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The Thermodynamics of Ligand Binding to the Aminoglycoside O-Nucleotidyltransferase(4′) and Variants Yields Clues about Thermophilic Properties

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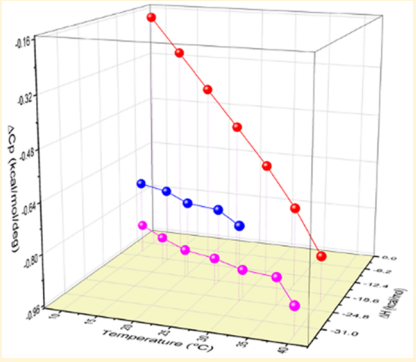
Supporting Information

ABSTRACT: The aminoglycoside nucleotidyltransferase(4′) is an enzyme with high substrate promiscuity and catalyzes the transfer of the AMP group from ATP to the 4′-OH site of many structurally diverse aminoglycosides, which results in the elimination of their effectiveness as antibiotics. Two thermostable variants carrying single-site mutations are used to determine the molecular properties associated with thermophilicity. The thermodynamics of enzyme−ligand interactions showed that one variant (T130K) has properties identical to those of the mesophilic wild type (WT) while the other (D80Y) behaved differently. Differences between D80Y and the T130K/WT pair include the change in heat capacity (ΔC_p), which is dependent on temperature for D80Y but not for WT or T130K. The change in ΔC_p with temperature ($\Delta\Delta C_p$) with D80Y is dependent on aminoglycoside only in H_2O and remains the same with all aminoglycosides in D_2O. Furthermore, the offset temperature (T_{off}), the temperature difference that yields identical enthalpies in H_2O and D_2O, becomes larger with an increase in temperature for WT and T130K but remains mostly unchanged for D80Y. Studies in H_2O and D_2O revealed that solvent reorganization becomes the major contributor to ligand binding with an increase in temperature for WT and T130K, but changes in low-frequency vibrational modes are the main contributors with D80Y. Data presented in this paper suggest that global properties associated with the enzyme−ligand interactions, such as the thermodynamics of ligand binding, may yield clues about thermophilicity and permit us to distinguish those variants that are simply a more thermostable version of the mesophilic protein.



Thermostable enzymes that are naturally found in thermophilic organisms and their ability to function at elevated temperatures are highly desirable for biotechnological and industrial applications. Thermophilic proteins may adopt various strategies to achieve thermal adaptation, yet they are structurally quite like their mesophilic variants. Therefore, understanding molecular properties that render proteins thermophilic is necessary. Numerous studies made comparisons to identify the differences in various structural and dynamic features of thermophilic and mesophilic enzyme homologues. Although several molecular aspects such as the presence of more polar interactions or H-bonds or tighter packing of the hydrophobic core have been suggested by earlier work,[1−6] more distributed effects and global dynamics of proteins playing a significant role were also suggested by other studies.[7,8]

These efforts, however, are somewhat complicated by the fact that they included only thermostable enzymes, some of which may simply be enzymes with melting temperatures (T_m) that are higher than those of mesophilic enzymes such as those with added stabilizing interactions in the form of disulfide bonds or ionic or hydrophobic interactions. Furthermore, these comparisons included proteins that have differences ranging from a few residues to tens of residues in their primary sequences as well as differences in their oligomeric state, which may introduce changes that are not necessarily specific for thermophilicity. In this work, we are separating the terms "thermostable" and "thermophilic" in the following manner; thermostable defines a variant of the mesophilic protein with increased thermostability achieved by either evolution-based or structure-based strategies leaving other properties of the protein like the mesophilic variant. Contrary to this, thermophilic proteins may have significantly different dynamic and thermodynamic properties relative to those of the mesophilic variants.

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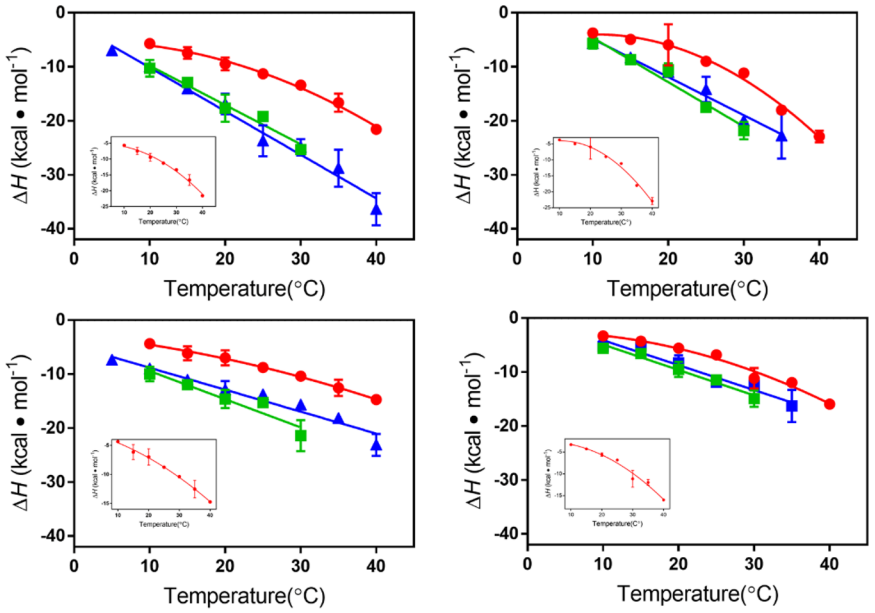

