

Binding of Nucleotides by T4 DNA Ligase and T4 RNA Ligase: Optical Absorbance and Fluorescence Studies

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ABSTRACT The interaction of nucleotides with T4 DNA and RNA ligases has been characterized using ultraviolet visible (UV-VIS) absorbance and fluorescence spectroscopy. Both enzymes bind nucleotides with the K_d between 0.1 and 20 μ M. Nucleotide binding results in a decrease of absorbance at 260 nm due to π -stacking with an aromatic residue, possibly phenylalanine, and causes red-shifting of the absorbance maximum due to hydrogen bonding with the exocyclic amino group. T4 DNA ligase is shown to have, besides the catalytic ATP binding site, another noncovalent nucleotide binding site. ATP bound there alters the π -stacking of the nucleotide in the catalytic site, increasing its optical extinction. The K_d for the noncovalent site is $\sim$ 1000-fold higher than for the catalytic site. Nucleotides quench the protein fluorescence showing that a tryptophan residue is located in the active site of the ligase. The decrease of absorbance around 298 nm suggests that the hydrogen bonding interactions of this tryptophan residue are weakened in the ligase-nucleotide complex. The excitation/emission properties of T4 RNA ligase indicate that its ATP binding pocket is in contact with solvent, which is excluded upon binding of the nucleotide. Overall, the spectroscopic analysis reveals important similarities between T4 ligases and related nucleotidyltransferases, despite the low sequence similarity.

INTRODUCTION

DNA ligase and RNA ligase from the bacteriophage T4 catalyze the formation of the phosphodiester bond between two molecules of nucleic acid. Several conserved sequence elements indicate that these enzymes belong to a superfamily of nucleotidyltransferases containing DNA, RNA ligases, and mRNA capping enzymes (Shuman and Schwer, 1995). Enzymic catalysis by the members of this superfamily proceeds in several steps and requires the presence of a nucleotide coenzyme/cosubstrate and a divalent metal cofactor (Sokolov, 1976; Shatkin, 1976).

In the first step of the catalytic cycle, nucleotidyltransferases form a covalent intermediate between the α -phosphate moiety of the nucleotide and the conserved lysine residue. Nucleotidyl transfer is reversible and in case of ATP- or GTP-dependent enzymes leads to the release of inorganic pyrophosphate. Although the metal cofactor is required for catalysis, several enzymes are known to bind nucleotide triphosphate in its absence (Hakansson et al., 1997; Subramanya et al., 1996).

X-ray diffraction analysis of several nucleotidyltransferases from this superfamily has been reported previously (Hakansson et al., 1997; Subramanya et al., 1996; Lee et al., 2000; Odell et al., 2000). Despite the low sequence homology of these enzymes, the topography of the nucleotide-binding sites showed considerable similarities. In all cases, the purine ring of the nucleotide is buried in a hydrophobic pocket of the protein and is stabilized by hydrogen bonding

with the exocyclic amino group, the ribose moiety, and the α -phosphate. For example, in the case of T7 DNA ligase, the adenine chromophore is inserted between the aliphatic side chains of Ile 220 and Lys 34 from one side and Tyr 149 from the other side. This Tyr ring is oriented parallel with respect to the adenine ring (ring-ring distance 4 Å) (Subramanya et al., 1996). In case of the mRNA capping enzyme from *Chlorella* virus PBCV-1, the guanine ring is positioned between residues Ile 216, Lys 82, and Phe 146 (stacking distance 3.7 Å) (Hakansson et al., 1997).

The T4 DNA and RNA ligases have not been crystallized, and hence relatively little is known about the topography of their ATP binding sites. Sequence alignment between the members of the superfamily indicated six conserved motifs, two of which (motifs IIIa and IV) could participate in hydrophobic interactions with the adenine chromophore (Subramanya et al., 1996; Timson et al., 2000). Accordingly, the adenine ring of the ATP bound to T4 DNA ligase is inserted between the Ile 346 (motif IV) and Trp 282 (motif IIIa). For T4 RNA ligase the alignment is less conclusive, indicating possible hydrophobic interactions with Tyr 229 (motif IV).

Optical spectroscopy is a powerful technique to study enzyme-substrate interactions. Unfortunately, these days it is rarely used, in fact, being superseded by x-ray crystallography. Even though one cannot solve the three-dimensional (3D) structure of a protein using optical spectroscopy, in many cases it is possible to zoom directly into the enzyme active site, making predictions on its composition and, e.g., solvent accessibility, and H-bonding interactions as, for example, illustrated in this paper. A great advantage of (optical) spectroscopy is its sensitivity combined with the possibility to detect structural changes occurring during catalysis. The combination of x-ray crystallography and (optical) spectroscopy constitutes the most promising ap-

Received for publication 4 June 2001 and in final form 31 August 2001.

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0006-3495/01/12/3545/15 \$2.00

TABLE 1 Nucleotide binding constants for T4 DNA ligase and T4 RNA ligase and the values of molar extinction coefficients at 260 nm

	K_1 (μM)				MgATP (μM)			MgATP ($\text{mM}^{-1} \text{cm}^{-1}$)	
	MgdATP	MgdCTP	MgAMP	ATP, no Mg	K_1	K_2	K_3	ϵ_b	ϵ_{b1}
T4 DNA ligase	$7.3 \pm 1.8^*$	$14.2 \pm 2.9^*$	$15 \pm 8^{\dagger}$	$<2^{\dagger}$	<0.15	100–250	$37.2 \pm 0.2^*$	12 ± 0.16	15.2 ± 0.3
T4 RNA ligase	$5 \pm 3^{\dagger}$	No binding [†]	$20 \pm 5^{\dagger}$	$<2^{\dagger}$				13.5 ± 0.2	ND

ND, not determined. Note that $[E]_{\text{active}}/[E]_{\text{total}} = 0.7 \pm 0.1$.

*From the pre-steady-state kinetic analysis (in preparation).

[†]Determined from equilibrium binding experiments monitoring the fluorescence emission at 340 nm.

proach to unravel the very details of the relation between enzyme structure and function.

It is known that the stacking interactions between two aromatic systems lead to a shift and a decrease of the intensity of the $\pi^* \leftarrow \pi$ transition bands for both chromophores. This so-called optical hypochromism is frequently observed in studies with nucleotides, nucleic acids, and proteins. For example, the absorbance of double-stranded DNA (dsDNA) at 260 nm increases up to 70% upon degradation to nucleosides or mononucleotides (Michelson, 1963). Optical hypochromicity of ATP bound to adenylate kinase and G-actin from rabbit muscle, the rec A protein from *Escherichia coli*, or the kinesin-related ncd motor domain protein from *Drosophila melanogaster* has been reported previously (Kachurin et al., 1990; Noda, 1973; Shimizu and Morii, 1998; West, 1970). In the latter enzyme, the adenine ring is positioned parallel to Tyr442, with a ring-ring distance of 4.3 Å. Optical difference spectroscopy revealed a 30% decrease of the 260-nm absorbance of tightly bound ADP.

Binding of substrate to an enzyme often leads to a change of the microenvironment of the aromatic residues of the protein, yielding characteristic difference spectra, which carry information on the nature of enzyme-substrate interactions. Interactions of proteins with sugars, nucleotides, DNA, and metal ions, including formation of charge-transfer complexes, have been previously characterized (for review see Demchenko, 1986).

In the present work we report optical effects observed upon binding of ATP, AMP, dATP, and dCTP to T4 ligases. The origin of the optical effects is discussed in relation to the known 3D structures of related nucleotidyltransferases.

MATERIALS AND METHODS

Enzymes and nucleotides

T4 DNA ligase and RNA ligase were purchased from MBI Fermentas (Vilnius, Lithuania). The proteins were essentially pure as judged by SDS-polyacrylamide gel electrophoresis; concentrations of the enzyme stock solutions were determined using the BCA protein determination kit (Pierce, Rockford, IL). All nucleotides were obtained as crystalline sodium salts from Amersham Pharmacia Biotech (Uppsala, Sweden).

T4 DNA ligase activity assay

The activity of T4 DNA ligase was measured at +20°C in a 15- μl reaction mixture containing 66 mM Tris-HCl, pH 7.8, 5 mM MgCl_2 , 1 mM dithiothreitol, 0.05 mg/ml bovine serum albumin (BSA), 1 mM ATP, and 1 μM of nicked dsDNA, which was prepared by annealing the Cy5-labeled 24-mer adjacent to the 5'-phosphorylated 24-mer on the complementary 72-mer template (sequences available upon request). T4 DNA ligase was added to a final concentration of 0.02 $\mu\text{g/ml}$ (0.36 nM). Aliquots of 0.5 μl were withdrawn in the course of time and mixed with 10 μl of the stop solution containing 10 mM NaOH, 10 mM EDTA, pH 9.5, and 5 mg/ml Blue Dextran 2000 in formamide. The samples were separated in polyacrylamide gels and analyzed on the ALF Express DNA sequencer (Amersham Pharmacia Biotech). The percentage of formed product in each sample was determined by integration of the peaks corresponding to the starting material, i.e., the Cy5-labeled 24-mer and the 48-mer ligation product.

