Thermodynamics of Human DNA Ligase I Trimerization and Association with DNA Polymerase β*

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The interaction between human DNA polymerase β (pol β) and DNA ligase I, which appear to be responsible for the gap filling and nick ligation steps in short patch or simple base excision repair, has been examined by affinity chromatography and analytical ultracentrifugation. Domain mapping studies revealed that complex formation is mediated through the non-catalytic N-terminal domain of DNA ligase I and the N-terminal 8-kDa domain of pol β that interacts with the DNA template and excises 5'-deoxyribose phosphate residue. Intact pol β, a 39-kDa bi-domain enzyme, undergoes indefinite self-association, forming oligomers of many sizes. The binding sites for self-association reside within the C-terminal 31-kDa domain. DNA ligase I undergoes self-association to form a homotrimer. At temperatures over 18 °C, three pol β monomers attached to the DNA ligase I trimer, forming a stable heterohexamer. In contrast, at lower temperatures (<18 °C), pol β and DNA ligase I formed a stable 1:1 binary complex only. In agreement with the domain mapping studies, the 8-kDa domain of pol β interacted with DNA ligase I, forming a stable 3:3 complex with DNA ligase I at all temperatures, whereas the 31-kDa domain of pol β did not. Our results indicate that the association between pol β and DNA ligase I involves both electrostatic binding and an entropy-driven process. Electrostatic binding dominates the interaction mediated by the 8-kDa domain of pol β, whereas the entropy-driven aspect of interprotein binding appears to be contributed by the 31-kDa domain.

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The DNA repair pathway termed base excision repair (BER) purifies genomic DNA of damaged nucleotides and abasic sites arising from a variety of exogenous and endogenous sources (1). For example, altered bases arising from deamination and from alkylation by both endogenous (e.g. S-adenosylmethylion) and endogenous alkylating agents (e.g. methyl methane sulfonate and vinyl chloride) are repaired by BER (2-4). The abasic site is generated from these base lesions by either spontaneous or enzymatic cleavage of the N-glycosidic bond. In both prokaryotic and mammalian cells, the abasic site is repaired by a mechanistically similar BER pathway (5, 6). In this pathway, the abasic site is usually cleaved by a class II AP endonuclease, followed by the sequential actions of a DNA polymerase, a 2-deoxyribose-5-phosphate lyase (dRP lyase), and finally a DNA ligase (5, 7). BER can be distinguished from other DNA excision repair pathways by the relatively small repair patch produced in double-stranded DNA after incision at the abasic site and also by the fact that base lesions repaired by the BER pathway are generally limited to modifications that are less bulky than those lesions repaired by the nucleotide excision repair pathway.

In mammalian cells, there are at least two BER pathways, which have been designated as follows: "short patch" or simple BER, in which the repair patch is a single nucleotide; and "long patch" or alternate BER, in which the repair patch is 2 to <13 nucleotides (5, 8). In the case of short patch BER, several lines of research have recently confirmed a role for DNA polymerase β (pol β) (5, 8-10). The N-terminal 8-kDa domain of pol β functions as a dRP lyase catalyzing a β-elimination reaction releasing dRP from the preincised AP site in double-stranded DNA (7, 11, 12). This domain is also capable of functioning as an AP site lyase catalyzing strand cleavage at intact AP sites in double-stranded DNA (12). Polymerase β and DNA ligase I have been found together in a BER-proficient complex isolated from bovine testis (13). Since purified human pol β and DNA ligase I interact in vitro (13), it seems likely that these enzymes also bind to each other within the naturally occurring BER-proficient complex. In addition, an interaction between pol β and AP endonuclease, another component of the BER-proficient complex, has been observed (14).

In this study, the interaction between pol β and DNA ligase I has been further investigated by two independent, complementary approaches. Initially, the regions of these enzymes that are required for stable complex formation were mapped by affinity chromatography. Subsequently, equilibrium experiments were conducted in the analytical ultracentrifuge to characterize the molecular species formed as a result of the stable interactions between pol β and DNA ligase I. Thermodynamic studies were also conducted in the analytical ultracentrifuge to gain further insight into the mechanism of binding. In contrast to the studies with pol β and DNA ligase I, we could not detect a stable complex between pol β and AP endonuclease in similar experiments. Together, our results suggest intriguing new implications as to how pol β and DNA ligase I function together to complete the base excision repair pathway.

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EXPERIMENTAL PROCEDURES
Preparation of Affi-Gel Affinity Resins and in Vitro Binding Assay
Affi-Gel 10 beads (Bio-Rad) with either recombinant human DNA ligase I or bovine serum albumin (BSA) as the ligand were prepared as described (15). The affinity beads (20 μl of beads with 0.15 nmol of either DNA ligase I or BSA as the ligand) were resuspended in 400 μl of binding buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) containing 2% dry milk and incubated for 15 min at 4 °C. After the addition of 0.15 nmol of intact human pol β, the 8-kDa N-terminal domain, or the 31-kDa C-terminal domain (16, 17), the incubation was continued for 1.5 h. Beads were collected by centrifugation, washed with binding buffer, and then resuspended in 15 μl of SDS-sample buffer. After heating at 70 °C for 5 min, proteins were separated by denaturing gel electrophoresis and transferred to a nitrocellulose membrane. After incubation with the pol β antibody, antigen-antibody complexes were detected by enhanced chemiluminescence (Pierce). Similar assays were carried out with different versions of the 8-kDa domain containing the single amino acid changes indicated in the figure legend.

Purification of GST and GST-pol β
Human pol β cDNA was subcloned into pGStag (18). After induction of plasmid-encoded GST and GST-pol β, cell extracts were prepared and protein expression was examined as described previously (19). Glutathione-Sepharose beads (Amersham Pharmacia Biotech) with equal amounts of either GST or GST-pol β were produced (19).

Pull-down Assays with Glutathione-Sepharose Beads
DNA ligase I polypeptides were labeled in vitro by coupled transcription and translation and partially purified by ammonium sulfate precipitation (19). Labeled polypeptides (5 μl), which were resuspended in buffer A (50 mM Hepes, pH 7.7, 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, and 0.1% Nonidet P-40), were incubated at room temperature for 30 min with glutathione-Sepharose 4B beads (20 μl with either GST-pol β or GST as the ligand) that had been diluted to a final volume of 150 μl with buffer A. After the beads were washed three times with 1 ml of buffer A, bound proteins were separated by SDS-PAGE. Labeled proteins were detected in the dried gel by autoradiography.