Figure 1. Binding enthalpy change as a function of temperature for WT (green), T130K (blue), and D80Y (red). The top panels show data for neomycin (left) and tobramycin (right) binding in H_2O. The bottom panels show data for neomycin (left) and tobramycin (right) binding in D_2O. Some of the error bars are smaller than the symbol size. Insets show data for D80Y with an expanded *Y* axis. Data for T130K are from ref 14.

To identify molecular properties associated with thermophilicity, we used the wild type (WT) and two thermostable, single-amino acid mutants (D80Y and T130K) of the aminoglycoside nucleotidyltransferase(4′) (ANT), which provides an excellent opportunity to distinguish the thermophilic variant from the thermostable one. This is because, although both mutants are thermostable, only one (D80Y) appears to be a thermophilic protein while the other (T130K) is simply a more heat stable variant of WT. Earlier computational work with these three variants suggested that global dynamics of the mesophilic and thermophilic variants differ and T130K is similar to WT.[9] The thermodynamics of enzyme−ligand interactions and effects of solvent on ligand binding, presented in this work, showed that while T130K shows behavior identical to that of WT, D80Y dramatically differs from both. Thus, data presented in this paper suggest that global properties associated with the enzyme−ligand interactions, such as the thermodynamics of ligand binding, may also yield clues about thermophilicity.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals, including the aminoglycosides, were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity available. Concentrated stock solutions of aminoglycosides were prepared after the removal of sulfate salt by ion exchange chromatography and then used in isothermal titration calorimetry (ITC) experiments. Concentrations of aminoglycoside solutions were determined by an enzymatic assay as described previously.[10] Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Inalco SAS. High-performance Ni-Sepharose and ion exchange matrix Macro Q were purchased from GE Healthcare and Bio-Rad Laboratories (Hercules, CA), respectively. Thrombin was obtained from EMD Millipore Corp.

Overexpression and Purification. WT ANT4 was originally isolated from mesophilic *Staphylococcus aureus*.[11] Thermostable T130K and D80Y were from *Bacillus stearothermophilus*[12] and were codon optimized for *Thermosynechococcus elongatus*.[13] The T130K gene was transferred from *T. elongatus* to *Escherichia coli*, and D80Y, WT, and T130K/D80Y (double mutant not used in this work) were generated via introducing site-directed mutations into T130K.[13] All three variants were overexpressed and purified as described previously.[13−15] All three ANT4 variants were stable for 3 weeks at 4 °C without a significant loss of activity (>90%). The protein concentration was determined by using extinction coefficients of 50880 M^{-1} cm^{-1} for D80Y and 49390 M^{-1} cm^{-1} for both WT and T130K.

Isothermal Titration Calorimetry (ITC). ITC experiments were performed using a VP-ITC microcalorimeter from GE Healthcare (Hercules, CA) over a temperature range of 10−40 °C. Proteins were dialyzed in a buffer system composed of 50 mM MOPS and 100 mM NaCl (pH 7.5 at 25 °C) in H_2O or D_2O (99.9%) prior to the ITC experiments. All aminoglycosides were diluted from concentrated stock solutions into the dialysate from enzyme preparations. The enzyme concentration in the calorimetry cell was 20 μM (monomer), while the aminoglycoside concentration in the syringe ranged from 400 to 800 μM. Enzyme and ligand solutions were degassed for 10−15 min before being loaded into the microcalorimeter. Ten microliters of the ligand was titrated into the enzyme in each injection. The experiments consisted of 27 injections separated by 240 s, and the cell stirring speed was 307 rpm. Most ITC experiments were repeated two or three times, and the data were fit using the global fit feature of SEDPHAT[16] considering the A + B ↔ AB heteroassociation model to determine the binding enthalpy (ΔH) and the association constant (K_a). The Gibbs energy (ΔG) and the entropy ($T\Delta S$) were determined from the relationships $\Delta G = -RT \ln K_a$ and $\Delta G = \Delta H - T\Delta S$, respectively. Data for T130K were taken from ref 14.