Optics and experimental setup

Ultraviolet (UV) spectroscopy was performed at $20 \pm 1^\circ\text{C}$ using a HP8353 UV-VIS spectrophotometer (Hewlett Packard, Palo Alto, CA). Electronic absorbance spectra were acquired with 3-s integration time to enhance the signal-to-noise ratio. Fluorescence measurements were performed on a Shimadzu RF 5001PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). All spectra, unless specified otherwise, were recorded in 66 mM Tris-HCl, pH 7.8, 5 mM MgCl_2 , and 0.05 mg/ml BSA. Amounts of T4 DNA (RNA) ligase, BSA, and nucleotides (ATP, AMP, dATP, and dCTP) used are given in the figure legends. For fluorescence measurements of the equilibrium binding of nucleotides to the T4 DNA and RNA ligases (see Table 1), small aliquots of nucleotide (1–4 μl) were added to the ligase solution (initial volume 270 μl , $10 \pm 2 \mu\text{M}$ enzyme). After each addition the reaction mixture was incubated for 1 min and the emission spectrum was recorded. The emission of protein in the absence of the nucleotide was taken as 100%. Binding data were corrected for dilution of the protein and for inner filter quenching of the protein fluorescence by the nucleotide. For the equilibrium binding of ATP to T4 DNA ligase studied by difference UV-VIS absorbance spectroscopy a more elaborate procedure was followed. Because a high accuracy of measurements is essential for a reliable interpretation of the difference spectra (Demchenko, 1986), we detail below our procedure for the preparation of samples and acquisition of the optical spectra.

Preparation of the cuvette

A 2-mm-pathway, 10- μl -volume airtight quartz cuvette (Hellma, Müllheim, Germany) was used for the optical absorbance measurements. Before the acquisition of each spectrum, the cuvette was washed with chromic acid and deionized water and dried under airflow delivered from a compressed air line filtered through a 0.2- μm gas filter. This treatment gave reproducible values for the water spectrum with a fluctuation in the 220–350-nm range of $\pm 1 \times 10^{-4}$ AU.

Preparation of the nucleotide and ligase stock dilutions

Nucleotide stocks (0.1–2 mM) were prepared in 66 mM Tris-HCl buffer, pH 7.8; their concentration was determined optically. The ligase stock was diluted in Tris-HCl buffer resulting in final enzyme concentrations of 50–60 μ M, 66 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 1 mM dithiothreitol, and 0.05 mg/ml BSA. For the binding reaction, 15–20 μ l of diluted ligase was mixed with 1.5–2 μ l of the nucleotide solution in a 500- μ l airtight PCR Eppendorf tube. As a reference, the ligase solution in the absence of the nucleotide was used. The dilution coefficients were determined by weighing the tube on a Mettler M3 micro-analytical balance (Mettler, Toledo, OH). Before addition of the reaction components, the outside of the tube was coated with an anti-static Lubsech Teflon spray (Siceront KF, Lyon, France), subsequently dried in an oven at 60°C for 30 min, and inserted in a home-made aluminum foil holster to avoid accumulation of static electricity on the tube during operation, which otherwise would interfere with weighing. While handling the tubes, anti-static gloves were used. This procedure yielded 2 μ g (~2 nl) precision or reproducibility of the measured values. The densities of deionized water, ligase-reaction buffers, and storage buffers were measured with a DMA 48 densitometer (Anton Paar K.G., Graz, Austria).

Recording of the optical absorbance spectra

The mixture of ligase with the nucleotide was incubated at room temperature for 15 min and rapidly transferred to the airtight cuvette, and the optical spectrum [enzyme+nucleotide, S_0] was recorded. The reference spectrum [free enzyme, S_{ref}] of ligase in the absence of nucleotide was obtained in the same manner. The difference spectrum S_n , enzyme-bound nucleotide, was calculated by subtracting the reference spectrum from the spectrum of the ligase nucleotide mixture appropriately correcting for the dilution factors of the ligase sample and the reference mixtures as determined by weighing. All S_n spectra were normalized to 1 M nucleotide concentration and 1 cm path length.

On the stability of DNA and RNA ligases at ambient temperature

It is known that preparations of T4 DNA (RNA) ligase gradually lose their activity when incubated at temperatures above 0°C (Silber et al., 1972; Weiss et al., 1968a). In this work, optical measurements have been performed at $+20 \pm 1^\circ\text{C}$ occasionally for a period up to 30 min. To minimize irreversible inactivation of the enzymes, BSA has been added to a concentration of 0.05 mg/ml. In the absence of BSA the activity of diluted T4 DNA ligase (0.33 μ g/ml, 5.9 nM) decreases by 30% in the first hour of incubation at $+20^\circ\text{C}$, whereas in the presence of BSA no significant decrease of activity was observed (Fig. 1).

RESULTS

Nucleotide binding model

Nucleotidyl transfer catalyzed by DNA and RNA ligases is conventionally described in terms of a bimolecular collision $E + \text{ATP} = E\text{-AMP} + \text{PP}_i$ (Weiss et al., 1968b; Modrich and Lehman, 1973; Doherty et al., 1996; Arabshahi and Frey, 1999; Timson et al., 2000). For the quantitative description of the reaction, however, a noncovalent enzyme-nucleotide intermediate E-ATP had to be invoked (Zagrebel'nyi et al., 1984). Moreover, we had to consider the possibility for the nucleotide to bind to a second site on

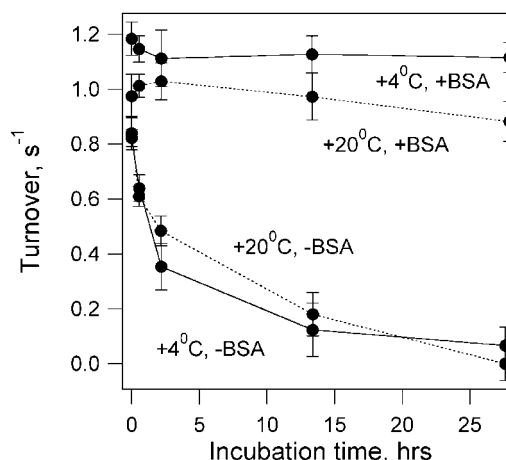
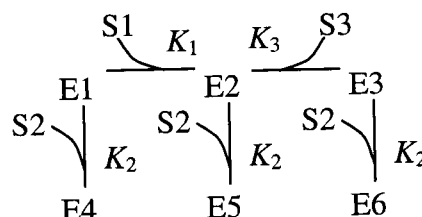


FIGURE 1 Stability of T4 DNA ligase. T4 DNA ligase was incubated both in the presence and the absence of 0.05 mg/ml BSA, at $+4^\circ\text{C}$ and $+20^\circ\text{C}$. The enzyme was diluted in the reaction buffer to a final concentration of 1 U/ml (~6 nM). The activity was measured after addition of 1 μ l of the T4 DNA ligase incubation mixture to 14 μ l of standard assay containing 1 μ M of the nicked dsDNA. Aliquots of 0.5 μ l were withdrawn in the course of time and processed as described in Materials and Methods. Each turnover number was determined from the linear part of the product formation curve. The zero point was obtained within 5 min after dilution.

the enzyme. To describe this binding we chose the following two-site model (Scheme I):



where S1 and S2 represent the nucleotide coenzyme (MgATP or MgGTP), S3 represents pyrophosphate, K_1 and K_3 are the observed dissociation constants for ATP and pyrophosphate at a given Mg^{2+} concentration, and K_2 is the dissociation constant of the nucleotide in the second binding site; E1 represents free enzyme, E2 enzyme:nucleotide, E3 enzyme-adenylylate, E4 enzyme:nucleotide, E5 enzyme:(nucleotide)₂, and E6 enzyme-adenylylate:nucleotide. Scheme I converges to the one-site model (Zagrebel'nyi et al., 1984), when the components 4–6 are excluded. Scheme I describes a one-site competitive binding model (if the components E5 and E6 are excluded) or a two-site binding model (when all components are considered).

Binding of ATP to T4 DNA ligase

The formation of the ligase-nucleotide complexes is accompanied by a decrease of the optical absorbance at 260 (Fig.