Protein Purification
Recombinant wild-type human pol β and its 8-kDa and 31-kDa domains were produced and purified as described (16, 17). AP endonuclease was purified as described previously (20). Human DNA ligase I was purified from baculovirus-infected cells (21).

Analytical Ultracentrifugation
Analytical ultracentrifugation was performed in a Beckman XL-A analytical ultracentrifuge using either a 4-hole or 8-hole rotor at rotor speeds appropriate to the study. All experiments were begun at 2 or 3 °C and incremented by 3 or 4 °C (except as noted) following attainment of equilibrium until either 38 °C was reached or clear signs of protein degradation occurred. A total of six types of equilibrium runs were performed as described below. Intact pol β with AP Endonuclease—An 8-hole rotor was used at the speed of 15,000 rpm. The centrifuge cells were flushed with argon prior to loading to prevent oxidation of the AP endonuclease by the atmospheric oxygen in the unfilled portion of the channels. Two concentrations of each AP endonuclease and pol β having absorbancies ~ 0.2 and 0.3 at 280 nm were run, the remaining three cells were loaded with mixtures of AP endonuclease and pol β in molar concentration ratios of 1:1, 1:2, and 2:1. These mixtures had absorbencies ~ 0.3 at 280 nm. Transmitted light intensity data were collected over a range of temperatures between 2 and 18 °C in steps of 4 °C.

Intact pol β with DNA Ligase I—Again, the 8-hole rotor was employed but at 10,000 rpm. Two cells were loaded with two concentrations of pol β having absorbencies ~ 0.22 and 0.34 at 280 nm; two other cells were loaded with DNA ligase I with concentrations having absorbencies ~ 0.2 and 0.3. The remaining three cells were loaded with pol β-DNA ligase I mixtures with molar concentration ratios of 1:1, 1:2, and 2:1, having absorbencies ~ 0.25. Transmitted light intensity data were again collected over a temperature range of 2–34 °C in steps of 4 °C. The data collected at 34 °C were discarded because protein degradation became apparent.

The 8-kDa N-terminal Domain of pol β—A 4-hole rotor was employed at 27,000 rpm, and the three cells were loaded with concentrations having absorbencies of 0.15, 0.22, and 0.3, respectively, at 280 nm. Analysis of the data clearly showed that the protein did not self-associate. Intensity scans were taken only at 2, 10, 18, 25, and 32 °C, and used for the experimental estimation of the protein fragment partitioning volume. It is assumed that these estimated values are more accurate than the compositional values since they were determined experimentally in the centrifuge, with appropriate buffers over the appropriate temperature range. They were subsequently used in the analysis of the experimental data from the fourth experimental study. The 8-kDa N-terminal Domain of pol β with DNA Ligase I—A 4-hole rotor was employed at 12,000 rpm, and the three cells were loaded with 1:1, 1:2, and 2:1 molar concentration ratios of DNA ligase I and the 8-kDa pol β. The absorbencies at 280 nm in all three cells were between 0.2 and 0.3. Intensity scans were taken at temperatures from 2 to 38 °C in steps of 3 °C. This proved to be the most stable mixture as no protein degradation was observed below 38 °C.

The 31-kDa C-terminal Domain of pol β—A 4-hole rotor was run at 17,000 rpm to equilibrium with three different protein concentrations having absorbencies ~ 0.25, 0.35, and 0.45 at 280 nm, respectively. Intensity data were collected from 2 to 30 °C in steps of 4 °C.

The last two experiments were performed, after the data from the previous experiments had been analyzed, to check the consistency of the emerging description of the binding sites on the pol β molecule.

Transmitted light intensity measurements were taken at a wavelength of 280 nm with a radial increment of 0.002 cm and with 16 replicates taken and averaged at each radial position. Equilibrium was considered to be attained when scans were invariant over 12 h. All protein solutions were in the same buffer: 25 mM Hepes, pH 7.5, 50 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol. In a first experiment, the buffer also contained 20% glycerol; this was found to retard equilibrium to an unacceptable degree due to the viscosity of the buffer. Therefore, buffers without glycerol were used for all subsequent experiments. Solution columns were, in all cases, approximately 5 mm in height. Both absorbency and intensity data were analyzed using the Windows version of the MLAB software (Civilized Software, Inc., Bethesda, MD) on Pentium-based personal computers. The analysis was performed by assuming appropriate models for concentration distributions, starting with the most consistent with current knowledge, and using non-linear least-squares curve fitting techniques to estimate model parameters such as equilibrium constants, molecular masses, and local species concentrations. The data presented in the figures are the results of fitting intensity data. The results of fitting absorbency data, which had been the customary approach in the past, did not change any of the qualitative conclusions reached.

Data Analysis
Transmitted light intensity data were collected in all cases, but both intensities and their corresponding derived absorbencies were used for the analysis. The reason for using both forms of the collected data lies in the recent demonstration that the customary analysis of absorbency data using least-squares estimation is not optimal due to the non-Gaussian character of the noise (22). The noise in the absorbency signal is the result of nonlinear transformation of the intensity signals, which are Gaussian. It has been shown that if intensity data were fitted directly, the estimation process would be optimal. A brief discussion of issues and mathematical methods are given in the Appendix, under “Direct Fitting of Transmitted Light Intensity in Analytical Ultracentrifugation.” Although simulated data clearly demonstrated the superiority of fitting intensity data, this is the first time where the methodology has been employed for actual collected data. For these reasons, both approaches were employed at every analysis step and the results compared. Where association was found to take place, both approaches were employed at every analysis step and the results compared. Where association was found to take place, both approaches were employed at every analysis step and the results compared. Where association was found to take place, both approaches were employed at every analysis step and the results compared.
The mathematical models which best fit the collected data for each experiment, are given below. These models were used for the estimation of the relevant association parameters.