Analytical Ultracentrifugation. Experiments were performed using a Beckman XL-1 analytical ultracentrifuge and an An-50Ti rotor. Proteins were dialyzed in 50 mM MOPS buffer (pH 7.5 at 25 °C) either with or without 100 mM NaCl to test

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the effect of salt on the monomer−dimer equilibrium; 400 μL of 1 μM enzyme and 390 μL of buffer were loaded into double-sector cells. Three hundred absorbance scans were collected at 230 nm and 50000 rpm over a temperature range of 10−40 °C. SEDNTERP[17] was used to calculate the buffer viscosity, buffer density, and protein partial specific volume. SEDFIT[18] was used to fit the sedimentation data to a continuous $[c(s)]$ distribution model.

RESULTS

The Thermodynamics of Ligand Binding Distinguishes D80Y from T130K and WT. The thermodynamics of the ligand binding to the mesophilic form (WT) and two thermostable variants (T130K and D80Y) of ANT was studied by ITC over a temperature range of 10−40 °C in H_2O and D_2O. Because of the high substrate promiscuity of the enzyme, four aminoglycosides were used in this study. The aminoglycosides used were representatives of neomycin (neomycin B and paromomycin) and kanamycin (kanamycin A, henceforth kanamycin and tobramycin) groups (Figure S1). In all cases, the binding was enthalpically favored. An example data set is shown in Figure S2. The entropic contribution was aminoglycoside- and enzyme-dependent and always unfavorable for WT and T130K. The only exceptions were for the binding of kanamycin and tobramycin to WT and the binding of neomycin to T130K at 10 °C. Contrary to this, a favorable entropic contribution was more significant with D80Y and in some cases remained favorable up to 25 °C. Exemplary data sets are presented in Tables S1−S4. In all cases, ΔG remained favorable ($\Delta G < 0$). While the binding enthalpy was becoming more favorable with an increase in temperature, the entropic contribution became more unfavorable. However, at all temperatures studied, the favorable enthalpy overcame the unfavorable entropic contribution to favor ligand binding. Figure 1 shows the enthalpy of binding of neomycin and tobramycin to all three variants as a function of temperature in H_2O and D_2O. The data acquired with paromomycin and kanamycin A are shown in Figure S3. These data show that, in the temperature range studied, ΔH decreases linearly with an increase in temperature for binding of all four aminoglycosides to WT and T130K. The decrease in ΔH was not linear with D80Y, indicating that the heat capacity change (ΔC_p), unlike the case for WT and T130K, is temperature-dependent. Despite aminoglycoside specific variations, the rate of decrease in ΔH with an increase in temperature was always faster in H_2O for WT and T130K in the temperature range tested. This was true for D80Y only at temperatures above ∼20 °C. Data acquired with neomycin and tobramycin are shown in Figure 2 to highlight this point for WT and D80Y. T130K behaved like WT, which was reported previously.[14] Nevertheless, several titrations were repeated with T130K to reproduce the earlier data. The temperature dependence of the enthalpy and ΔC_p for binding of neomycin to all three variants is shown as a three-dimensional plot in Figure S4.

Differences observed in binding enthalpies in H_2O and D_2O ($\Delta\Delta H = \Delta H_{H_2O} - \Delta H_{D_2O}$), as shown in Figure 3, enhance the distinction between D80Y and the WT/T130K pair. Of the two thermostable variants, T130K behaves like mesophilic WT and D80Y shows different behavior. $\Delta\Delta H$ becomes more negative with an increase in temperature in a linearly dependent manner and becomes positive at low temperatures for both WT and T130K. In contrast, $\Delta\Delta H$ shows a nonlinear

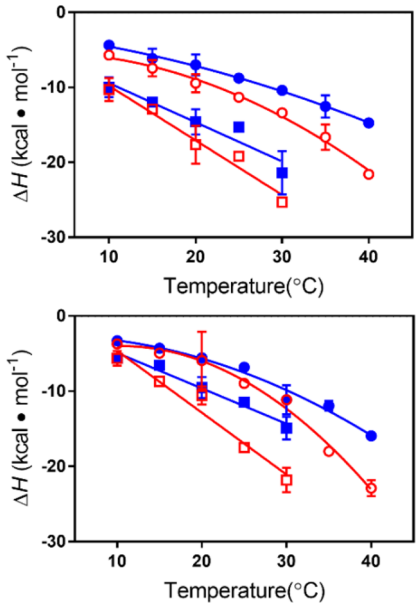

Figure 2. Comparison of binding enthalpies in H_2O (empty symbols) vs D_2O (filled symbols). Binding of neomycin (top) and tobramycin (bottom) to WT (squares) and D80Y (circles).

correlation with temperature and remains negative with D80Y (Figure 3). The only exception to this is binding of kanamycin to D80Y.