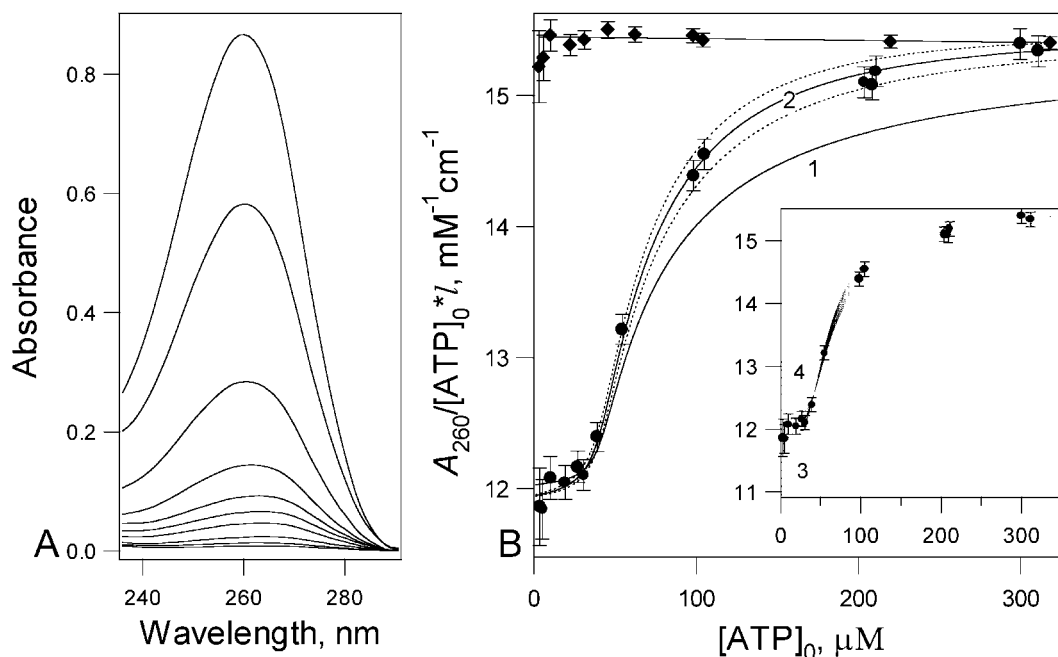


FIGURE 2 Equilibrium binding of ATP to T4 DNA ligase. (A) Difference spectra [enzyme+ATP minus free enzyme]. Concentrations of ATP (from bottom to top spectrum) are as follows: 3.6, 5.39, 10.55, 19.52, 27.12, 39.12, 54.67, 98.4, 203.5, and 299.78 μM and 59 μM of T4 DNA ligase. (B) Molar extinction of ATP at 260 nm as a function of its concentration: $\blacklozenge$, ATP in the absence of enzyme; $\bullet$, in the presence of T4 DNA ligase. Binding trace 1 is calculated using Eq. 1 for the one-site model (Scheme 1); components E4–E6 are excluded. Binding trace 2 is calculated for the two-site model with $K_2 = 150 \mu\text{M}$. Upper dotted trace is for the two-site model, $K_2 = 100 \mu\text{M}$; lower trace, 250 μM . Other fitting parameters are shown in Table 1. The binding traces in the inset of B are shown to distinguish between the absorbance of the enzyme-nucleotide complexes E2 and E3. Trace 3 is obtained by taking the molar extinction coefficient for E2 as 8.6 vs. 15.4 $\text{mM}^{-1} \text{cm}^{-1}$ for E3. For trace 4 the values are opposite. Intermediate traces are calculated by taking the sum of the extinction values constant a 24 $\text{mM}^{-1} \text{cm}^{-1}$.

2) and around 298 nm (Fig. 3). Characteristic difference spectra are shown in Figs. 4 and 5.

The binding curves in Fig. 2 were calculated using Eq. 1, which is the derivation of the Lambert-Beer law:

$$\frac{1}{l} \frac{A_{260}}{[\text{ATP}]_0} = \epsilon_f^{260} - \frac{[\text{E2}]_{\text{eq}} + [\text{E3}]_{\text{eq}} - [\text{E3}]_0}{[\text{ATP}]_0} (\epsilon_f^{260} - \epsilon_b^{260}) - \frac{[\text{E5}]_{\text{eq}} + [\text{E6}]_{\text{eq}}}{[\text{ATP}]_0} (\epsilon_f^{260} - \epsilon_{b1}^{260}), \quad (1)$$

where A_{260} is the difference absorbance at 260 nm minus 320 nm [enzyme+nucleotide minus free enzyme]; $[\text{ATP}]_0$ is the total concentration of nucleotide in the reaction mixture; $[\text{E3}]_0$ is the initial concentration of enzyme-adenylylate; l is the light path length; $\Delta\epsilon_f^{260}$ is the molar extinction of free ATP at 260 nm; $\Delta\epsilon_b^{260}$ is the molar extinction of the enzyme-bound nucleotide in the one-nucleotide occupied enzyme species at 260 nm; and $\Delta\epsilon_{b1}^{260}$ is the molar extinction of the enzyme-bound nucleotide in the two-nucleotide occupied enzyme species.

Trace 1 calculated from the one-site model does not cover the experimental data (Fig. 2). Introduction of distinct values for the extinction coefficients of components E2 and E3 or a variation of K_1 and K_3 values does

not improve the fitting. In fact, at concentrations of ATP in excess of the enzyme Eq. 1 assumes the form $f([\text{ATP}]) \sim \epsilon_f^{260} - \text{Const}/[\text{ATP}]_0$, approaching its maximal value $\Delta\epsilon_f^{260}$ hyperbolically, i.e., not steep enough to fit to the experimental data points. The same holds for the one-site competitive binding model (trace not shown). On the other hand, trace 2 calculated with the two-site model fits to the data adequately (Fig. 2). It appears that the molar extinction coefficient at 260 nm of the nucleotide bound in the high-affinity catalytic site in the one-nucleotide occupied enzyme is nearly 25% lower than that of the free ATP, whereas the molar extinction coefficient of the nucleotide in the two-nucleotide occupied enzyme is very close to that of the free nucleotide, $\epsilon_{b1}^{260} \approx \epsilon_f^{260}$ (Table 1). In addition, the K_d for the second site, the low-affinity noncovalent ATP binding site, is between $100 < K_2 < 250 \mu\text{M}$ (Fig. 2, dashed traces; Table 1). It can be further concluded that the noncovalent complex E2 and the enzyme-adenylylate E3 have a very similar molar extinction at 260 nm (Fig. 3, inset). The lower trace 3 is calculated assuming that the extinction of AMP in the complex E3 is 8.6 $\text{mM}^{-1} \text{cm}^{-1}$, whereas the extinction of ATP in E2 is equal to the ϵ_f^{260} value. The upper trace 4 was calculated assuming that these values are opposite.

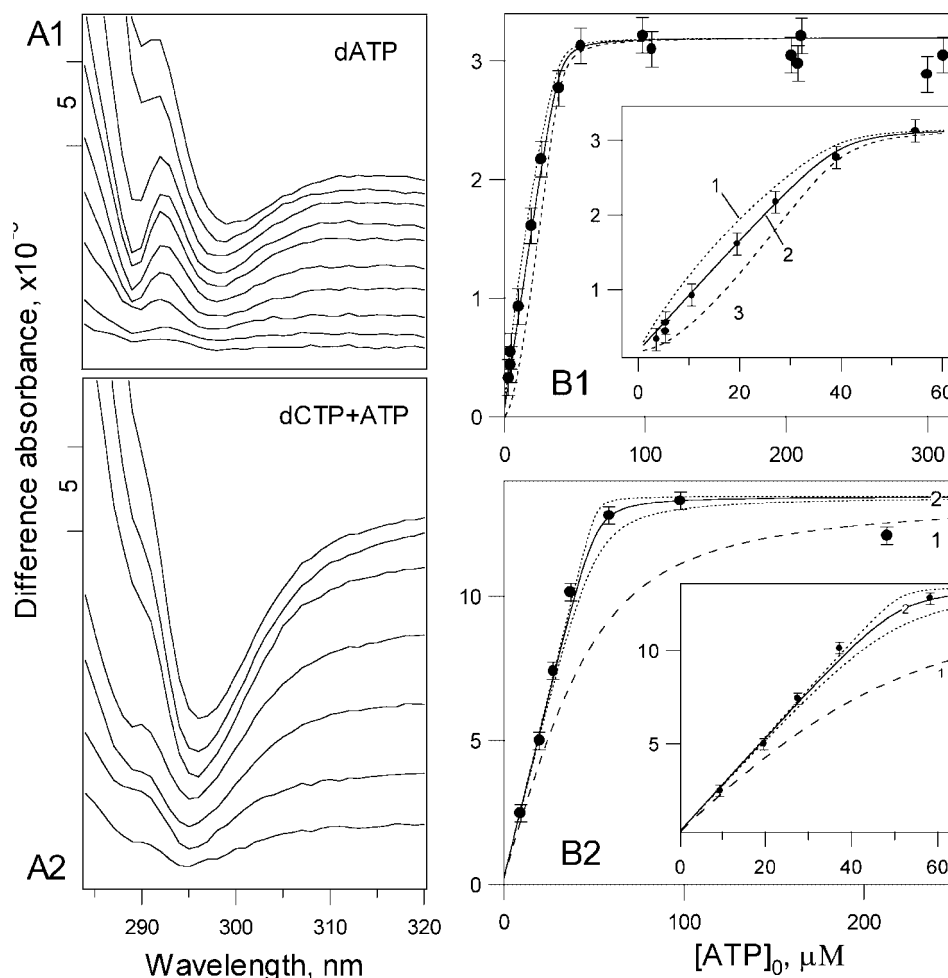


FIGURE 3 Binding of ATP to T4 DNA ligase and the effect of dCTP: absorbance at 296–298 nm. (A1) Difference spectra [enzyme+ATP minus free enzyme]. Concentrations of ATP and enzyme are shown in the legend to Fig. 2. (A2) Difference spectra [enzyme+dCTP+ATP minus enzyme+dCTP]. Concentrations of ATP (from bottom to top trace) are as follows: 9.47, 19.61, 27.51, 37.18, 58.27, 98.18, and 212.62 μM and 317.41 μM of dCTP and 74.7 μM of T4 DNA ligase. The spectra in A1 and A2 are essentially linear above 320 nm with the value of difference absorbance $\Delta A = 0 \pm 1 \times 10^{-3}$; the spectra are offset vertically for clarity of presentation. (B1) Difference absorbance 320 nm minus 298 nm obtained from spectra shown in A1 and five additional measurements. Binding traces 1–3 are calculated for the one-site model: trace 1, molar extinction at 298 nm $\Delta\epsilon_{\text{b}}^{298} = 0$ for E2 and $\Delta\epsilon_{\text{b}}^{298} = 0.66 \text{ mM}^{-1} \text{ cm}^{-1}$ for E3; trace 3, $\Delta\epsilon_{\text{b}}^{298} = 1.02$ for E2 and $\Delta\epsilon_{\text{b}}^{298} = 0 \text{ mM}^{-1} \text{ cm}^{-1}$ for E3; trace 2, $\Delta\epsilon_{\text{b}}^{298} = 0.4 \text{ mM}^{-1} \text{ cm}^{-1}$ for both E2 and E3. (B2) Difference absorbance at 330 nm minus the minimum at 295–297 nm (see A2). Binding traces 1 and 2 are calculated for the one-site competitive binding model using $K_2 = 14.2 \text{ }\mu\text{M}$, $\Delta\epsilon_{\text{C}}^{296} = 0.94 \text{ mM}^{-1} \text{ cm}^{-1}$, $\Delta\epsilon_{\text{C1}}^{296} = 0.4 \text{ mM}^{-1} \text{ cm}^{-1}$; trace 1, $K_1 = 1.1 \text{ }\mu\text{M}$; trace 2, $K_1 = 0.05 \text{ }\mu\text{M}$. Dotted lines show the traces calculated with $K_1 = 0.15 \text{ }\mu\text{M}$ (lower trace), $K_1 = 0.01 \text{ }\mu\text{M}$ (upper trace).