**Intact pol β**—The association model that emerged for the intact pol β data was that of an indefinite isodesmic self-association. The mathematical model for this type of association is given in Equation 1.

\[
c_{\text{pol}β}(r) = \frac{c_{\text{pol}β}\exp(A_M M β \delta r^2)}{1 - c_{\text{pol}β}\exp[lnK_{12} - ln(E_{M}^{β}_{280}/2) + A_M M β \delta r^2]} + \epsilon
\]  
(Eq. 1)

\(c_{\text{pol}β}(r)\) is the total pol β monomer concentration at radius \(r\), \(c_{\text{pol}β}\) is the monomer concentration at \(r_0\), an arbitrarily chosen reference radius, \(M_β = 38185\) Da is the monomer molecular mass, \(lnK_{12}\) represents the natural logarithm of the equilibrium constant, on a molar scale, for the binding of a monomer to any size oligomeric pol β chain, \(E_{M}^{β}_{280} = 22,080\) is the molar extinction coefficient of pol β at 280 nm; \(A_M = (1 - \sigma_{M}^{β}/2RT)\), where \(\sigma\) is the compositional partial specific volume of the solute molecule at the temperature \(T\); \(\rho\) is the specific mass of the buffer at that temperature, \(\omega\) is the rotational speed used in rad/s, \(R\) is the gas constant, and \(T\) the absolute temperature; also, \(\delta r^2 = r^2 - r_0^2\) and \(\epsilon\) is the base-line offset correction due to finite absorbency of the buffer and other measurement factors. \(\lnK_{12}\), \(c_{\text{pol}β}\), and \(\epsilon\) were used as the fitting parameters.

**DNA Ligase I**—The association model that emerged from the DNA ligase I (L) data was \(3L_β \rightleftharpoons L_β\). Such a monomer-trimer system is mathematically modeled in Equation 2.

\[
c_{\text{L}β}(r) = c_{\text{L}β}\exp(A_L M_L \delta r^2) + c_{\text{L}β}\exp[lnK_{31} - lnE + 3A_L M_L \delta r^2] + \epsilon
\]  
(Eq. 2)

\(\lnE = ln(E_{M}^{β}_{280}/3)\) is the conversion factor between molar and absorbency scale of \(lnK_{31}\), where \(E_{M}^{β}_{280} = 64620\) is the DNA ligase I molar extinction coefficient at 280 nm. The rest of the terms are self-explanatory or are defined in connection with Equation 1.

**DNA Ligase I with the 8-kDa Domain of pol β**—In addition to the monomeric forms of these proteins, the DNA ligase I trimer and some form of a hetero-oligomer of the two proteins were observed. The large size difference between the monomers and the fact that very small amounts of monomers remained unbound made the problem mathematically ill-conditioned. This necessitated the use of mass conservation principles to mathematically eliminate one of the reference concentrations. The mathematical form for this type of system may be written as shown in Equation 3.

\[
c_{\text{L}β}(r) = c_{\text{L}β}\exp(A_L M_L \delta r^2) + c_{\text{L}β}\exp[lnK_{31} - lnE + 3A_L M_L \delta r^2] + c_{\text{L}β}\exp[lnK_{31} - lnE + 3A_L M_L \delta r^2] + \epsilon
\]  
(Eq. 3)

\(\lnK_{31}\) is, on molar scale, the natural logarithm of the association constant for the trimerization of the DNA ligase I itself which was discussed in the previous section, \(lnK_{31}\) is the corresponding molar scale constant for the formation of the hetero-\(n\)-mer complex \(L_{n} β\), \(c_{\text{L}β}\) is the reference concentration for the DNA ligase I and \(c_{\text{L}β}\), is the reference concentration for the 8-kDa domain of pol β as expressed in terms of that of the DNA ligase I based on mass conservation principles; \(E_{M}^{β}_{280} = ln(E_{M}^{β}_{280} E_{M}^{β}_{1280}^N(n(E_{M}^{β}_{280} E_{M}^{β}_{280})))\) is the conversion factor between molar and absorbency scales for \(lnK_{31}\), where \(E_{M}^{β}_{280}\) and \(E_{M}^{β}_{1280}\) and the remaining symbols have been defined previously. The value of \(n\) determines the size of the hetero-oligomer formed and clearly, \(c_{\text{L}β}\) is also a function of \(n\) and of \(lnK_{31}\). Here, \(c_{\text{L}β}\), \(lnK_{31}\), and \(\epsilon\) were the fitting parameters.

**DNA Ligase I with Intact pol β**—The system described under "DNA Ligase I with the 8-kDa Domain of pol β" above had to be modified slightly to allow for the indefinite self-association of the pol β as described under "Intact pol β." Thus, the appropriate mathematical model is shown by Equation 4.

\[
c_{\text{L}β}(r) = c_{\text{L}β}\exp(A_L M_L \delta r^2) + c_{\text{L}β}\exp[lnK_{31} - ln(E_{M}^{β}_{280}/3) + 3A_L M_L \delta r^2] + c_{\text{L}β}\exp[lnK_{31} - ln(E_{M}^{β}_{280}/3) + 3A_L M_L \delta r^2] + \epsilon
\]  
(Eq. 4)

\(\lnK_{31}\) is the trimerization constant for the DNA ligase I, and \(\lnK_{31}\) is the association constant for binding of a pol β monomer to its oligomeric chain, both of which are known as discussed in the previous sections;
Identification of the Regions Required for Complex Formation between DNA Ligase I and pol β by Affinity Chromatography—Analysis of the structure of pol β, initially by controlled proteolysis and more recently by x-ray crystallography, has shown that this enzyme is composed of an 8-kDa N-terminal domain that binds to DNA template and carries dRP lyase activity and a 31-kDa C-terminal domain that contains the DNA polymerase active site. In this study, using solution conditions similar to those previously employed for the isolation of macromolecular complexes containing pol β and DNA ligase I (13), we found that both intact pol β and the 8-kDa N-terminal domain bind to Affi-Gel 10 beads with DNA ligase I as the covalently attached ligand, but were not retained by beads with BSA as the ligand (Fig. 1A). In contrast, no significant binding of the 31-kDa domain to either of the affinity beads was detected (Fig. 1A).