The temperature dependence of ΔC_p for the formation of the binary D80Y−aminoglycoside complexes showed aminoglycoside specific variation; however, it was always stronger in H_2O than in D_2O. Figure 4 illustrates this for the formation of D80Y−neomycin and D80Y−tobramycin complexes. The change in ΔC_p as a function of temperature ($\Delta\Delta C_p$) is determined from slopes of such plots, and the values are listed in Table 1. Variation of $\Delta\Delta C_p$ with different aminoglycosides was more significant in H_2O and showed a 2.6-fold difference between the lowest and highest values. In D_2O, apart from neomycin, $\Delta\Delta C_p$ was very similar for all three aminoglycosides (Table 1). In all cases, $\Delta\Delta C_p$ was more negative in H_2O.

The differential temperature dependence of ΔC_p between H_2O and D_2O also indicates temperature-dependent effects on transfer between these solvents. ΔC_p values for transfer ($\Delta C_{p,tr} = \Delta C_{p,H_2O} - \Delta C_{p,D_2O}$) for the binary D80Y−aminoglycoside complexes are shown in Figure 5. $\Delta C_{p,tr}$ for the transfer from D_2O to H_2O is negative above 20 °C for all four aminoglycosides and becomes positive between 10 and 20 °C, except for that of kanamycin, which is very small. There is no such variation for WT or T130K, and $\Delta C_{p,tr}$ is always negative with all aminoglycosides except for that for the binding of paromomycin to WT, which is approximately zero (Table 2).

Analytical centrifugation studies showed that all three ANT4 variants exhibit a similar effect of temperature on the monomer−dimer equilibrium over the studied temperature range (Figure S5). The increase in temperature results in a stronger tendency of all forms to dimerize. With the exception of small differences observed in the monomer−monomer association constants, there were no differences between variants and the presence of salt increased the monomer−monomer affinity by a factor of ∼10 with all three variants.[14] No detectable temperature-dependent compaction or expansion was detected for any of the three variants.

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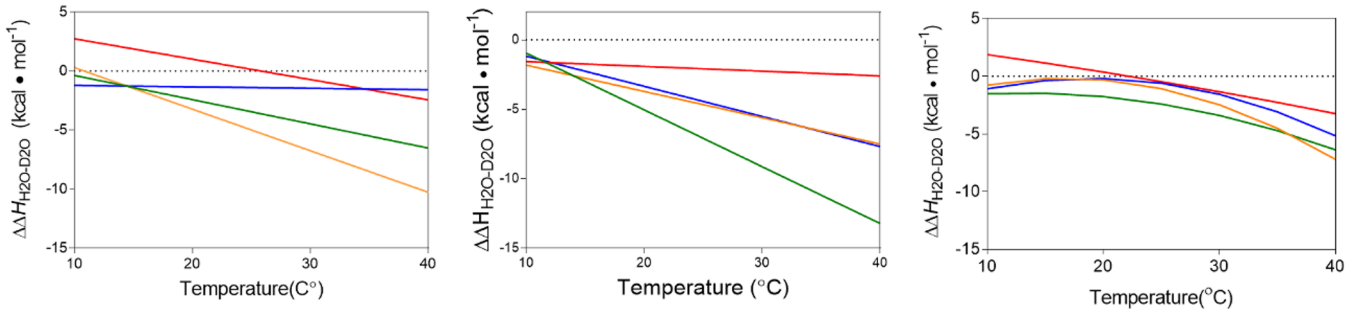

Figure 3. Temperature dependence of $\Delta\Delta H$ for WT (left), T130K (middle), and D80Y (right) with neomycin (green), paromomycin (blue), tobramycin (orange), and kanamycin (red). Curves in D80Y plots do not represent any kind of fitting; they simply are connecting data points. Data for T130K are reprinted with permission from ref 14. Copyright 2014 American Chemical Society.

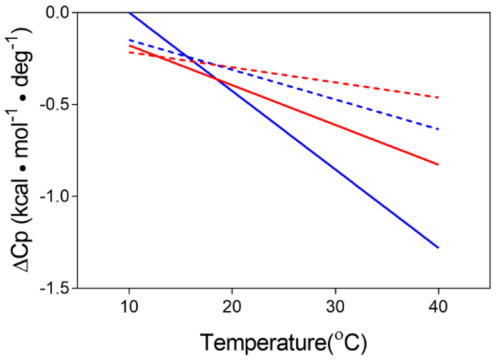

Figure 4. Change in heat capacity as a function of temperature for binding of tobramycin (blue) and neomycin (red) to D80Y in H_2O (solid lines) and D_2O (dashed lines).