The traces converge to the experimental data when E2 and E3 have the same molar extinction.

ATP binding curves shown in Fig. 3 were calculated using Eq. 2:

$$A_{298} = [\Delta\epsilon_{\text{b}}^{298}([E2]_{\text{eq}} + [E3]_{\text{eq}} - [E3]_0) + \Delta\epsilon_{\text{b1}}^{298}([E5]_{\text{eq}} + [E6]_{\text{eq}})]l, \quad (2)$$

where A_{298} is the absorbance at 320 nm minus 298 nm, $\Delta\epsilon_{\text{b}}$ and $\Delta\epsilon_{\text{b1}}$ are the difference extinction coefficients of the one- and two-site occupied enzyme-nucleotide species versus free enzyme, respectively.

In contrast to the data obtained at 260 nm, the one-site model is sufficient to describe binding in this case. The two-site model can be used as well if $\Delta\epsilon_{\text{b}} = \Delta\epsilon_{\text{b1}}$. Although the decrease of the difference absorbance at 298 nm at high ATP concentration (see Fig. 3) could indicate a possible redistribution between differently absorbing one- and two-site occupied species, it is more simply explained by the non-zero absorbance of the unbound ATP at this wavelength. A large background absorbance of ATP at 260 nm (Fig. 2 A) leads to an increase of the apparent intensity of the peak at 292 nm and a red-shifting of the trough minimum at 298 nm (Fig. 3 A).

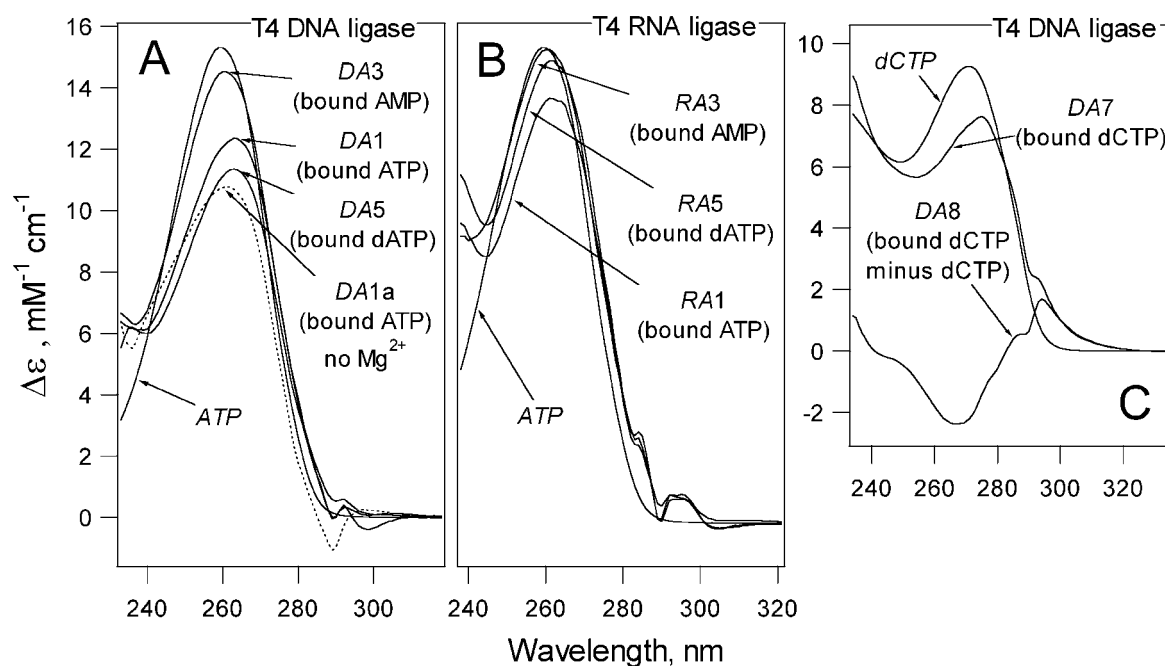


FIGURE 4 Optical difference spectra [enzyme+nucleotide minus free enzyme], showing both the decrease and the red-shifting of the absorbance of the ligase-bound nucleotides. (A and C) T4 DNA ligase (DA^* spectra); (B) T4 RNA ligase (RA^* spectra). ATP: [buffer+ATP minus buffer]; dCTP: [buffer+dCTP minus buffer]; DA1, RA1: [enzyme+ATP minus free enzyme] in the presence of Mg^{2+} ; DA1a: the same, but without Mg^{2+} and in the presence of 10 mM EDTA; DA3, RA3: [enzyme+AMP minus free enzyme]; DA5, RA5: [enzyme+dATP minus free enzyme]; DA7: [enzyme+dCTP minus free enzyme]; DA8: Difference spectrum DA7 minus dCTP. Concentrations of the components in the enzyme-nucleotide mixture were as follows: T4 DNA ligase, 72.4 μM (80 μM for DA1a); ATP, 30.1 μM (31.5 μM for DA1a); AMP, 32.2 μM ; dATP, 29.8 μM ; dCTP, 31.5 μM ; T4 RNA ligase, 96 μM ; ATP, 28.1 μM ; AMP, 32.5 μM ; dATP, 30.4 μM .

It can also be concluded that the complexes E2 and E3 have the same difference molar extinction at 298 nm, $\Delta\epsilon = 0.4 \text{ mM}^{-1} \text{ cm}^{-1}$ (Fig. 3 B1, trace 2). This value indicates that a single tryptophan residue is involved in the optical changes at this wavelength (Demchenko, 1986).

It is known that the purified T7 DNA ligase and the human DNA ligase I contain up to 30% of adenylylated enzyme (Doherty et al., 1996; Montecucco et al., 1990). In the case of T4 DNA ligase, fitting to the data shown in Figs. 2 and 3 indicates that only 70% of the total enzyme in the reaction mixture participates in binding, also suggesting the presence of the pre-adenylylated ligase.

Binding of ATP to the complex of T4 DNA ligase and dCTP

Even though dCTP is unable to participate in or to inhibit the ATP-pyrophosphate exchange reaction catalyzed by T4 DNA ligase (Weiss et al., 1968a), it still forms an optically active complex with the enzyme (Fig. 4 C). Binding of ATP to this complex results in a decrease of the characteristic absorbance of the dCTP:ligase complex at 294 nm and the appearance of a trough at 298 nm, which is characteristic for the T4 DNA ligase complex with ATP (Fig. 5 C), suggesting that these two nucleotides bind to the same site. A

titration of the ligase:dCTP complex with ATP was performed to study the interaction between these two nucleotides in the active site of the ligase. In Fig. 3 A2, difference spectra [enzyme + ATP + dCTP minus free enzyme minus dCTP] are presented. The trough at 295–297 nm is roughly threefold deeper than in Fig. 3 A1, [enzyme + ATP minus free enzyme]. In Fig. 3 B2 we show the corresponding binding traces, calculated using Scheme I and Eq. 3:

$$A_{296} = [\Delta\epsilon_C^{296}([E4]_0 - [E4]_{eq}) + \Delta\epsilon_{C1}^{296}([E2]_{eq} + [E3]_{eq} - [E3]_0) + \Delta\epsilon_{C2}^{296}([E5]_{eq} + [E6]_{eq})]l, \quad (3)$$

where the $[E4]_0 \approx [E]_0 - [E3]_0$ is the initial concentration of the ligase:dCTP complex before addition of ATP, $\Delta\epsilon_C$ is the difference extinction of the ligase:dCTP complex versus free enzyme, $\Delta\epsilon_{C1}$ is the difference extinction of one-nucleotide-occupied enzyme, and $\Delta\epsilon_{C2}$ is the difference extinction of the two-nucleotide-occupied enzyme at 296 nm. It is immediately evident from the data in Fig. 3 that the one-site model cannot describe the binding. Otherwise, the spectra in Fig. 3, A1 and A2, and data in Fig. 3 B1 and B2, would be identical. On the other hand, the one-site competitive binding model fits to the data readily. Because dCTP binds T4 DNA ligase with a relatively low K_d (see Table 1) we were able to estimate the K_d for ATP in the catalytic site