To further define the interaction between the 8-kDa domain and DNA ligase I, we examined binding of several altered versions of the 8-kDa domain to the DNA ligase-I-affinity beads. The structures of these altered versions of the 8-kDa domain with single amino acid changes closely resemble that of the wild type protein, as judged by circular dichroism (23). Changing a lysine residue at position 35, 60, 68, or 72 to alanine abolished the interaction between the 8-kDa domain and DNA ligase I. In addition, the substitution of glutamate with glutamine at position 71 and the substitution of lysine with alanine at position 84 also strongly reduced binding. An example of these experiments is shown in Fig. 1B. With the E71Q and K84A residues of the 8-kDa domain, specific binding to the DNA ligase-I-affinity beads was observed with higher protein concentrations of the 8-kDa domain (data not shown). These results suggest that the surface-exposed, charged residues known to be involved in the 8-kDa domain’s interaction with DNA and the dRP lyase activity (23) are also involved in binding to DNA ligase I.

DNA ligase I is composed of a C-terminal catalytic domain and an N-terminal domain that is dispensable for DNA ligase I catalytic activity, but is required for the enzyme’s function in vivo (23, 24). In experiments with in vitro translated DNA ligase I polypeptides, we observed specific binding of full-length DNA ligase I and a fragment corresponding to the N-terminal 118 amino acids of DNA ligase I to GST-pol β beads, but not to control GST beads (Fig. 1C). In contrast, a fragment corresponding to the C-terminal catalytic domain of DNA ligase I did not bind to the GST-pol β or GST beads (Fig. 1C). Thus, we conclude that the 8-kDa domain of pol β mediates the interaction with DNA ligase I and that pol β binds to the N-terminal domain of DNA ligase I. To further characterize the stable complexes formed between pol β and DNA ligase I, we performed the series of equilibrium ultracentrifugation studies described below.

Intact pol β—It is well known that pol β can self-associate under conditions of lower ionic strength, such as buffer with 150 mM KCl (24). Under the conditions used here, single species analysis of the data revealed the existence of higher oligomers. To resolve the actual species formed, we fit a variety of models describing self-associations such as nB ⇌ Bn or nB ⇌ 2Bn ⇌ B2n for n = 2, 3, or 4 and where B is the protein monomer. For some of the models, the quality of the fit was reasonable and the fit tended to improve with the complexity of the model. It was observed, however, that with increasing temperature, models that included higher oligomers fit the data better. These observations led to the interpretation that intact pol β undergoes indefinite self-association whereby oligomers of all sizes are formed. In particular, if the free energy of association is constant for the addition of each additional monomer to the oligomeric chain, the association is termed isodesmic. The corresponding mathematical model (Equation 1) was fitted to the collected data. The fit was excellent at all temperatures with an association constant in the range 8,800 to 10,100 M⁻¹. Fig. 2A shows a plot of Gibbs free energy of association versus temperature, where it is seen that the addition of each monomer to the oligomeric chain requires between −4.9 and −5.4 kcal/mol. The plots in Fig. 2B summarize the thermodynamic parameters of the association. There was a significant temperature effect: changes in both entropy and enthalpy decreased with temperature, and both are negative above 290 K. A possible interpretation scenario may be proposed here, based on the association strength variations with increased temperature; starting at low temperatures, the association strength increases, which means that higher oligomers will be forming at least up to

**Fig. 2.** Changes in thermodynamic parameters accompanying the indefinite isodesmic self-association of human pol β in solution relative to 0 °C. Association Gibbs free energy (A) and thermodynamic parameters (B) of pol β are shown. ΔHf,B are enthalpic changes, TΔSf,B are entropic changes, and ΔGf,B are changes in heat capacity. Note the different scale on the right vertical axis for entropy and heat capacity changes.
domains of pol β and DNA Ligase I

SCHEME 1. Diagram illustrating self-associative behavior of human DNA ligase I and DNA pol β. A, human DNA ligase I; B, DNA pol β. Protein-protein interaction regions in the 8-kDa and 31-kDa domains of pol β are illustrated. Two forms of pol β monomer are shown.

about 20 °C. Entropic changes are positive but decreasing with temperature. This is interpreted as an indication of changes in protein hydration upon self-association, since the association itself increases order and, therefore, decreases entropy. Above 18–20 °C, however, the association strength stays constant. The negative entropy change may be attributed to another effect such as a different structural form of the monomer, which could reflect, for example, a temperature-driven conformational change. This scenario is consistent with the pol β association with DNA ligase I (see below). Also, the specific heat capacity changes are negative and increase in magnitude with temperature. This is interpreted as an indication of changes in the standard thermodynamic parameters. The very large \( T \Delta S^0 \) term combined with the positive heat capacity change, \( \Delta C_p \), are consistent with an association that is affected through electrostatic type forces such as salt-bridge formations. The small positive entropy changes, however, indicate some water displacement from the hydration layer upon association. If the association is effected through salt-bridge formation, the small enthalpic increases along with the small but positive heat capacity changes are consistent with partially exposed salt groups whose burial upon association keeps thermal interactions weak.

Mixtures of DNA Ligase I and the 8-kDa Domain of pol β—Since the 8-kDa domain of pol β does not self-associate, whereas DNA ligase I forms a trimer, it was initially assumed that the DNA ligase I trimer will persist in the presence of the 8-kDa domain which may or may not attach itself to the DNA ligase I trimer. Therefore, a sequence of models was employed to fit the experimental data. These models were of the forms 3L + B \( \rightleftharpoons \) L\(_3\)B + B, 3L + B \( \rightleftharpoons \) L\(_3\)B, and 3L + 3B \( \rightleftharpoons \) L\(_3\)B\(_3\), where L represents DNA ligase I and B the 8-kDa domain. The quality of the fit favored the latter model, but due to the small size difference between L (102 kDa) and the BL complex (110 kDa) and the fact that the strong interaction resulted in very small amounts of unbound monomers for at least one of the loaded concentration ratios, the mathematical optimization problem turned out to be ill-conditioned. This was reflected in the high sensitivity of the optimization process to the initial parameter...
estimates and to inconsistent reference concentration ratios at the outcome. This problem was resolved by using the mass conservation principle (26). Since the mass of the solute can be measured accurately by integrating the zero-time absorbency scans, one or more of the concentrations at the reference radii can be expressed in terms of the remaining fitting parameters. Intensity scans are useful in this instance since the radial locations of the cell menisci and bottoms can be ascertained with greater accuracy than from absorbency scans. Here, one of the reference concentrations was solved in terms of the association constant and the other reference concentration. Thus, the final equilibrium model that was used for parameter estimation is mathematically expressed in Equation 3. The quality of fit was easily superior for 3:3 heterohexamer formation, and the analysis was completed using \( n = 3 \) at all temperatures. Values for the equilibrium constants were used to compute the Gibbs free energy change for heterohexamer formation as shown in Fig. 4A. It is seen that Gibbs free energy varies between \(-32\) kcal/mol and \(-35\) kcal/mol as the temperature increases to 32 °C. The energy for the attachment of each 8-kDa monomer to the DNA ligase I trimer may then be computed after the DNA ligase I trimerization energy is subtracted; the resulting values vary from \(-6.65\) kcal/mol to \(-6.85\) kcal/mol with increasing temperature. At 32 °C, this gives a \( K_a \) \( \sim 10^5 \) M\(^{-1}\) for binding of each 8-kDa monomer to each DNA ligase I binding site (Scheme 2).