Table 1. Change in ΔC_p as a Function of Temperature and Its Aminoglycoside-Dependent Variation

	$\Delta\Delta C_p(H_2O)$[a] (cal mol^{-1} deg^{-2})	$\Delta\Delta C_p(D_2O)$[a] (cal mol^{-1} deg^{-2})
D80Y–tobramycin	−42	−16
D80Y–paromomycin	−37	−15
D80Y–neomycin	−22	−8
D80Y–kanamycin	−16	−14

[a]All R^2 values for curve fitting were >0.999.

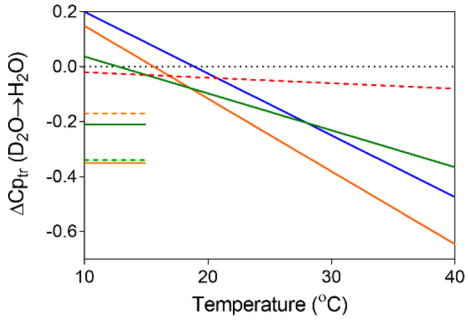

Figure 5. Temperature dependence of $\Delta C_{p,tr}$ ($\Delta C_{p,H_2O} - \Delta C_{p,D_2O}$) for binding of neomycin (green), paromomycin (blue), tobramycin (orange), and kanamycin (red) to D80Y. The constant $\Delta C_{p,tr}$ values for the binding of tobramycin (orange) and neomycin (green) to WT and T130K are shown for comparison as short lines.

Table 2. $\Delta C_{p,tr}$ Values for WT and T130K

	$\Delta C_p(H_2O)$	$\Delta C_p(D_2O)$	$\Delta C_{p,tr}$ ($\Delta C_{p,H_2O} - \Delta C_{p,D_2O}$)
neomycin–WT	−0.73 ± 0.07	−0.52 ± 0.09	−0.21 ± 0.09
paramomycin–WT	−0.63 ± 0.08	−0.62 ± 0.08	−0.01 ± 0.08
tobramycin–WT	−0.82 ± 0.1	−0.47 ± 0.05	−0.35 ± 0.05
kanamycin–WT	−0.59 ± 0.03	−0.42 ± 0.05	−0.17 ± 0.05
neomycin–T130K[a]	−0.81 ± 0.06	−0.47 ± 0.05	−0.34 ± 0.06
paramomycin–T130K[a]	−0.67 ± 0.05	−0.41 ± 0.05	−0.26 ± 0.05
tobramycin–T130K[a]	−0.55 ± 0.01	−0.39 ± 0.04	−0.16 ± 0.04
kanamycin–T130K[a]	−0.35 ± 0.03	−0.29 ± 0.03	−0.06 ± 0.03

[a]Data from refs 14 and 15.

DISCUSSION

Differences in the temperature dependence of the binding enthalpy separated D80Y from the other thermostable variant T130K, which behaved like WT. Unlike WT and T130K, there was no linear correlation between the binding enthalpy and temperature with D80Y, which was indicative of the temperature dependence of ΔC_p. In our earlier work, a hint of curvature with one aminoglycoside was detected but was not investigated further.[9] In this work, the extended temperature range and the use of a couple of different aminoglycosides confirmed it and allowed that behavior to be investigated. The extent of curvature in plots of ΔH versus temperature for the formation of D80Y–aminoglycoside complexes was different for each ligand and did not seem to be correlated with any structural features of the ligand itself (Figure S1). The curvature in plots of ΔH versus T of D80Y starts well below the T_m of D80Y (Figure S3), suggesting that unfolding coupled to binding[19] may not be the reason but two partially overlapping enthalpic processes with different negative ΔC_p values may instead be at play. The binding enthalpy of all four aminoglycosides was always more favorable in H_2O at temperatures above 15 °C with all three enzymes. At lower temperatures, binding of some of the aminoglycosides was enthalpically more favored in D_2O with WT and T130K. Contrary to this, apart from kanamycin, ligand binding to D80Y at low temperatures was still enthalpically more favorable in H_2O. These observations suggest that, in D_2O, the bound state is stabilized at low temperatures and unbound states are stabilized above 15–20 °C with WT and T130K.[20]