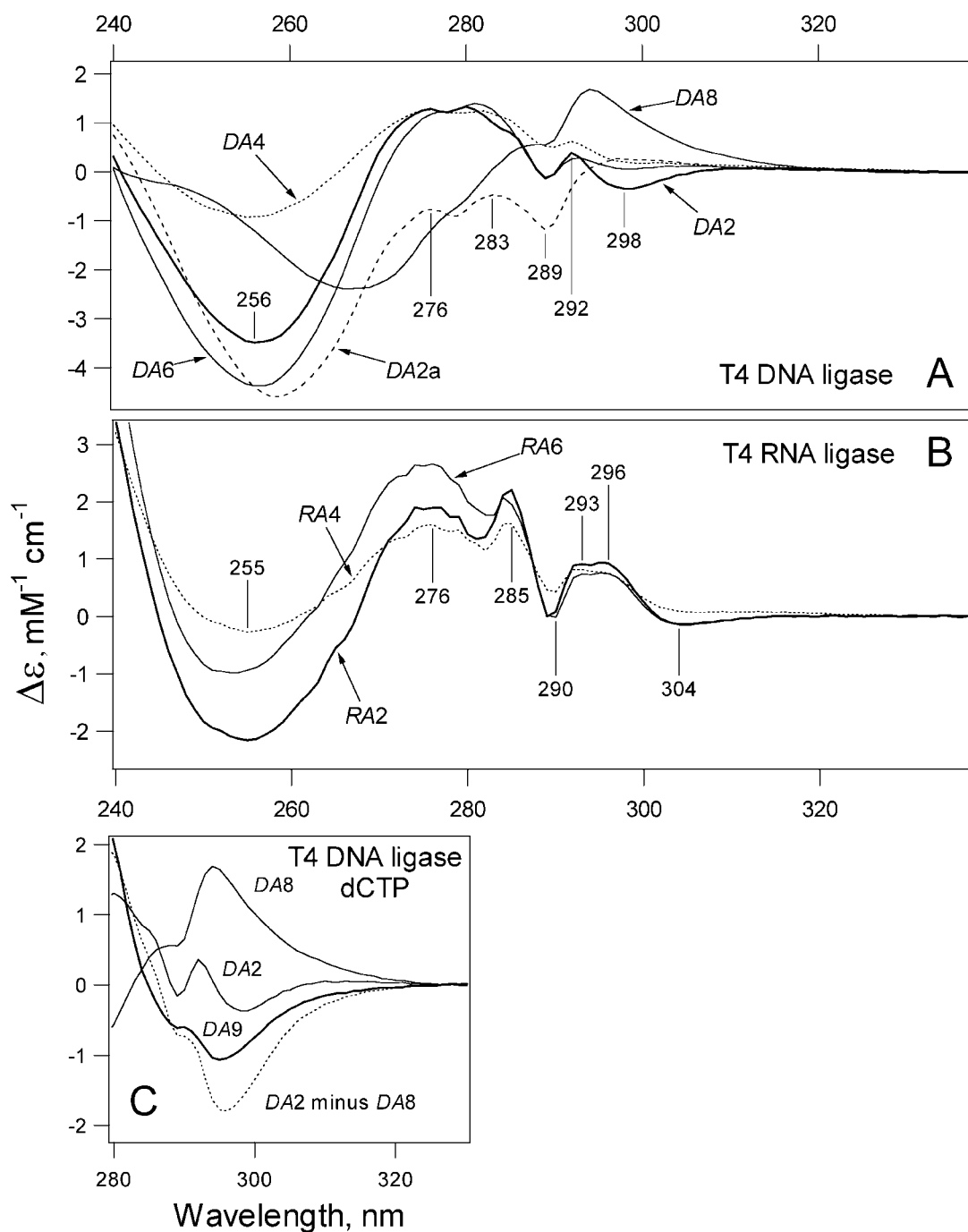


FIGURE 5 Optical difference spectra [ligase+nucleotide minus free ligase minus nucleotide], showing mainly the effects on the aromatic residues. (A and C) T4 DNA ligase; (B) T4 RNA ligase. Spectra DA2, DA2a, RA2, [ligase+ATP minus free ligase minus ATP] (DA1, DA1a, RA1 minus ATP); DA4, RA4: [ligase+AMP minus free ligase minus AMP] (DA3, RA3 minus A0); DA6, RA6: [ligase+dATP minus free ligase minus dATP] (DA5, RA5 minus spectrum of free dATP); DA8: [ligase+dCTP minus free ligase minus dCTP] (DA7 minus dCTP); DA9: difference spectrum [enzyme+dCTP+ATP minus enzyme+dCTP]. The concentrations of enzymes and nucleotides are given in the legend to Fig. 4.

from competitive binding studies. At 5 mM Mg^{2+} this value is below 150 nM (Fig. 3 B2, trace 2). The two-site binding model, in principle, can also be used for fitting of the data, if $\Delta\epsilon_{C1} = \Delta\epsilon_{C2}$.

Summarizing, we conclude that T4 DNA ligase has two interacting nucleotide binding sites. One is a catalytic site, binding to which leads to the formation of the enzyme-adenylylate complex. The other one is a noncovalent bind-

TABLE 2 Optical hypochromicity and red-shift of the absorbance maximum of ligase-bound nucleotides

	Optical hypochromicity (%)		Red-shift (nm)	
	T4 DNA ligase	T4 RNA ligase	T4 DNA ligase	T4 RNA ligase
ATP	23*/30 [†]	12*	4/2 [†]	2.5
AMP	6*	No effect	1	1
dATP	27*	2.5*	4	2.5
dCTP	21 [‡]	No effect	4	No shift

*At 260 nm.

[†]In the absence of Mg²⁺ and presence of 10 mM EDTA.[‡]At 271 nm.

ing site, which is located in close vicinity of the catalytic site. The K_d for ATP for the catalytic site is less than 150 nM, whereas for the second noncovalent binding site the K_d is approximately three orders of magnitude higher than this value, between 100 and 250 μ M.

UV-VIS spectral properties of enzyme-nucleotide complexes

Binding of nucleotides by T4 DNA or RNA ligase changes the electronic absorbance and fluorescence properties of both enzyme and nucleotide. These changes are evidently caused by the specific ligase-nucleotide interactions, because mixing of ATP with excess of a control protein (BSA) does not change the spectra (not shown). Two main structural changes contribute to the optical changes observed upon formation of the ligase-nucleotide complexes: 1) perturbation of the environment of the nucleotide, which leads to a decrease and shifting of the absorbance bands between 259 and 275 nm (Shimizu and Morii, 1998; Benz and Roberts, 1973), and 2) perturbation of the environment of the aromatic residues of the ligase, which leads to the appearance of so-called fine structure between 282 and 320 nm and a decrease and shift of the excitation/emission bands (Demchenko, 1986).

Perturbation of the environment of the nucleotide

Binding of nucleotides to T4 DNA ligase results in a considerable hypochromic effect (Fig. 4; Table 2), implying a π -stacking interaction between the nucleotide and an aromatic residue of the enzyme (Toulme, 1978). The hypochromic effect decreases in the sequence ATP > Mg-dATP > MgATP > Mg-dCTP > MgAMP. Compared with DNA ligase, T4 RNA ligase affects the optical absorbance of nucleotides to a lesser extent (Table 2). In addition, the hypochromicity of dATP bound to T4 RNA ligase is four-fold weaker than that of ATP, whereas the spectrum of dCTP in the presence of the enzyme does not change at all (Table 2).

Binding of nucleotides to both T4 ligases shifts the absorbance maximum of the nucleotide to the red, but to a different extent. T4 DNA ligase induces a 4-nm red-shift of (d)ATP spectra compared with the 2.5-nm shift induced by RNA ligase (Fig. 4; Table 2). Binding of AMP by both enzymes results in weaker observed red-shifts and hypochromicity, in part due to the fact that under the conditions of the experiment $\sim 60\%$ of the binding site is occupied.

It is interesting that the shifts of the absorbance bands of ATP and dATP bound to T4 RNA ligase are of the same magnitude, even though the hypochromicity differs by more than a factor of four. This indicates that the optical hypochromism and band shifting have a different origin. One of the explanations for the observed red-shift of a chromophore is a change of the polarizability of the environment, which is directly related to a change of the refractive index of the media (Demchenko, 1986). In other words, the red-shift increases with the refractive index. However, it appears that this effect becomes less significant with an increase of the molar extinction of the chromophore, as shown below. The change of the refractive index of the medium, i.e., water (1.33) to the protein (1.44–1.49 (Doty and Geiduschek, 1953; Yanari and Bovey, 1960)) leads to a 4–5-nm red-shift and a 17% increase of tyrosine absorbance ($\epsilon^{274.5} = 1.42$). For tryptophan ($\epsilon^{279.9} = 5.55$) this effect is already less pronounced, with a 2-nm red-shift and a 10% absorbance increase (Demchenko, 1986). We have determined that the absorbance of ATP ($\epsilon^{259} = 15.4$) in a 60% sucrose solution ($n = 1.44$) is red-shifted less than 1 nm and increased in intensity less than 1% (data not shown). This indicates that the polarizability of the environment plays an insignificant role in shifting the absorbance spectrum of the ligase-bound nucleotide.

