-38 (4)	-90 (70)	n/a	n/a
	283	-18 (4)	-31 (1)	50 (20)		
	288	-24 (5)	-31.7 (0.7)	30 (20)		
	293	-39 (2)	-32.1 (0.4)	-25 (7)		
	298	-35 (5)	-33 (1)	-10 (20)		
	303	-41 (7)	-35 (2)	-20 (30)		
	308	-42 (4)	-33.8 (0.6)	-30 (10)		
	313	-46 (5)	-33.7 (0.4)	-40 (20)		
EcPL FADH ⁻	318	-56 (2)	-34.0 (0.3)	-70 (6)		
	323	-61 (10)	-37 (2)	-80 (30)		
	283	-34.9 (2)	-33.3 (0.8)	-6 (8)	2.6 (0.1)	4 × 10 ⁶
	288	-40.4 (0.7)	-33.6 (0.2)	-24 (3)	2.6 (0.1)	3 × 10 ⁶
	293	-46.8 (3)	-34.3 (0.6)	-43 (10)	3.2 (2)	4 × 10 ⁶
	298	-52.7 (3)	-34.5 (0.8)	-61 (10)	2.4 (1)	3 × 10 ⁶
	303	-57.9 (1)	-34.6 (0.6)	-77 (5)	2.4 (0.5)	2 × 10 ⁶
	283	-26.2 (2)	-34.1 (0.7)	28 (8)		
EcPL FADH ⁻	290	-33.9 (2)	-34.3 (0.8)	1 (7)		
	298	-42.6 (5)	-35.1 (0.7)	-25 (20)		
	305	-53.3 (3)	-36.3 (0.5)	-56 (9)		

$T, \text{ K}$	$H^\circ, \text{ kJ mol}^{-1}$	$G^\circ, \text{ kJ mol}^{-1}$	$S^\circ, \text{ J K}^{-1} \text{ mol}^{-1}$	$k_{\text{cat}} \text{ min}^{-1}$	cat eff, min^{-1}
310	-55.0 (4)	-36.7 (0.5)	-59 (10)		

^aThermodynamic data taken from ref 15.

^bError from standard deviation of replicate trials in parentheses.

^cPropagated error in parentheses.

Table 2.

Analysis of the Electrical and Nonelectrical Interactions for Binding of SsPL

	SsPL FADH ⁻		EcPL FADH ^{-e}		SsPL FADH ⁻		EcPL FADH ^{-e}	
<i>T</i> , °C	45	25	25	45	25	25	25	25
<i>Z</i> , ψ^a (Figure 5)	-0.63 (0.2)	-0.71 (0.2)	N/A	-1.2 (0.2)	0.2 (0.4)	N/A		
Log <i>K</i> _{nonelect} ^a (Figure 5)	5.69 (0.07)	5.58 (0.07)	N/A	5.68 (0.09)	5.7 (0.1)	N/A		
<i>K</i> _{nonelectrical}	4.88×10^5	3.8×10^5	5.5×10^5	4.7×10^5	4.5×10^5	1.2×10^5		
<i>G</i> _{nonelect} ^b , kJ mol ⁻¹	-34.6 (0.06)	-31.8 (0.03)	-27.0 (0.2)	-35.4 (0.04)	-30.5 (0.06)	-29.0 (0.1)		
<i>Z</i> ^c	0.9 (0.3)	1.0 (0.3)	3.3	1.6 (0.3)	0 (0.5) ^f	2.0		
<i>H</i> _{nonelect} ^d , kJ mol ⁻¹	-58 (10)	-39 (10)	-32.8 (4)	-56 (2)	-35 (5)	-26 (2)		
<i>S</i> _{nonelect} ^e , J K ⁻¹ mol ⁻¹	-70 (30)	-20 (30)	-20 (10)	-9 (20)	-70 (7)	10 (6)		

^aError in parentheses from least-squares analysis of data.^bPropagated error in parentheses.^cAssumed $\psi = 0.70$, ref 28.^dValue from Table 1.^eTaken from ref 15.^fValue was -0.3. Interpreted as 0 contacts.

Table 3.

Homologous Amino Acids for Substrate-Protein Interactions

AnPL ^a	EcPL ^b	SsPL ^b
V148	V150	N141
G150	R152	T143
R232	R227	K204
E283	E275	Q242
W286	W278	W245
N349	N342	N308
R350	R343	R309
M353	M346	M312
W392	W385	W351
N407	N401	D365
Q411	Q405	Q369
K414	K408	T372
Q461	R458	V317

^a Identified using ref 26.^b Alignment done using ref 33.

Table 4.

Comparison of SsPL, StPL, and EcPL Compositions

	SsPL	StPL	EcPL
number of salt bridges ^a	N/A	47 (2E0I)	32 (1DNP)
positively charged aa ^b	71	62	53
negatively charged aa ^b	63	62	51
grand average of hydropathicity ^b	−0.64	−0.507	−0.395

^aRef 37.^bRef 26.