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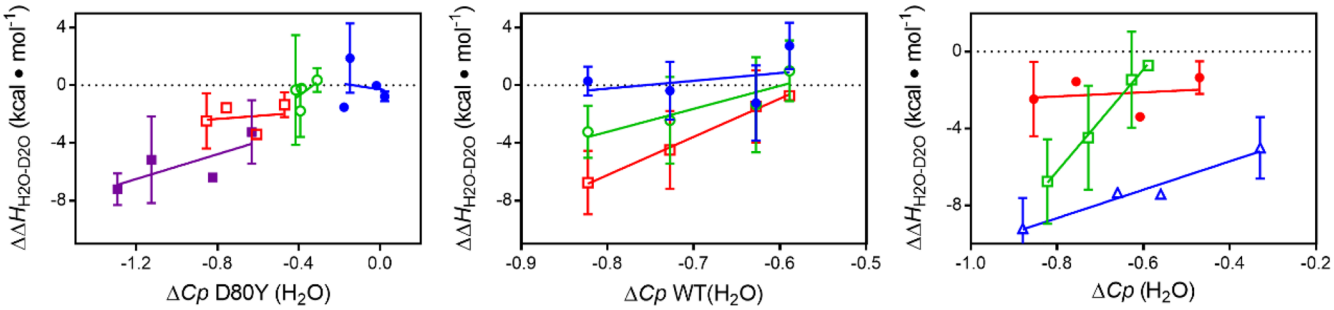

Figure 6. $\Delta\Delta H$ vs ΔC_p at different temperatures. Data for 10 °C (blue), 20 °C (green), 30 °C (red), and 40 °C (purple) are shown for D80Y (left) and WT (middle). Each data point at a given temperature represents a different aminoglycoside. Data acquired at 30 °C are replotted for all three enzymes for comparison (right): WT (green), T130K (blue), and D80Y (red). Data for T130K are from ref 14.

In the case of D80Y, there is no such temperature-dependent shift and unbound states are always stabilized relative to enzyme−aminoglycoside complexes.

It is not clear why kanamycin binding to D80Y is showing a pattern different from those of other aminoglycosides. However, we note that kanamycin is the only aminoglycoside within this set with an -OH group at the 2′ site (Figure S1). We have earlier shown that the presence of -NH_2 versus -OH at the 2′ site can have a significant effect on thermodynamics and even determines substrate behavior. For example, kanamycin B is a substrate for the aminoglycoside N^3-acetyltransferase-VIa (AAC-VIa) while no detectable activity was observed with kanamycin A.[21] Their enthalpies of binding to different aminoglycoside-modifying enzymes can be different by as much as 6.9 kcal/mol.[22] The only difference between these two aminoglycosides is the presence of -NH_2 at the 2′ site in kanamycin B and an -OH in kanamycin A (Figure S1).

Differences between the two thermostable variants were enhanced by the temperature dependence of $\Delta\Delta H$. While $\Delta\Delta H$ can be positive at low temperatures with WT and T130K, it is always, except for that of kanamycin, negative with D80Y. The transfer of hydrophobic groups from D_2O to H_2O occurs with a positive ΔH that becomes more negative with an increase in temperature.[23] $\Delta\Delta H$ values for WT and T130K are consistent with this. The same is true for D80Y only above ∼20 °C. Again, kanamycin is the exception with D80Y. Furthermore, a linear correlation is observed between $\Delta\Delta H$ and temperature with WT and T130K, which was nonlinear with D80Y (Figure 3).

The negative $\Delta C_{p,tr}$ with all variants is also consistent with the transfer of hydrophobic groups from D_2O to H_2O.[23,24] However, this is true for D80Y only above 20 °C, and at lower temperatures, $\Delta C_{p,tr}$ becomes positive (again, except for that of kanamycin) in an aminoglycoside-dependent manner. This is also consistent with the effect on the transfer of polar groups from D_2O to H_2O and, again, separates D80Y from WT and T130K. It is likely that protein dynamics and protein−solvent interactions have different temperature dependence in D80Y as compared to WT and T130K. These thermodynamic data are consistent with the differences in the temperature dependence of global protein dynamics between D80Y and WT and T130K as determined by molecular dynamics (MD) simulations.[9]

Variable $\Delta C_{p,tr}$ values for D80Y suggest that, at higher temperatures, the transfer of hydrophobic groups from D_2O to H_2O is impacting this parameter while at low temperatures (<15 °C) the effect on polar groups becomes dominant for D80Y. Because $\Delta\Delta H$ always remains negative for D80Y, this observation suggests that the temperature dependence of $\Delta\Delta H$ alone cannot be attributed to the transfer of hydrophobic groups from D_2O to H_2O. These observations are consistent with multiple overlapping events affecting the behavior of D80Y in solution and temperature-dependent alteration of their dominance in thermodynamics of D80Y−aminoglycoside interactions. Earlier, it was shown by MD simulations that the second principle component is the most effective in the dynamics of D80Y. This is unlike the case for WT and T130K, in which the first principle component was the most effective for both enzymes.[9] This was observed even though the motion of protein appears to be similar in all three enzymes. Our data suggest that the thermodynamic parameter ΔC_p for ligand binding separated D80Y from the WT/T130K pair.