Perturbation of the environment of the aromatic residues

Optical absorbance spectroscopy

The π -stacking between tryptophan and a nucleotide leads to a decrease of the tryptophan absorbance at 280 nm for 1–2 mM⁻¹ cm⁻¹ (Ishida et al., 1982; Porschke, 1980; Morita, 1974). In addition, a charge transfer band is observed between 295 and 340 nm with $\epsilon \approx 0.3$ mM⁻¹ cm⁻¹ (Ishida et al., 1982; Morita, 1974). The absorbance at 280 nm of the T4 DNA ligase-ATP complex in the absence of Mg²⁺ is ~ 1 mM⁻¹ cm⁻¹ less than in its presence, which could indicate tryptophan-ATP stacking in the absence of a divalent cation (Fig. 5, compare DA2 and DA2a). However, because none of the spectra in Fig. 5 shows a distinct charge-transfer band, it can be concluded that the tryptophan-nucleotide stacking does not occur in the two T4 ligases. This is in contrast to what could be concluded from the sequence alignment in Subramanya et al. (1996). On the other hand, stacking with tyrosine or phenylalanine would

TABLE 3 Position and intensity of the tryptophan transitions in proteins

$\pi^* \leftarrow \pi$ transitions	Position in proteins,* $\lambda_{\max}$ (nm)	Extinction in solution, [†] ϵ (mM ⁻¹ cm ⁻¹)	T4 DNA ligase-ATP complex		T4 RNA ligase-ATP complex	
			$\lambda_{\max}$ (nm)	$\Delta\epsilon$ (mM ⁻¹ cm ⁻¹)	$\lambda_{\max}$ (nm)	$\Delta\epsilon$ (mM ⁻¹ cm ⁻¹)
¹ L _b	280–286	5.6	NV	NV	285	~0.4 [‡]
	287–295	4.5	292	~0.2 [‡]	293, 296	~0.8 [‡]
¹ L _a	289–302	0.5	298	–0.4 [‡]	302	–0.16 [‡]

NV, not visible.

*Adapted from Strickland et al. (1971) and Demchenko (1986).

[†]From Demchenko (1986).[‡]Difference extinction (320 nm as a reference).

be consistent with our observations due to their low extinction at 280 nm. In addition, no charge transfer band is observed for complexes of these two amino acids with ATP (Toulme, 1978). Because the tyrosine stacking is expected to yield a tyrosine perturbation spectrum, which in our case is not observed either, we favor for both T4 ligases a π -stacking between ATP and phenylalanine, as observed for mRNA CE PBCV-1 (Hakansson et al., 1997). In the case of T4 DNA ligase the stacking residue could be Phe 279 (motif IIIa).

The electronic transitions of aromatic amino acid residues are known to shift upon interaction with the environment. Exposing a tryptophan residue to a more hydrophobic environment leads to a red-shift of the ¹L_b transition band, which is observed in difference spectra as two peaks between 284 and 286 nm (difference extinction between 0.1 and 0.2 mM⁻¹ cm⁻¹) and 292 and 295 nm (0.3 and 0.4 mM⁻¹ cm⁻¹) (Table 3) (Herskovits and Sorensen, 1968; Demchenko, 1986). The difference spectra DA2 [ligase+ATP minus free ligase minus ATP], DA4 [ligase+AMP minus free ligase minus AMP], and DA6 [ligase+dATP minus free ligase minus dATP] (Fig. 5) show that a single tryptophan residue is perturbed after binding of ATP to T4 DNA ligase. For T4 RNA ligase, two tryptophan residues are involved, because the peak at 285 nm is double in extinction and two peaks, at 293 and 296 nm, are present (Table 3; Fig. 5 B, spectra RA2, RA4, and RA6). In both cases the absence of the characteristic absorbance peak between 286 and 289 nm implies no involvement of tyrosine residues (Demchenko, 1986).

As can be seen from Fig. 5, A and B, the binding of ATP decreases the absorbance of both ligases around 298–300 nm, indicating a blue-shift of the 0–0 ¹L_a band of the perturbed tryptophan residue. This effect correlates with a weakening of the hydrogen bonding interactions between the ring nitrogen proton of tryptophan and a proton acceptor group (Lin and Sakmar, 1996; Strickland et al., 1972). The latter could be carbonyl oxygen from a peptide bond or a side-chain amide (difference absorbance minimum at 295–298 nm), aromatic nitrogen of histidine (296–299 nm), or carboxylate ion (297–302 nm) (Strickland et al., 1972). On the other hand, binding of AMP, or ATP in the absence of Mg²⁺ ions, or dATP does not significantly change the

absorbance around 300 nm, whereas binding of dCTP increases the absorbance in this region (compare DA2 [ligase+ATP minus free ligase minus ATP] with DA4 [ligase+AMP minus free ligase minus AMP], DA2a [ligase+ATP minus free ligase minus ATP in the absence of Mg²⁺], DA6 [ligase+dATP minus free ligase minus dATP], and DA8 [ligase+dCTP minus free ligase minus dCTP]).

Fluorescence spectroscopy

The excitation/emission spectra of T4 ligases are shown in Fig. 6. To characterize the ATP-dependent quenching of fluorescence we recorded two excitation spectra measuring emission at 308 and 340 nm and two emission spectra with excitation at 275 and 292 nm before and after addition of ~0.8 mol of ATP per mol of the enzyme. A decrease of emission of ~25% per mol of bound enzyme was observed for both T4 ligases. T4 RNA ligase, in contrast to T4 DNA ligase, showed a 4-nm blue-shift of the emission maximum and a 1-nm red-shift of the excitation maximum (Fig. 6 A2, compare RF5, RF7 with RF6, RF8 and RF1 with RF2). This indicates that the interactions of the emitting tryptophan residues of T4 RNA ligase with the solvent change significantly upon binding of the nucleotide, a point that is more clearly illustrated by comparing the difference excitation/emission spectra [free enzyme minus enzyme+nucleotide] (Fig. 6, F9–F12). The quenched emission of T4 RNA ligase (maximum at 350 nm, RF11) is 12-nm red-shifted with respect to the emission of the free enzyme (maximum at 338 nm, RF5). According to the discrete-state model (Burstein et al., 1973) the tryptophan residue, which emits at 350 nm, is exposed to the solvent, whereas the residue emitting at 338 nm is located at the surface of the protein in a region of protein-bound water dipoles with low mobility. A similar conclusion can be drawn from the recent more elaborate classification of tryptophan fluorescence shifts in proteins by Vivian and Callis (2001). On the other hand, the difference emission spectrum of T4 DNA ligase is not shifted with respect to the emission of the nucleotide-free enzyme. Both the quenched tryptophan residue (DF11, DF12) and the residues in the free enzyme (DF5, DF7) have emission maxima at 337–339 nm, indicating that they are shielded

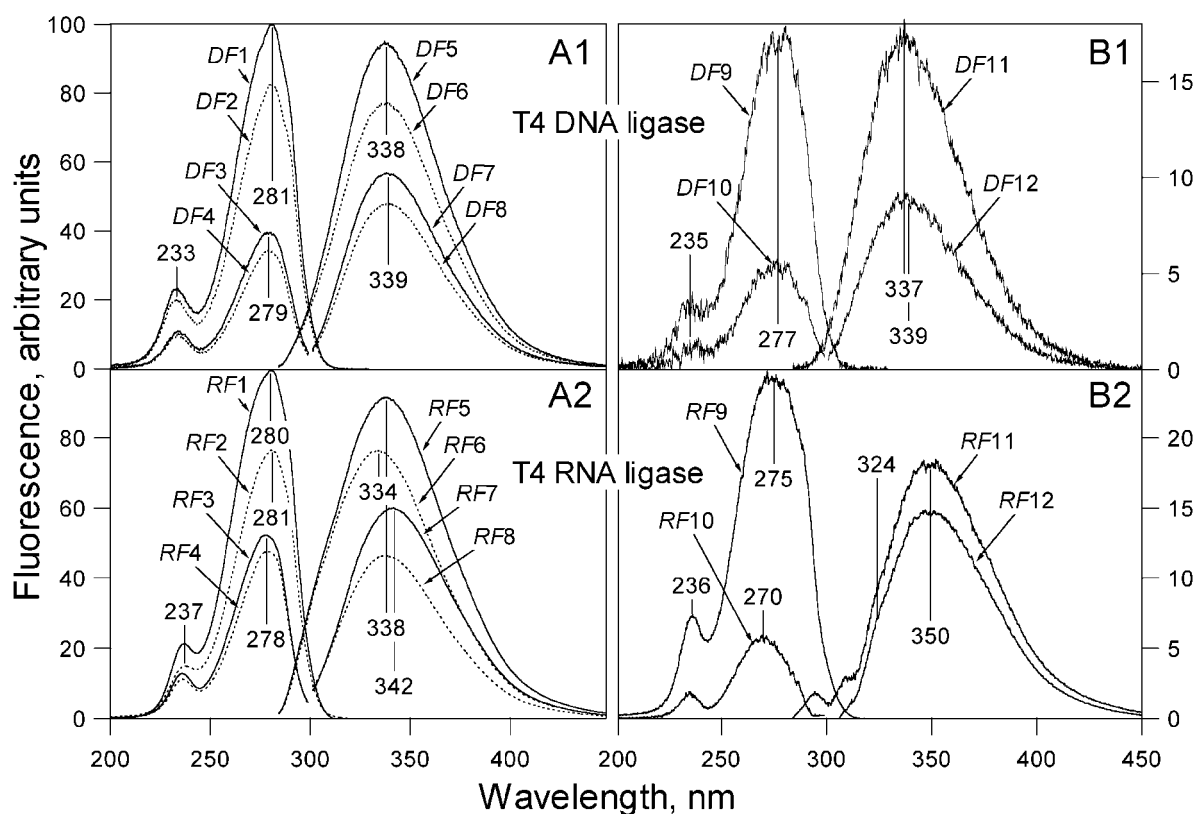


FIGURE 6 Uncorrected fluorescence spectra of T4 DNA ligase (A1 and B1; DF* spectra) and T4 RNA ligase (A2, B2; RF* spectra). Here are shown the spectra [free enzyme] (solid line), [enzyme+ATP] (dotted line) (A1 and A2) and difference spectra [free enzyme minus enzyme+ATP] (B1 and B2). F1, [free enzyme] excitation spectrum obtained by measuring emission at 340 nm; F2, the same as F1 but [enzyme+ATP]; F9, difference spectrum [F1 minus F2]; F3, [free enzyme] excitation spectrum obtained by measuring emission at 308 nm; F4, the same as F3 but [enzyme+ATP]; F10, spectrum [F3 minus F4]; F5, [free enzyme] emission spectrum obtained by excitation at 275 nm; F6, the same as F5 but [enzyme+ATP]; F11, difference [F5 minus F6]; F7, [free enzyme] emission spectrum obtained by excitation at 292 nm; F8, the same but [enzyme+ATP]; F12, difference [F7 minus F8]. T4 DNA (RNA) ligase was diluted up to 6 μ M (13 μ M) in 540 μ l of reaction buffer, pH 7.8 (see Materials and Methods). An aliquot of 270 μ l was withdrawn to record the [free enzyme] spectra. To the remainder, 1 mM ATP was added to a final concentration of 5 μ M (10 μ M), and after 3 min of incubation at room temperature the [enzyme+ATP] spectra were acquired. A1, B1, and A2, B2 are shown on the same relative scales, in which the maximum excitation intensity of free enzyme (measuring emission at 340 nm) is taken as 100.

