Standard Free Energy for the Hydrolysis of Adenylylated T4 DNA Ligase and the Apparent pKₐ of Lysine 159⁎

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Equilibrium constants for the adenylylation of T4 DNA ligase have been measured at 10 pH values. The values, when plotted against pH, fit a titration curve corresponding to a pKₐ of 8.4 ± 0.1. The simplest interpretation is that the apparent pKₐ is that of the 6-amino group of the AMP-accepting residue Lys159. Based on the pH dependence of the equilibrium constants, the value at pH 7.0 is 0.0213 at 25 °C, corresponding to ΔG° = +2.3 kcal mol⁻¹. From this value and the standard free energy change of −10.9 kcal mol⁻¹ for the hydrolysis of ATP to AMP and PPᵢ, we calculate that ΔG° for the hydrolysis of the adenyl-DNA ligase is −13.2 kcal mol⁻¹. The presence of conserved basic amino acid residues in the catalytic domain, which are proximal to the active site in the homologous catalytic domain of T7 DNA ligase, suggests that the pKₐ of Lys159 is perturbed downward by the electrostatic effects of nearby positively charged amino acid side chains. The lower than normal pKₐ 8.4 compared with 10.5 for the 6-amino group of lysine and the high energy of the α,β-phosphoanhydride linkage in ATP significantly facilitate adenylylation of the enzyme.

DNA ligases catalyze phosphodiester-bond formation between the adjacent 5'-phosphate and 3'-hydroxyl ends of the nicked DNA chains (1, 2). DNA ligases were first identified in 1967 (3–8) and shown to play essential roles in DNA replication, repair, and recombination. Recently, interest in DNA ligases has been expanded by new information regarding the connections between human cancers and DNA repair systems (9, 10). The two types of DNA ligases are ATP-dependent and NAD⁺-dependent, respectively. ATP-dependent ligases are from eukaryotic cells, certain prokaryotes, and bacteriophages of the T series; NAD⁺-dependent enzymes are from other prokaryotic cells. DNA ligases require a divalent cation for activation.

EXPERIMENTAL PROCEDURES

Materials—ATP, CHES,† HEPES, dithiothreitol, ovalbumin, and DNase were purchased from Sigma. The following were obtained from the vendors indicated: Mg acetate from Aldrich; pET21 vector from Novagen; YM30 filtration membrane from Amicon; [3H]ATP from ARC; Ultrafree-MC 30K filter unit from Millipore; and Bio-Safe II from Research Products International Corp.

Preparation of DNA Ligase—T4 DNA ligase was purified from Escherichia coli BL21(DE3) transformed with plasmid pEAW128. To construct pEAW128, the gene encoding T4 DNA ligase was inserted into pET21A (Novagen) in two PCR fragments. Genomic DNA of E. coli strain BNN67, in which the T4 ligase gene is inserted as a bacteriophage lysogen (15), was used as the template for both PCR reactions. Genomic DNA was prepared using a published procedure (16). The 256 base pairs of the T4 ligase gene defining the N terminus of the protein from the initiation codon to the MfeI site were inserted into pET21A, replacing sequences from the Ndel to the BamHI sites. The 5'-PCR primer corresponded to bases 1–18 of the T4 ligase gene, with 5'-CTCAGGACCAT added to the 5' end to provide a clamp and an Ndel site.

DNA ligase reacts with ATP in the first step to displace PPᵢ, and generate an AMP enzyme (adenyl enzyme) complex in which the AMP is linked to the 6-amino residue of the enzyme through a phosphoramidate bond (13). The AMP enzyme then binds nicked DNA and transfers the AMP group to the 5'-phosphoryl group in the nick. The resulting adenosine-5'-phosphophosphoryl moiety in the nick activates the phosphate group at the 5' terminus of the DNA by supplying AMP as a good leaving group. The final step is a nucleophilic attack by the 3'-hydroxyl group on this activated phosphorus atom to displace AMP and form the 3'-phosphodiester bound.