The changes in standard thermodynamic parameters associated with this interaction are plotted in Fig. 4B. Since the thermodynamics shown in this plot include energies for both the DNA ligase I trimerization and the 8-kDa binding to the DNA ligase I, the difference between this and the DNA ligase I trimerization thermodynamics (Fig. 3B) may be attributed to the 8-kDa binding to the DNA ligase I. The comparison at physiological temperatures indicates that this last interaction results in an overall lower entropy system compared with the DNA ligase I trimer by itself. Therefore, the negative heat capacity change associated with the 8-kDa binding is more likely due to burial of active sites rather than hydrophobic effects. Water release into the bulk phase plays a more significant role at lower temperatures. The spatial placement of the three bound 8-kDa domain onto the DNA ligase I trimer cannot be accurately deduced from the above data, and Scheme 2 should be interpreted as one showing stoichiometry rather than spatial arrangement; an equally, if not more plausible scenario could have the 8-kDa domain of pol \( \beta \) binding on the inside surface of the DNA ligase I trimeric structure.
Mixture of DNA Ligase I and Intact pol β—Species analysis of the collected equilibrium data at lower temperatures failed to indicate the presence of species larger than 300 kDa, which corresponds to the DNA ligase I trimer. This suggested that interactions between pol β and DNA ligase I, if they occurred, would probably be limited to a one-to-one molecular species. Initially, the data were fit assuming models that included no heterogeneous complex formation, but allowed for the DNA ligase I trimer and various pol β oligomers. The quality of fits was poor and deteriorated at higher temperatures where it became clear that a species somewhat heavier than the DNA ligase I trimer was present near the cell bottom. Similar results were obtained with a model that assumed a single pol β molecule binding to the DNA ligase I trimer. When the 3:3 association model was included at higher temperatures, all fits were excellent and all statistical measures of the quality of fit were excellent. In contrast, below 18 °C the association constants for 3:3 complex formation dropped to zero. Further analysis revealed that the best fits were obtained when a 1:1 pol β-DNA ligase I complex was included in the model. The concern that such species would not be distinguishable among the various pol β oligomers was alleviated by the fact that the size of the 1:1 complex lies between the (pol β)₃ and (pol β)₄ oligomers and the method is sensitive enough to distinguish species whose molecular masses differ by more than 3 or 4%. With the 1:1 heterodimer as the model, all statistical measures of the quality of fit improved and were excellent. The above results are consistent with the interpretation that a stable hexamer forms at higher temperatures (>18 °C) while at lower temperatures, a single pol β molecule binds a single DNA ligase I molecule to form a stable heterodimer.

At lower temperatures, therefore, one-to-one pol β-DNA ligase I binding is the thermodynamically preferred association (Scheme 3). Below 18 °C the pol β-DNA ligase I binding is stronger than that of pol β-pol β self-association. Interestingly, intact pol β-DNA ligase I binding is weaker (ΔG° ≈ −5.4 to −6.3 kcal/mol) than binding between DNA ligase I and the 8-kDa domain (ΔG° ≈ −6.6 to −6.9 kcal/mol). Moreover, in the presence of the DNA ligase I trimer, binding of the pol β monomer appears to be altogether precluded (Scheme 3).

We propose that below approximately 18 °C a heterodimer of pol β monomer and DNA ligase I monomer forms, whereas above that temperature the association mode changes and three pol β monomers attach themselves to the DNA ligase I trimer to form the B₃L₃ complex (Scheme 3). The energy for B₃L₃ complex formation might be expected to be equal to 3 times that of BL formation, plus additional energy required for the DNA ligase I trimerization and any other binding within the B₃L₃ complex. To gain further insight into various bonds that form in the 3:3 complex, the free energy values for the BL heterodimer at temperatures up to 18 °C were multiplied by a factor of 3, and the resulting values were plotted together with the values obtained for the 3:3 complex at temperatures above 18 °C (Fig. 5). The shift between the two sets of data represents the additional energy discussed above; the magnitude of this shift is approximately −15 kcal/mol and is similar to the energy for the formation of the DNA ligase I trimer (−14 kcal/mol). From these observations, it appears that pol β binds to the DNA ligase I trimer at >18 °C, and this binding competes with the pol β self-association. The slight change in slope after 18 °C (Fig. 5) is an indication of a small amount of additional energy that is probably a reflection of entropic rearrangement within the complex. This is consistent with the pol β conformational change proposed in connection with pol β self-association (Scheme 1).