from the solvent by the protein matrix. In addition, T4 DNA ligase displays a 2-nm red edge excitation shift of the quenched fluorescence (excitation at 292 vs. 275 nm),

which is absent in RNA ligase (Table 4). The red edge excitation shift is caused by the interaction of the tryptophan chromophore with the slow relaxing dipoles such as protein-

TABLE 4 Fluorescence emission properties of T4 DNA and RNA ligase

	Source of tryptophan fluorescence	$\lambda_{\max}$ (nm)	$\Delta\lambda$ (nm)	275/292-nm red-edge shift
T4 DNA ligase	[Free enzyme], DF7 [†]	339	56	1
	[enzyme + MgATP], DF8	339	57	1
	DF7 - DF8 = DF12	339	54	2
T4 RNA ligase	[Free enzyme], RF7	342	60	4
	[enzyme + MgATP], RF8	338	59	4
	RF7 - RF8 = RF12	350	59	0
Proteins in general [§]	Class I, buried	330-332	48-49	0 [‡]
	Class II, surface	340-342	53-55	2-7 [‡]
	Class III, exposed	350-353	59-61	0 [‡]

*Measured by excitation at 292 nm; $\Delta\lambda$, band width at half-maximal intensity.

[†]See Fig. 6.

[‡]Adapted from Demchenko (1986).

[§]From Burstein et al. (1973).

bound water. The residues exposed to the solvent produce no shift because the solvent dipoles relax faster than the excitation state lifetime (Demchenko, 1986).

In contrast to the quenched emission, the fluorescence of the nucleotide-free T4 DNA ligase is shifted with red edge excitation by 1 nm, whereas for RNA ligase a 4-nm shift is observed (Fig. 6, compare *D(R)F5* with *D(R)F7*; Table 4). This indicates that on average T4 RNA ligase contains surface tryptophan residues that are accessible to water molecules with restricted mobility, whereas DNA ligase contains residues that are buried in the protein matrix. Residues buried in a hydrophobic nonpolar environment do not experience dipole-dipole interactions and hence produce no shift (Demchenko, 1986).

T4 RNA ligase displays a difference emission profile with two minor peaks at 295 and 309 nm and a clear shoulder at 324 nm (Fig. 6 *B2*, *RF11*, *RF12*). The first two could originate from a tyrosine residue, because they are absent or less expressed in the fluorescence spectrum derived from the excitation at 292 nm (*RF12*). The shoulder at 324 nm, however, is present in emission spectra obtained by excitation at both 275 (*RF11*) and 292 nm (*RF12*) and might derive from a tryptophan residue, perhaps the one that is quenched upon binding of ATP. If this optically active tryptophan residue is not completely quenched and the remaining emission is blue-shifted to 330–335 nm as a result of exposure to a less polar environment, then in difference spectra it would appear as an apparent shoulder at 320–325 nm.

Both T4 ligases in complex with ATP experience red-shifting of the excitation maximum, because the difference excitation spectra are blue-shifted with respect to the excitation spectra of the nucleotide-free enzymes. The difference is only 2–4 nm for T4 DNA ligase (281/277 nm for *DF1/DF9* and 279/277 for *DF3/DF10*). For RNA ligase it amounts to 5–8 nm (280/275 nm for *RF1/RF9* and 278/270 for *RF3/RF10*), and, similarly to the red-shift of the tryptophan absorbance (Fig. 5 *B*), this indicates a change of the environment of the emitting tryptophan residue to less polar.

The difference excitation spectrum of T4 RNA ligase, which was recorded by measuring the emission at 308 nm, has a maximum at 270 nm (Fig. 6 *B2*, *RF10*). This is close to the excitation maximum of tyrosine residues in proteins (average 270–275 nm), which might indicate fluorescence resonance energy transfer between a tyrosine and the optically active tryptophan residue.

DISCUSSION

Equilibrium binding of nucleotides

T4 DNA and RNA ligases are two-substrate enzymes, acting according to a ping-pong mechanism (Rae et al., 1975), which implies the presence of separate binding sites for

DNA (RNA) and for ATP. The 3D structures of several related nucleotidyltransferases that might serve as model proteins for the T4 ligases indicate that the hydrophobic ATP binding site is located inside the bigger positively charged cavity where the DNA (RNA) binds (Subramanya et al., 1996; Odell et al., 2000; Lee et al., 2000). Binding of ATP by the T4 ligases changes the optical absorbance spectra in a manner indicating hydrophobic interactions between ATP and the protein. On the other hand, negligible changes are observed upon binding of dsDNA by T4 DNA ligase, implying that binding is mainly due to electrostatic interactions (data not shown). In the presence of 5 mM Mg^{2+} , ATP predominantly exists as a negatively charged species, MgATP^{2-} . Therefore, in the absence of dsDNA and the presence of excess ATP, the latter could occupy the DNA binding site. The close vicinity of the DNA and ATP binding sites (cf. Subramanya et al., 1996; Odell et al., 2000; Lee et al., 2000) may allow conformational interactions between the two. Optical spectroscopy is, in principle, sensitive enough to detect such perturbations. Thus, a two-site nucleotide-binding model should be considered to accurately describe ATP-ligase interactions.

Both T4 DNA and RNA ligases are capable of synthesizing dinucleoside polyphosphates, for example Ap_4A , with low reaction rates ($k_{\text{cat}} \approx 10^{-2} \text{ s}^{-1}$). This reaction occurs as a displacement of AMP in the E-AMP complex by ATP and is inhibited by the DNA or RNA (Sillero and Sillero, 2000; Madrid et al., 1998; Atencia et al., 1999). This experimental observation supports a two-site nucleotide-binding model and suggests that the second nucleotide molecule binds in the DNA (RNA) binding site of the enzyme-AMP complex.

The binding traces of MgATP^{2-} by T4 DNA ligase are calculated for two different wavelengths. Data derived from the absorbance at 260 nm indicate the presence of two ATP binding sites. A one-site model is unable to fit the experimental data (Fig. 2). The results can be readily explained with a two-site model if the molar extinction of the ATP bound to the first site ($12 \pm 0.16 \text{ mM}^{-1} \text{ cm}^{-1}$) would increase to a value of $15.2 \pm 0.3 \text{ mM}^{-1} \text{ cm}^{-1}$ upon occupation of the second site. However, the data obtained from the wavelength region between 298 and 300 nm can be adequately described with a one-site model. This discrepancy could arise from the fact that the difference spectrum is composed of the absorbance changes of two different chromophores. One of them, the nucleotide, senses the ATP bound in the second site whereas the other, tryptophan, apparently does not. As indicated below, a weakening of the hydrogen-bonding interactions of the tryptophan residue causes the decrease of absorbance at 298 nm. The decrease of absorbance at 260 nm originates from π -stacking of the adenine ring of nucleotide and an aromatic residue of the enzyme, but not tryptophan, because the characteristic charge-transfer band between 295 and 340 nm is absent. If binding of ATP in the second site would cause a misalign-

ment between the stacked chromophores in the catalytic site, it would result in an increase of the absorbance at 260 nm. However, if the second ATP does not affect the hydrogen-bonding state of the optically active tryptophan residue, the difference between the one- and two-nucleotide-occupied enzyme species cannot be monitored at 298 nm.

Perturbation of the environment around the nucleotide

Analysis of the 3D structures of related enzymes shows that the ATP chromophore is inserted between an aromatic residue on one side and a hydrophobic patch of amino acid residues on the other side, forming the walls of the binding cleft (Hakansson et al., 1997; Subramanya et al., 1996). This patch is conserved in T4 ligases, indicating that hydrophobic interactions might influence the optical properties of the ligase-bound ATP. In general, the effect of a hydrophobic protein environment is similar to the effect of a hydrophobic solvent, resulting in a 10–15% decrease and a 1–3-nm blue-shift of the absorbance band of the adenine nucleotide at 259 nm (Charney and Gellert, 1964; Delabar et al., 1972). In our case, the decrease is larger and the absorbance maximum is red-shifted, indicating that additional interactions are involved. In the case of cytidine, however, the effect of a hydrophobic solvent results in a hypochromicity of ~25% and a 5-nm red-shift of the absorbance maximum at 270 nm (Charney and Gellert, 1964), which is similar to what we observed for the ligase-bound dCTP. Thus, a hydrophobic environment alone is sufficient to explain the observed optical properties of the dCTP bound to T4 DNA ligase.