The second and the third steps (Eqs. 1b and 1c) are thermodynamically spontaneous reactions (i.e. −ΔG°*). However, the adenosine-5'-phosphoramidate formed in the first step is expected to be a high energy intermediate. The standard free energy change for the hydrolysis of E-Lys-NH-AMP (nucleoside-5'-phosphoramidate) has not been known; however, it could be calculated from the equilibrium constant for adenylylation of the enzyme at pH 7.0 and the standard free energy change of −10.9 kcal mol⁻¹ for the hydrolysis of ATP to AMP and PPᵢ (14). In this study, we report the equilibrium constants for the adenylylation of T4 DNA ligase at ten different pH values, the apparent pKₐ for dissociation of the protonated lysine residue of the T4 DNA ligase, and the standard free energy change for the hydrolysis of adenylylated T4 DNA ligase.

E-Lys-NH₂ + ATP ↔ E-Lys-NH-AMP + PPᵢ (Eq. 1a)
The 3'-PCR primer corresponded to bases 261–247 of the T4 ligase gene, with 5'-CCCGATCC added to the 5' end to provide a BamHI site and clamp. With the first fragment inserted, the modified pET21A was digested at MfeI and HindIII, and the remaining base pairs of the gene were inserted. The 5'-PCR primer for this second fragment corresponded to bases 254–285 of the T4 ligase. The 3'-primer corresponded to bases 1460–1455 of the T4 ligase gene, with 5'-CCTAGCTTCA added to provide a clamp and HindIII site.

DNA ligase was purified by a modification of the procedure of Davis et al. (15). Cells were disintegrated by addition of DNase and then sonicated for 4 min in 1 min bursts. At the early stage of purification two ammonium sulfate fractionations were performed. The last step of the procedure, hydroxyapatite chromatography, was omitted because the enzyme was essentially pure as indicated by gel electrophoresis. Purified enzyme was concentrated in an Amicon ultrafiltration device using the YM30 filtration membrane.

Equilibrium Constant for Adenylylation of DNA Ligase—The equilibrium constants ($K_{eq}$) for the reaction of DNA ligase and [3H]ATP to produce the covalent AMP enzyme and pyrophosphate according to Eq. 2 were measured at ten different pH values.

$$\text{DNA ligase} + \text{Mg}^2+\text{[H]}\text{ATP} \leftrightarrow \text{DNA ligase}^\sim \text{[H]}\text{AMP} + \text{Mg}^2+\text{PP}_i$$

($\text{Eq. 2}$)

The buffers used for the different pH values were as follows: CHES for pH values 10.0, 9.5, 9.0; TAPS for pH values 8.5, 8.4, 8.2; and HEPES for pH values 8.0, 7.8, 7.5, 7.6. Each reaction mixture (50 µl) consisted initially of 50 mM buffer, 10 mM dithiothreitol, 0.0025–1 mM of Mg$^2+$[H]ATP, 1 mM Mg-acetate, and 0.012 mM DNA ligase. The reactions proceeded for 20 min at 25 °C. The reactions were stopped by addition of 550 µl of 15 mM EDTA. The solutions were filtered through Ultrafree-MC (30-kDa cutoff) filter units, which separated the enzyme from the small molecules. Ultrafree-MC filter units with a low binding reagent of 550 µl was added to the upper chamber inside the vial. After 1 h, 600 µl of the 50 mM buffer and 15 ml of Bio-Safe II counting mixture were added into the scintillation vial, which was then counted for [3H]AMP ligase. Nonspecific binding to the filter was determined in the same way using samples in which chicken ovalbumin was substituted for DNA ligase. To confirm that the reactions had reached equilibrium in 20 min, two of the reactions (0.04 and 0.1 mM ATP at pH 8.5) were carried out for three different reaction periods (20, 30, and 40 min). The results showed no significant differences in the production of [3H]-labeled ligase after 20 min.