Further insight into the nature of the shift from BL to B₃L₃ formation may be gained by examining the corresponding changes in the standard thermodynamic parameters and in particular by examining the entropic changes accompanying the formation of the various complexes. At 18 °C, pick a representative temperature, the ΔS° change accompanying the B₃L₃ formation is about 0.35 kcal/mol, a large entropic increase in the system. The corresponding values for the BL formation is less than 0.01 kcal/mol, that of the B₈L₈ is about 0.09 kcal/mol, and that for the DNA ligase I trimerization about 0.07 kcal/mol. Based on these comparisons, the probable emerging scenario is that when a pol β monomer binds to a DNA ligase I monomer at lower temperatures, not much spatial rearrangement of atoms or of hydration takes place. At physiological temperatures, when the 8-kDa domain of pol β binds to the DNA ligase I trimer there is actually a decrease in entropy, which signifies increased system order through the formation
of the larger complexes which are electrostatically driven and possibly through additional hydration of the newly formed complex. The additional entropic change, however, accompanying the formation of the pol β-DNA ligase I \( \text{B}_{x}\text{m}_{y} \) heterodimer is probably an indication of a hydrophobic reaction whereby a large number of complexed water molecules are displaced; that displacement becomes favored only when the temperature is raised to about 18 °C. If the pol β conformational change at around 18 °C as suggested above is true, then that change may be exposing pol β hydrophobic domains.

**Mixture of 31-kDa C-terminal Domain with DNA Ligase I**—Since the binding site for the DNA ligase I lies on the 8-kDa domain of pol β, it should be expected that the remaining protein domain would not have affinity for DNA ligase I. This was indeed the case. Consistently excellent mathematical fits were obtained when no heterogeneous associations were included in the mathematical model. This is consistent with the complete absence of interprotein binding by the 31-kDa domain and with localization of DNA ligase I binding sites in pol β, as already discussed above (data not shown).

**AP Endonuclease and AP Endonuclease-pol β Mixture**—After equilibrium was established at a given temperature, the data were analyzed while equilibrium was being attained at the next higher temperature. Therefore, these experiments were discontinued at 18 °C as it became apparent that AP endonuclease does not self-associate and does not associate with pol β. Species analysis of the AP endonuclease and the AP endonuclease-pol β mixture data clearly showed that AP endonuclease remained a monomer while in the mixture and that no species could be detected with a molecular mass of \( \sim 77 \) kDa or its integer multiples, which would be the mass of a 1:1 or higher complex (data not shown). These data are consistent with earlier experiments that failed to detect an association between pol β and AP endonuclease (13, 14).

**DISCUSSION**

Repair of a uracil-containing DNA substrate by the “short patch” or simple BER pathway can be catalyzed by a protein complex isolated from bovine testis nuclear extract (13). Two of the components of this complex, pol β and DNA ligase I, probably participate in the repair of other base lesions, in particular alkylated bases and AP sites. Interactions between pol β and AP endonuclease (14) and between pol β and DNA ligase I (13) may underlie the stability and activity of BER complexes. Using affinity chromatography, we mapped the regions of pol β and DNA ligase I that are required for the stable interaction of these enzymes. Interestingly, this interaction occurs between the non-catalytic N-terminal domain of DNA ligase I and the 8-kDa domain of pol β that binds to gapped DNA and possesses dRP lyase activity (7, 11, 12, 27).

A series of equilibrium experiments was conducted in the analytical ultracentrifuge to further investigate associative behavior in solution of human AP endonuclease, pol β, and DNA ligase I. AP endonuclease was found to neither self-associate nor associate with pol β. In contrast, pol β was found to be a dynamic molecule that both self-associates and associates with DNA ligase I. Intact pol β undergoes indefinite isodesmic self-association, forming oligomers of many sizes; the 8-kDa N-terminal domain did not self-associate, whereas the 31-kDa C-terminal domain self-associates indefinitely. We interpret these data to mean that intact pol β possesses two binding sites that can mediate pol β self-association, that these binding sites are energetically identical, and that the sites are located in the 31-kDa C-terminal domain of pol β, rather than in the 8-kDa N-terminal domain (Scheme 1). These conclusions are consistent with a previous study reporting the self-association of rat pol β at higher protein concentrations (>10 μM) and low ionic strength (24).

DNA ligase I formed a stable trimer in the low ionic strength buffer used in the equilibrium ultracentrifugation experiments. This could be explained by the presence of two binding sites on the DNA ligase I monomer for binding to itself. The fact that the trimer is a stable complex and that no additional monomers attach to it suggests non-symmetrical positioning of the binding sites on the molecule’s surface, as illustrated in Scheme 1. In agreement with the domain mapping studies (Fig. 1), both intact pol β and its 8-kDa domain formed stable complexes with DNA ligase I in solution. The 31-kDa domain of pol β appears to influence the interprotein association, but itself does not possess a DNA ligase I binding site. Three 8-kDa domain molecules associate with the DNA ligase I trimer, to form a stable heterohexamer at all temperatures (Scheme 2). The association of the 8-kDa domain with DNA ligase I was not accompanied by an increase in entropy and is consistent with binding through electrostatic forces. In support of this notion, changing any one of several basic amino acid residues on the surface of the 8-kDa domain to a non-polar residue inactivated DNA ligase I binding (Fig. 1B).

The interaction of the intact pol β molecule with DNA ligase I depended critically on the temperature of the solution. At lower temperatures, we observed a 1:1 interprotein association only, while above about 18 °C three pol β molecules bound to three DNA ligase I molecules forming a stable heterohexamer (Scheme 3). This shift in association mode for binding above 18 °C was accompanied by an average increase in entropy, consistent with water dispersion upon hydrophobic interactions. The implication of this result is that a conformational change in the 31-kDa domain of pol β is temperature-mediated. This conformational change allows DNA ligase I trimerization to dominate the overall association mode of the system and allows the 8-kDa domain in intact pol β to bind to the DNA ligase I trimer. This is illustrated in Scheme 3. The proposed conformational change in pol β has implications for regulating assembly of DNA ligase I into the stable trimer. At lower temperature, pol β does not bind stably to the DNA ligase I trimer. Instead, only the 1:1 pol β/DNA ligase I interprotein complex is formed.

To complete short patch BER, a single nucleotide is inserted, the 5’ dRP group is removed and the resultant nick is ligated.
Since these first two steps are catalyzed by pol β and the final one by DNA ligase I, it will be of interest to determine how the formation of a stable complex, either the 1:1 or 3:3, heterodimer or heterohexameric, respectively, between these enzymes influences these reactions. In this regard, it is noteworthy that DNA ligase I, which initiates the ligation reaction by transferring an AMP moiety from itself to the phosphate at the 5' terminus, binds to the domain of pol β that interacts with and processes the 5' terminus in the steps prior to ligation.