Different ΔC_p values in H_2O and D_2O suggest the contribution of solvent reorganization to binding is temperature-dependent.[20] The contribution of the solvent to binding can also be shown by plots of $\Delta\Delta H$ versus ΔC_p.[20] Such a plot, representing data acquired at different temperatures, is shown in Figure 6 for WT and D80Y. Each of the four data points for a given temperature represents data acquired with a different aminoglycoside. For WT, the correlation between $\Delta\Delta H$ and ΔC_p becomes stronger with an increase in temperature, similar to what was observed with T130K.[14] This is not the case with D80Y. As shown in Figure 6, the correlation is still weak even at higher temperatures. Figure 6 shows the same plot at 30 °C for all three enzymes for comparison. A plot of the correlation coefficient against temperature for all three variants is shown in Figure 7. These data show that the contribution of solvent rearrangement to ΔC_p increases with an increase in temperature and becomes the major effector above 20 °C for WT and T130K. Data acquired with D80Y show no such systematic

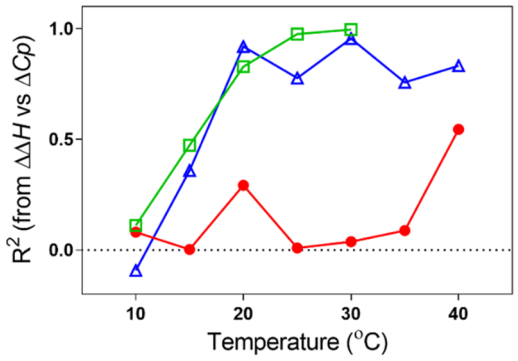

Figure 7. Temperature-dependent variation of the correlation coefficient from least-squares fits of $\Delta\Delta H$ vs ΔC_p data for WT (green), T130K (blue), and D80Y (red).

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change, and even at 40 °C, the correlation is still weak. Data also suggest that such a correlation may start to be established for D80Y at temperatures above 40 °C. However, we did not test binding at higher temperatures because even a small degree of protein unfolding can have a significant impact on the observed ΔH and cause curvature in plots of ΔH versus temperature, thus rendering data analysis difficult if not impossible.

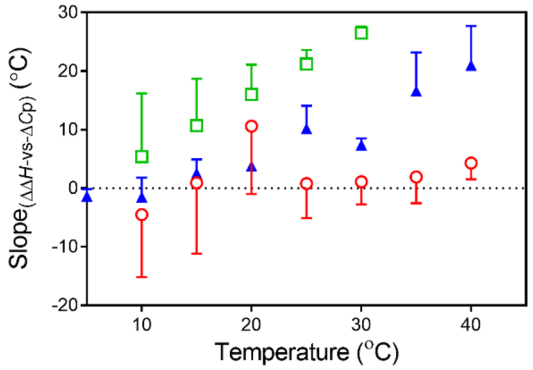

Figure 8. Slopes of $\Delta\Delta H$ vs ΔC_p for WT (green), T130K (blue), and D80Y (red).

Another contrasting aspect of these data is shown in Figure 8 as the temperature dependence of the slopes determined from plots of $\Delta\Delta H$ versus ΔC_p. The slopes have units of degrees Celsius and yield the offset temperature, the temperature difference at which we can observe the same binding enthalpy in H_2O and D_2O.[20] A steady increase observed with WT and T130K is not visible with D80Y. These data are indicative of the fact that D80Y does not follow the pattern observed with WT and T130K. This is very likely to be a result of differential solvent−protein interactions and differences in the global dynamics of these proteins.

Solvent reorganization and changes in low-frequency vibrations of proteins are the major contributors to ΔC_p.[25] Data acquired with D80Y suggest that unlike WT and T130K, changes in the low-frequency vibrational modes of the protein are the major sources of the observed change in heat capacity. If the solvent contribution becomes more dominant at temperatures above 40 °C, this will still separate D80Y from T130K. Even though T130K is thermostable, the thermodynamics of ligand binding and solvent effects mirror those of WT. Thus, T130K may simply be a more heat stable variant of WT but does not exhibit thermophilic properties. These data are consistent with MD simulations that also highlighted differences in protein dynamics and its temperature dependence between D80Y and the WT/T130K pair.[9]

We note that these data should not be interpreted as temperature dependence of ΔC_p as a unique property of thermophilic proteins. Such temperature dependence has been observed with DNA−ligand interactions.[27] The observed temperature dependence of ΔC_p for D80Y−aminoglycoside interactions suggests that two linked processes with different temperature dependencies and negative ΔC_p values are involved. This was not observed with WT or T130K.