Apparent equilibrium constants ($K_{eq}$) for adenylylation of DNA ligase (Eq. 2) at different pH values were calculated by using the following equation:

$$K_{eq} = \frac{[\text{E-AMP}]_{\text{eq}}[\text{Mg}^2+\text{PP}_i]}{[\text{E}] \times [\text{AMP}]_{\text{eq}} \times [\text{ATP}]_{\text{eq}}}$$

($\text{Eq. 3}$)

In a given measurement of $K_{eq}$, the values of [E-AMP] were obtained as ligase-bound tritium at each concentration of Mg$^2+$[H]ATP, and $K_{eq}$ was the maximum amount of ligase-bound tritium.

**Evaluation of the Apparent $K_{eq}$ for Lys$^{99}$—** The $K_{eq}$ for the dissociation of Lys$^{99}$ in DNA ligase refers to the process of Eq. 4.

$$\text{E-Lys}^{99} - \text{NH}_2^+ + \text{H}^+ \leftrightarrow \text{E-Lys}^{99} - \text{NH}_2^+ + \text{H}^+$$

($\text{Eq. 4}$)

Assuming that only the unprotonated form of Lys$^{99}$ reacts with MgATP, the apparent dissociation constant for Lys$^{99}$, $K_s$, is given by Eq. 5, where $K_{eq}$ is the apparent equilibrium constant at pH = x, and $K_{eq}$ is the pH-independent overall equilibrium constant.

$$K_{eq} = \frac{K_{eq}}{K_s + [\text{H}^+]} K_{eq} + 10^{-pH}$$

($\text{Eq. 5}$)

The apparent equilibrium constants, $K_{eq}$, were measured as described under “Experimental Procedures.” The results were then plotted as $K_{eq}$ versus pH. Then from fitting the data to Eq. 5 the line was computed as well as the apparent $pK_o$ for Lys$^{99}$. The $pK_o$ value resulting from this fitting procedure was 8.40 ± 0.09.

**RESULTS AND DISCUSSION**

**Equilibrium Constants for the Formation of E-Lys$^{99}$-NH$_2$AMP—** The covalent intermediate is produced in the reaction of DNA ligase with ATP according to Eq. 1a. The apparent equilibrium constant at a given pH = x, $K_{eq}$, can be measured by use of radiolabeled ATP as described under “Experimental Procedures.” The equilibrium constants for adenylylation of DNA ligase were determined at ten different pH values and have been plotted against pH in Fig. 1.

**TABLE I**

| Reaction | $\Delta G^{\circ}$ (kcal mol$^{-1}$) |
|----------|----------------------------------|
| E-Lys-NH$_2$AMP + MgPP$_i$ = E-Lys-NH$_2$ + MgATP | -2.3 (Eq. 6) |
| MgATP + $\text{H}_2\text{O} = \text{AMP} + \text{MgPP}_i$ | -10.9$^*$ (Eq. 7) |
| E-Lys-NH$_2$AMP + $\text{H}_2\text{O} = E$-Lys-NH$_2 +$ AMP | -13.2 (Eq. 8) |

*$^*$ See Ref. 14.

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**FIG. 1.** $K_{eq}$ versus pH for adenylylation of DNA ligase. The apparent equilibrium constants, $K_{eq}$, were measured as described under "Experimental Procedures." The results were then plotted as $K_{eq}$ versus pH. Then from fitting the data to Eq. 5 the line was computed as well as the apparent $pK_o$ for Lys$^{99}$. The $pK_o$ value resulting from this fitting procedure was 8.40 ± 0.09.
value of $pK_a$ for Lys$^{159}$ in the active site of the T4 DNA ligase, from 10.5 to 8.4, could well be caused by electrostatic destabilization of the lysine-6-ammonium group by the nearby positively charged, basic amino acids. The positive electrostatic field would favor proton dissociation and a lowered $pK_a$. This effect might not be confined to Lys$^{159}$ and would perturb the acid dissociation constants of the other basic residues as well, it is only the effect on the $pK_a$ of Lys$^{159}$ that would be detected by our chemical test of adenylating the enzyme.