In addition to participating in BER, DNA ligase I is the enzyme responsible for joining Okazaki fragments during DNA replication. In this process, DNA ligase I binds to proliferating cell nuclear antigen, an interaction that is also mediated by the non-catalytic N-terminal domain of DNA ligase I (15). Thus, the demonstration here that DNA ligase I forms a stable trimer in solution is particularly intriguing since proliferating cell nuclear antigen functions as a homotrimer that encircles DNA and tethers interacting proteins such as DNA polymerases and DNA ligase I to their DNA substrate. This raises the possibility that structurally similar multiprotein complexes may be involved in the gap-filling and ligation steps of lagging strand DNA replication and short patch BER. Further studies of these protein complexes and their interaction with DNA substrates should be informative.

**APPENDIX**

The measuring optical system in the analytical ultracentrifuge is the dual beam spectrophotometer which measures the transmitted light through the reference and the sample sectors of each loaded cell. Normally, the reference sector contains a buffer and the sample sector contains the solution, in the same buffer, of the macromolecules under investigation. The transmitted light intensity data of the reference sector, \( I_0 \), and of the sample sector, \( I_r \), are customarily converted into light absorbances, \( A \), using the simple relation, \( A = \log_{10} (I_0/I_r) \). It is these transformed data that are fit to assumed concentration distribution models to obtain estimations of parameters such as molecular weights and association constants. This has been the established practice in the field due to the simple fact that absorbance is directly proportional to concentration whose distribution in the centrifugal field contains the information sought. Such apparently innocent transformation, however, has significance when viewed from the signal processing perspective. As with every measurement, the collected data are contaminated with random noise whose sources are rather diverse, including the noise in the electronic circuits, which contaminates both the light source and the sensor, the inaccuracies in the exact measurement position and, in all likelihood, the noise associated with the practical impossibility of obtaining absolutely clean quartz windows throughout both the reference and the sample window sectors. All these noise sources add stochastic noise to the data, and the noise from each source may even have different statistical characteristics. Some of these sources will add Gaussian noise, but other sources may result in different noise probability distributions. It is well known, however, from the central limit theorem (29) that, when a number of stochastic processes with differing statistical distributions are added together, the character of the resulting signal tends to be Gaussian or normally distributed. Therefore, it is expected that the raw collected data by the optical system in the centrifuge will be contaminated by Gaussian noise.

When scanning the cells in the XL-A ultracentrifuge, it is normal practice to scan each radial position a number of times and average the results. This reduces the noise in the recorded data. Again, the customary practice is to request the final recorded data in the form of absorbances to be analyzed later. The transformation of the intensity data into absorbances is a non-linear transformation and, as such, the noise characteristics are not preserved (29). At this point, the question of curve-fitting method for noisy data arises. The final decision for the most probable behavior of the solute in the particular solution is based on the quality of the fit as described by various statistical measures. The method used most often for curve fitting is that of least squares (30), which seeks estimates of the unknown model parameters so that the sum of squares of the deviations of the actual data from the model curve is minimized. In addition, it is known that optimal least-squares estimation is achieved if the data are weighted by the inverse of the variance. Hence, weighted least squares has been the method of choice. This, however, requires an iterative scheme as the optimal weights are not known at the outset. It is known from estimation theory (31) that the above method gives unbiased estimations of the unknown parameters under one of the following two conditions; either the noise probability distribution is Gaussian, or else no statistical information about the characteristics of the noise is available.

The claim about the nature of the noise in intensity and absorbance data was born out of actual data collected from the centrifuge and fitted by Gaussian distribution functions (32). It was found that the noise in the intensity data is indeed closely Gaussian with standard deviation that increases linearly with signal strength and that the absorbance data noise deviates in a statistically significant manner from being Gaussian.

Least-squares method or any other method chosen to perform curve-fitting is effectively acting as a noise filter to eliminate measurement noise in order to extract the useful information contained in the signal. It has been proven that, if the noise probability distribution is known, the method referred to as the maximum likelihood estimation (33) results in the absolutely optimal de-noising of the signal and in unbiased parameter estimation. In addition, if the noise is Gaussian, the least-squares method and maximum likelihood are exactly equivalent. For the case of absorbance data, however, it was shown previously that the resulting probability distribution of the non-linear transformation of Gaussian intensity data results in a new distribution that is neither Gaussian and not even symmetric. Therefore, using the least-squares estimation method would result in statistically biased parameter estimators. The mathematical details connected to the above discussion may be found in a previous publication (32). It is, therefore, proposed that the intensity data be used directly in a least-squares scheme. One of the methods proposed there requires that the reference data be first smoothed using a cubic spline function series, which can be optimally fit by the weighted least-squares method. This will result in an algebraic description of the reference sector data \( I_r(r) \). The sample side intensity data can subsequently be fit with the function \( I_s(r) = I_r(r) 10^{C(r,a)} \) where \( C(r,a) \) is the total concentration distribution of the assumed interaction model for the macromolecules under investigation; here, \( r \) is the radial position and \( a \) is the vector of parameters whose values are to be estimated by the fitting.

For the purpose of demonstrating the difference between fitting absorbance or intensity data, we performed simulations of a variety of systems and juxtaposed the results. In these simulations, a system was assumed and a mathematical model for the concentration distribution along the radius was written for a given set of system parameters such as molecular masses and association constants. Then, noise with characteristics similar to those observed in the XL-A was added to those mathematical models to simulate collected data in intensity form which were also converted into absorbances. Finally, the method of weighted least squares was applied with both the traditional absorbance data and with the intensity data to
investigate how well the two approaches recover the initial system parameter values.

The following systems were investigated.

**Solutions of Simple Monomers**—Molecular masses were recovered from simulated data with an error of the order of 3–5% from absorbance data, while the error with intensity data was consistently under 0.5%. This is significant, especially when the analysis is required to distinguish between species which are similar in size. Absorbance data would require at least a 10% difference in size, while intensity could distinguish sizes differing by as little as 1–2%.

**Monomer-Dimer Association**—Here, the association constant was the main parameter of interest. The estimation error here depended on the strength of association. For the strongest association, the error when using absorbance data was of the order of 7–8%, and, for the weakest association, it was of the order of 5–6%. In contrast, the error when using the intensity data directly always stayed below 2–3%.