According to the 3D data of the model proteins, the adenine ring of the enzyme-bound ATP is interacting with the aromatic amino acid residue by π -stacking. The π -stacking results in parallel orientation of the $\pi^* \leftarrow \pi$ transition dipole moments of the aligned chromophores. The dispersion interactions between non-identical transitions decrease the dipole strength of the lowest-energy transitions of interacting species (Urry, 1985; Volkov and Pechenaia, 1982; Kondo et al., 1970; DeVoe and Tinoco, 1962). As a result, a 1–3-nm blue-shift and up to 30% hypochromicity of the 259-nm absorbance band of (deoxy)adenosine could be expected, depending on the ring-ring distance between the bound nucleotide and the aromatic residue. For both T4 ligases we observed a 25–30% decrease of the nucleotide absorbance, which is consistent with the π -stacking effect, but the opposite band shift indicates that other interactions are also involved.

In 3D structures of related enzymes the exocyclic amino function of ATP forms hydrogen bonds with proton-accepting residues. In general, donation of electron density via a hydrogen bond to an aromatic conjugated system stabilizes the $\pi^* \leftarrow \pi$ transitions leading to a red-shift of the transition maximum. This effect has been studied with various aro-

matic compounds (for review see Demchenko, 1986), indicating that a shift of several nanometers is easily attainable. Therefore, hydrogen bonding of the enzyme with the exocyclic amino group of the nucleotide would be consistent with the observed red-shift for the ligase-bound ATP. Although an overall increase of the absorbance maximum of 10–15% is expected due to hydrogen bonding, the combined influence of stacking and hydrophobic interactions could result in a net decrease of absorbance.

Summarizing, the simplest explanation for the spectral changes of ligase-bound ATP would be the combined action of π -stacking, hydrophobic interactions, and hydrogen bonding. On the other hand, a change to a more hydrophobic environment alone is sufficient to interpret the spectrum of ligase-bound dCTP.

Perturbation of the environment of the tryptophan residues

General remarks

The absorbance of tryptophan at 280 nm is a superposition of two strongly overlapping $\pi^* \leftarrow \pi$ transitions, $^1L_a \leftarrow ^1A_1$ and $^1L_b \leftarrow ^1A_1$ (Rehms and Callis, 1987; Albinsson et al., 1989). The 1L_a transition band is broad and featureless and forms the entire envelope of the absorbance peak at 280 nm, whereas the 1L_b is narrower and possesses a distinct vibrational structure. For pure tryptophan in water, two 1L_b vibronic lines can be observed at 280 and 288 nm (0–0 band). A change to a more hydrophobic environment shifts the 1L_b band several nanometers to the red. In proteins, this yields characteristic perturbation difference spectra with maxima at 284–286 and 292–295 nm (Demchenko, 1986). The 1L_a excited state possesses a twofold stronger permanent dipole moment compared with the 1L_b . As a result, the energy of the 1L_a transition of tryptophan is more strongly dependent on the environment of the indole ring being particularly sensitive to hydrogen bonding interactions (Strickland et al., 1972), because the 1L_a excited state induces a positive charge on the ring nitrogen (Callis, 1997). Hydrogen bonding with proton acceptors increases the electron density on the ring nitrogen, decreasing the dipole moment and red-shifting the 1L_a transition. In difference spectra of proteins this leads to an increase of absorbance around 300 nm, whereas a weakening of hydrogen bonding interactions leads to a blue-shifting of the 1L_a band and a decrease of the absorbance at this wavelength (Lin and Sakmar, 1996).

Origin of the tryptophan perturbation spectra in T4 ligases

Two kinds of tryptophan residues could contribute to the perturbation spectra observed upon binding of a nucleotide to the ligase. One would be a residue outside the substrate-binding site, whose optical properties change due to a sub-

stantial conformational rearrangement of the protein in the substrate-bound form. In our work we found no evidence for such changes. Usually, the absorbance of several residues is expected to change, producing complex difference spectra with a high extinction, e.g., when a decrease of polarity around tryptophan residues is accompanied by an increase of polarity around tyrosine residues or vice versa (Demchenko, 1986). Also, it is unlikely that the microenvironment of a remote tryptophan would induce opposite optical effects upon binding of ATP and dATP compared with dCTP (by T4 DNA ligase).

The second kind of tryptophan residue would be one in the active site of the enzyme. Its environment can change from polar to nonpolar, for example, as a result of shielding from the solvent after substrate binding or due to direct hydrophobic interactions with the substrate. In the ATP binding pocket of two model enzymes the tryptophan residue is located perpendicularly to the adenine ring at a distance of 3.4–3.5 Å (Trp 167, T7 DNA ligase; Trp 190, mRNA CE). This residue is shielded from the solvent by the bound nucleotide and is located on the N1-C2 side of the adenine ring in both cases. The ring nitrogen of this tryptophan residue forms a hydrogen bond with the carbonyl oxygen of a peptide bond (3.06–3.08 Å to Phe 146, mRNA CE), which might be weakened upon binding of a nucleotide (4.21 Å to Thr 167, T7 DNA ligase) (for crystal structures see Subramanya et al., 1996; Hakansson et al., 1997).

Binding of ATP to both T4 ligases yields tryptophan perturbation spectra characteristic of a change of the microenvironment of this residue to more hydrophobic. In addition, the decrease of absorbance at 298 nm indicates a weakening of hydrogen bonding of the optically active tryptophan residue. The difference fluorescence spectra show that the emission of a tryptophan residue is quenched upon binding of the nucleotide. Therefore, there are sufficient arguments to propose that the optically active tryptophan residue is located in the ATP binding site close to the bound nucleotide, but not in stacking interaction, similar to the particular tryptophan residue of the model proteins alluded to above.

Differences between the T4 DNA and RNA ligases

The tryptophan difference spectra of T4 DNA and RNA ligase indicate, besides similarities, also marked differences between these enzymes.

For example, the active site of the T4 RNA ligase undergoes a more drastic polar-to-nonpolar transition upon binding of the nucleotide. Upon binding of ATP, the tryptophan absorbance of T4 RNA ligase shifts further to the red (292 nm for T4 DNA ligase versus 293 and 296 nm for RNA ligase). For both ligases, the difference excitation spectra display a red-shift of the excitation maximum, indicating an exposure of the emitting residue to a more hydrophobic

environment, but in the case of T4 RNA ligase the shift is more pronounced (5–8 nm vs. 2–4 nm, Fig. 6). The difference emission (Fig. 6 B1 and B2) shows that the quenched fluorescence of T4 RNA ligase originates from a surface tryptophan residue, which is exposed to the solvent. On the other hand, the analogous tryptophan residue of T4 DNA ligase is inaccessible to the solvent and interacts only with the protein-bound water molecules.

The 3D structures of the enzyme-nucleotide complexes of T7 DNA ligase and mRNA capping enzyme from *Chlorella* virus PBCV-1 show the presence of two hydrogen bonds with the 2'- and 3'-hydroxyl groups of the ribofuranosyl moiety of the bound nucleotide, suggesting that in the case of deoxyribonucleotide only one bond might be formed with the 3'-hydroxyl moiety. This could be the reason why the affinity of dATP for T4 DNA ligase is nearly two orders of magnitude lower than for ATP (Table 1). In the case of T4 RNA ligase the hypochromicity of bound dATP is four- to fivefold lower than that of ATP, whereas both show the same red-shift of the absorbance maximum and similar intensity of the tryptophan perturbation spectrum. dCTP does not change its spectrum in the presence of the enzyme. Assuming that T4 RNA ligase forms only one hydrogen bond with the nucleotide ribose via the 2'-hydroxyl group, the deoxyribose moiety of enzyme-bound dNTP would not be hydrogen-bonded at all, which may explain the differences between the spectra of the ligase-bound ATP and dATP.

Summarizing, we may conclude that the tryptophan residue in the ATP binding pocket of T4 DNA ligase is inaccessible to the solvent but is in contact with protein-bound water. Binding of the nucleotide changes the environment of this tryptophan to more hydrophobic, probably due to the exclusion of bound water or as a result of direct interactions with the nucleotide (but not through stacking). In the case of T4 RNA ligase, two of three tryptophan residues are located on the surface of the protein, inaccessible to the solvent but in contact with the protein-bound water molecules. The third residue is located in the ATP binding site of the enzyme and is exposed to solvent. Binding of the nucleotide excludes the solvent from the binding pocket, changing the environment of the exposed tryptophan residue to more hydrophobic.

It can be concluded that binding of ATP by DNA and RNA ligase from bacteriophage T4 involves several adenine-ligase interactions such as π -stacking, hydrophobic, and hydrogen-bonding interactions. In addition, the tryptophan residue is present in the active site of both ligases, and the binding of the nucleotide influences the hydrogen bonding of this residue. Collectively, these findings show that despite the low sequence homology, the ATP binding sites of T4 ligases are similar to those of related enzymes with known 3D structures. Pursuing the analogy further, a common protein fold might be responsible for a common catalytic mechanism in these four enzymes or, more generally,

in the nucleotidyltransferases of the entire superfamily. This suggestion supports predictions made on the basis of a structural genomic analysis of weakly related nucleotidyltransferases (Shuman and Schwer, 1995; Aravind and Koonin, 1999; Koonin and Gorbalenya, 1990).

We thank Prof. W. R. Hagen for critically reading the manuscript.

This work was supported by the Association of Biotechnology Centers in The Netherlands (project I.2.8) and by The Netherlands Research Council for Chemical Sciences with financial aid from The Netherlands Technology Foundation (grant 349-3565).

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