**Standard Free Energy Change for Hydrolysis of Adenylylated DNA Ligase**—The recommendation of the International Union of Pure and Applied Chemistry is that $\Delta G^{\circ}$ refers to total concentrations of the ionic species of each component of the reaction at pH 7.0, 25 °C, and 1 mM free Mg$^{2+}$ (21). In this study, standard free energy changes follow this recommendation. The standard free energy change for hydrolysis of adenylylated DNA ligase, $\Delta G^{\circ}$, can be calculated from the free energies of the reaction studied here, Eq. 1a, and that for the hydrolysis of ATP to AMP and PP$_1$ (22). To determine the $\Delta G^{\circ}$, the value for $K_{diss}$ at pH 7 for the adenlyylation of DNA ligase was calculated using Eq. 5 and found to be $K_{diss} = 0.0213$. Then the $\Delta G^{\circ}$ for Eq. 1a was calculated as $-7.3$ kcal mol$^{-1}$ from $\Delta G^{\circ} = -RT \ln K_{diss}$. The standard free energy change ($\Delta G^{\circ}$) for hydrolysis of adenylylated DNA ligase, Eq. 8, can be calculated as the sum of Eqs. 6 and 7 as shown in Table I. The value of $\Delta G^{\circ}$ is $-13.2$ kcal mol$^{-1}$, which indicates that E-Lys-NH-AMP is a high-energy compound. As we expected (14) the standard free energy of hydrolysis of adenylylated DNA ligase is in the same range as the value of $\Delta G^{\circ}$ for the hydrolysis of UMP-imidazolide to UMP and imidazole, which is $-14.7$ kcal mol$^{-1}$ (22).

Although adenylyl-DNA ligase is high energy, it is not so high as to be inaccessible by cleavage of the phosphoanhydride bond linking P$_\alpha$ and P$_\beta$ in Mg-ATP, which is $2.3$ kcal mol$^{-1}$ lower in energy when expressed as the standard free energy of hydrolysis. The difference can be overcome by the millimolar concentration of Mg-ATP in a cell and the very favorable free energy changes for the coupled reactions Eqs. 1b and 1c. The low apparent $pK_a$ of Lys$^{159}$ is a significant factor in bringing the value of $\Delta G^{\circ}$ near enough to that of Mg-ATP for cleavage to AMP and Mg-PP$_1$. If the apparent $pK_a$ of Lys$^{159}$ had been normal for the 6-aminogroup (10.5), $\Delta G^{\circ}$ for the hydrolysis of adenylyl-DNA ligase would have been $-16.1$ kcal mol$^{-1}$, a value that would make the formation of the adenylyl-DNA ligase intermediate very difficult. Therefore, it seems that electrostatic $pK_a$ perturbation of Lys$^{159}$ at the active site of DNA ligase is significant in facilitating the action of this enzyme.

Another factor favoring the adenlylation of DNA ligase is the energy of the $\alpha,\beta$-phosphoanhydride linkage in ATP. The standard free energy of hydrolysis has recently been found to be $-10.9$ kcal mol$^{-1}$ (14), which is significantly more negative than the $-7.8$ kcal mol$^{-1}$ for hydrolysis of the $\beta,\gamma$-phosphoanhydride linkage (21). The combined effects of the high energy $\alpha,\beta$-phosphoanhydride linkage in ATP and the low value of $pK_a$ for Lys$^{159}$ in T4 DNA ligase facilitate the formation of the intermediate AMP ligase.

Another high energy intermediate in the action of DNA ligase is the adenlylated DNA produced by transfer of the AMP-group from the adenylyl-DNA ligase to the 5'-P of nicked DNA. The resulting phosphoanhydride linkage is a P$_1$-P$_2$-dialkyl diphosphate, and as such can be expected to display a similar value of $\Delta G^{\circ}$ to that for the hydrolysis of UDP-glucose to UMP and glucose-1-phosphate, which is $-10.3$ kcal mol$^{-1}$ (14). This is $2.9$ kcal mol$^{-1}$ less negative than $\Delta G^{\circ}$ for the hydrolysis of adenylyl-DNA ligase, so that the transfer of the AMP group from the adenlylated enzyme to nicked DNA appears to be spontaneous.

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