CONCLUSIONS

In this work, we showed that two thermostable variants of ANT, each with a single-site mutation, may provide clues to distinguish thermophilic proteins from variants that simply become thermostable by an additional stabilizing interaction such as addition of a disulfide bond or an ionic interaction but are otherwise identical to mesophilic WT. Both T130 and D80 are away from the subunit−subunit interface and accessible to solvent.[26] Figure 9 shows a close-up of the location of both residues. It is likely that substitution of lysine at position 130 may allow an ionic interaction between this residue and Asp127 or Glu130 or backbone oxygen of Ser90 that is in the proximity of T130 and increase the thermostability of this variant without affecting other properties significantly. D80Y, on the other hand, does not appear to be positioned to engage in such a bond formation interaction. However, replacement of an ionic side chain with Tyr may potentially increase the number of hydrophobic interactions leading to thermostability. Thus, D80 may be a node impacting protein dynamics and its interactions with solvent and ligands leading to thermophilic behavior.

Protein function in a thermophilic environment may require more than just an increased T_m, features such as different protein dynamics and/or solvent−protein interactions or interactions with other molecules and ligands. Our earlier work showed significant differences in the dynamic properties of apoenzymes that separated D80Y from WT and T130K.[9] This work shows that the thermodynamics of enzyme−ligand

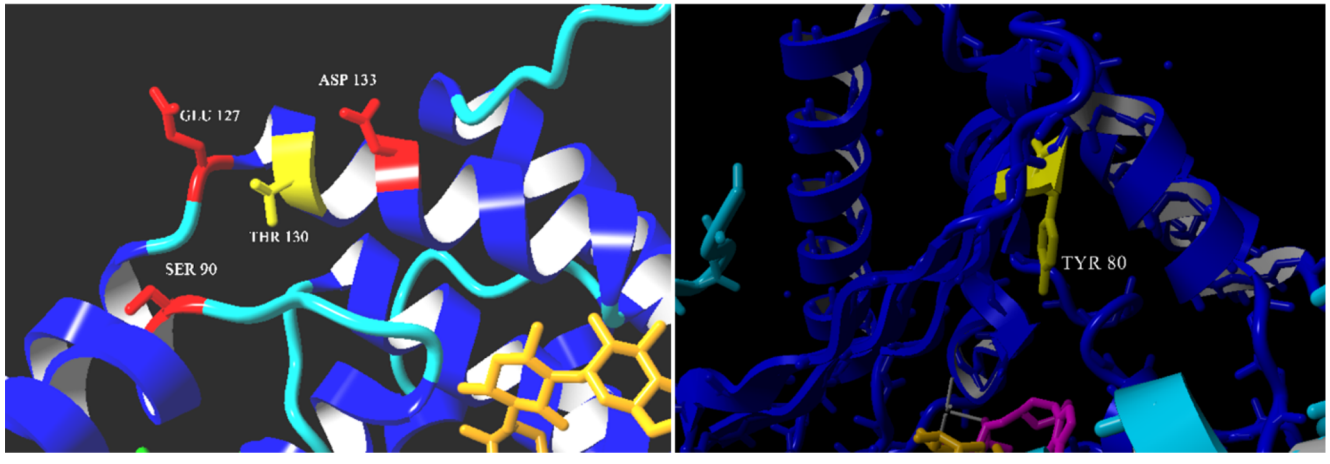

Figure 9. Close-up of the protein structure around residues 130 (left) and 80 (right) from the crystal structure of D80Y.[26]

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interactions also provide features that separate the two thermostable variants from each other. We should note that the additivity of the melting temperatures of T130K and D80Y as observed in the double mutant also suggests different mechanisms for achieving thermostability for these two variants.[9] Because the thermodynamics of enzyme−ligand interactions of T130K is identical to that of WT, we believe that its T_m is increased by interactions of K130 with one of the candidate sites as described earlier without affecting other molecular properties of WT. D80Y, on the other hand, has significantly different thermodynamics (and a T_m higher than that of T130K) and is therefore denoted as "thermophilic". There are currently accepted properties that are associated with thermophilic proteins such as deletion of loops at the surface, an increased fraction of aliphatic hydrophobic side chains, charged residues, and an increased number of polar interactions.[3,28] We note that replacement of D80 with Y is also against the current paradigm of thermophilic proteins having more polar interactions because an ionic side chain is replaced with a phenolic one. Thus, it appears that determinants of thermophilicity are broader and may not be easily determined by a sequence of structural comparisons. The general applicability of these results to other systems in terms of one thermodynamic parameter or another of protein−ligand interactions remains to be seen.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b01201.

Aminoglycoside structures, ITC titrations, additional plots of ΔH versus T, tables of thermodynamic data, and temperature-dependent analytical ultracentrifugation data (PDF)

Accession Codes

WT, P05057; T130K, P05058.

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Notes

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