**Two Different Proteins Forming a Heterodimer**—The association constants were recovered with an error of about 5% when using absorbances, while the error was reduced to 2–3% with intensity data fitting.

Thus, the preformed simulations clearly demonstrate the advantage of fitting transmitted light intensity data directly. There are additional advantages in using intensity data that were discovered in the process of the investigation. First, the smoothing of the reference data using the spline functions is virtually "cleaning" the reference sector window of particles that might adhere to the quartz window and cause unwanted signal fluctuations in that sector. Such fluctuations are often clearly seen in intensity data as local aberrations from the expected smooth sensitivity profile of the sensor photomultiplier cathode. The transformation into absorbances hides these fluctuations, which are often a source of a significant portion of the error. Another advantage is that with intensity data it is much easier to locate both the meniscus and the bottom of the cell in order to eliminate data that are contaminated by edge effects or by the region of non-linear sensitivity of the sensors.

In the present investigation, both absorbance and intensity fitting were used throughout the study but the final results presented are those obtained using intensity. For the purposes of supporting the above arguments, Fig. A1 (top panel) shows an example of intensity data fitting of the pol β isodemic association. It includes a representative example of direct intensity data fit (top panel) and the corresponding fitting errors (bottom panel). This figure is provided for the benefit of investigators who are accustomed to viewing absorbance data and have a well developed intuition for that presentation format. Fig. A2 shows a plot of the Gibbs free energy of association, obtained using Equation A1, superimposing the results of the complete pol β study using both absorbance and intensity data. Also shown are the fits obtained using the thermodynamic equations (Equations A2–A4 or A5 and A6–A8) to obtain the standard thermodynamic parameter changes. The differences are striking but consistent with the theory discussed above since the standard deviation of the estimation error will be minimal when using intensities as compared with using absorbencies. In addition, with such pronounced differences in the values of the standard thermodynamic parameter changes, the possibility exists of differing qualitative interpretations of the thermodynamic nature of interactions. This makes another strong argument for analyzing transmitted light intensity data directly.

**Fig. A1, Fitting transmitted light data directly.** Intensity data fit for DNA ligase I trimerization at 10 °C (top panel). Upper two curves represent reference sector data, and the lower two curves represent sample sector data. **Bottom panel** shows sample data fitting errors for the fit shown in top panel. Correspondences are established by the data point symbols used. For this particular data set, which is one of the "cleanest" in the whole investigation, there was 4% difference in estimated value for the association constant between absorbance and intensity fitting.

**Fig. A2.** Gibbs free energy of association as a function of temperature using absorbance data fitting (■) and two variants of direct intensity data fitting (□ and △). Points were computed from the equilibrium constants using Equation A1 and the resulting set of points was fit using Equations A2–A4 or A5 and A6–A8 to obtain the standard thermodynamic parameter changes for association. Note the clear superiority of the fits on the data obtained using intensity fits like those in Fig. A1.
Thermodynamic Analysis Methods

Thermodynamic analysis of the association can be performed based on the relationship between the association constant $K_{ab}$ for the interaction $a + b \Leftrightarrow ab$ and the Gibbs free energy change, $\Delta G^0(T)$.

$$\Delta G^0(T) = RT\ln(K_{ab}) \quad \text{(Eq. A1)}$$

The classical thermodynamic definition of $\Delta G^0(T)$ and the associated parameters is shown by Equations A2–A4.

$$\Delta G^0(T) = \Delta H^0(T) - T\Delta S^0(T) \quad \text{(Eq. A2)}$$

$$\Delta H^0(T) = \Delta H^0(T_0) + \Delta C_p^0(T - T_0) \quad \text{(Eq. A3)}$$

$$\Delta S^0(T) = \Delta S^0(T_0) + \int_{T_0}^{T} \frac{\Delta C_p^0(T)}{T} dT = \Delta S^0(T_0) + \Delta C_p^0 \ln(T/T_0) \quad \text{(Eq. A4)}$$

$\Delta H^0(T)$ is the enthalpy change for the association and represents thermal energy changes taking place due to binding, $\Delta S^0(T)$ is the entropic change taking place due to positional and conformational changes of the associating subunits, $\Delta H^0(T_0)$ is the standard enthalpy change at reference temperature $T_0$, $\Delta C_p^0$ is here assumed to be temperature invariant; inclusion of its temperature dependence in these functions does not improve the quality of the fits. The reference temperature is arbitrary but 0°C, 37°C, or a temperature in the middle of the range examined and for which data are available are usual such choices. The latter is mathematically more desirable for enhancing the quality of curve fitting, but other reference temperatures may be more desirable on physical grounds. The thermodynamic computations begin by calculating the $\Delta G^0(T)$ values at the temperatures for which meaningful data have been collected. These data are fit using Equations A2–A4 as the mode with the least-squares method to obtain estimates of the values of the fitting parameters, which are the standard enthalpy, entropy, and heat capacity changes with respect to the chosen reference temperature. A problem with the assumption of temperature invariant heat capacity change is that it violates the requirement that the slope of $\Delta G^0(T)$ at $T = 0$ K should be zero. This is overcome by the thermodynamic analysis proposed by Chun (34) in connection to the Plank-Benziinger thermal work function. There, the $\Delta G^0(T)$ values are fit for the parameters $\alpha$, $\beta$, and $\gamma$ of the Gibbs polynomial function whose slope at 0 K is always zero.

$$\Delta G^0(T) = \alpha + \beta T^2 + \gamma T^3 \quad \text{(Eq. A5)}$$

The thermodynamic parameters $\Delta H^0(T)$, $\Delta S^0(T)$, and $\Delta C_p^0$ are then given by Equations A6–A8.

$$\Delta H^0(T) = -\alpha - \beta T^2 - 2\gamma T^3 \quad \text{(Eq. A6)}$$

$$T\Delta S^0(T) = -2\beta T^2 - 3\gamma T^3 \quad \text{(Eq. A7)}$$

This type of analysis allows easy evaluation of the temperature invariant enthalpy, $\Delta H^0(0) = \alpha$, which represents the chemical bond energy at 0 K, a fundamental quantity.

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