Structural and kinetic aspects of chemical reactions in DNA duplexes. Information on DNA local structure obtained from chemical ligation data

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ABSTRACT

Chemical ligation of oligonucleotides in double-stranded helices has been considered in its structural-kinetic aspect. A study was made of (i) two series of DNA duplexes with various arrangements of reacting groups in the ligation junction induced by mispairing or by alteration of furanose structure (the replacement of dT unit with rU, aU, IU, xU, dxT ones) and of (ii) eight synthetic water-soluble carbodiimides with different substituents at N1 and N3 atoms. We assumed that some information on the local structure of modified sites in the duplex can be obtained from kinetic parameters of oligonucleotide coupling reaction. The ratio of kinetic constants \( k_3/(k_2 + k_3) \) for productive and nonproductive decomposition of the activated phosphomonoester derivative apparently reflects the reaction site structure: for a given duplex this parameter is virtually independent of the condensing agent composition. Based on the analysis of the chemical ligation kinetics a suggestion has been made about the conformation of some modified units in the double helix.

INTRODUCTION

The interest in chemical reactions in spatially organized nucleotide systems, stirred up by the works on RNA self-cleavage and RNA self-splicing, has markedly increased in recent years. This class of reactions includes chemical ligation, i.e. covalent joining of oligonucleotide blocks on a complementary template under the action of chemical agents [1]. Studies on the chemical ligation patterns allow valuable information to be obtained on the reactive ability and local structure of double-helical nucleic acids. Such approach are also quite promising in respect of designing the nonenzymic means of assembling extended DNAs including those with preset modifications.

Obviously the efficiency of chemical ligation is associated with mutual arrangement of the reacting groups in the double helix.

We have earlier shown that a local structural alterations at the nick, caused e.g. by a mismatched base pair or a modified sugar residue, greatly affects the reaction rate [2].

The present work analyzes the interrelationship between the kinetic parameters of chemical ligation and the structure of the reaction site. To this end, we have introduced a parameter which is a relation of the rate constants for individual reaction steps. This parameter is shown to be a structural invariant of a given duplex, i.e. to be practically independent of the composition of chemical agents. The latter were a set of specially synthesized water-soluble carbodiimides differing in the nature of substituents at N1 and N3:

\[
\begin{align*}
C_2H_5NCNCH_2CH_2CH_2NH(CH_3)_2Cl & \quad \text{CDI 1} \\
CH_3NCNCH_2CH_2NH(CH_3)_2Cl & \quad \text{CDI 2} \\
C_2H_5NCNCH_2CH_2NH(CH_3)_2Cl & \quad \text{CDI 3} \\
CH_3NCNCH_2CH_2NH(CH_3)_2Cl & \quad \text{CDI 4} \\
C_2H_5NCNCH_2CH_2N(CH_3)_3I & \quad \text{CDI 5} \\
CH_3NCNCH_2CH_2N(CH_3)_3I & \quad \text{CDI 6} \\
C_2H_5NCNCH_2CH_2N(CH_3)_3I & \quad \text{CDI 7} \\
CH_3NCNCH_2CH_2N(CH_3)_3I & \quad \text{CDI 8}
\end{align*}
\]

The substrates were DNA duplexes la (synthesis of a phosphodiester bond) and la' (synthesis of a pyrophosphate bond):

\[
\begin{align*}
5' & \quad A-A-C-C-T-A-C-C-T^1 pG-G-T-G-T \\
3' & \quad T-G-G-A-T-G-G-A - C-C-A-C-C-A \\
\text{duplex la}
\end{align*}
\]

\[
\begin{align*}
5' & \quad A-A-C-C-T-A-C-C-Tp^1 pG-G-T-G-T \\
3' & \quad T-G-G-A-T-G-G-A - C-C-A-C-C-A \\
\text{duplex la'}
\end{align*}
\]

Then the kinetic aspects of chemical ligation were studied in duplexes with a variable reaction site, using such modifications as would alter the relative positions of the groups while leaving

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their chemical nature unchanged. Two families of DNA duplexes of different primary structure were constructed (Scheme 1).

Modifications at the nick (indicated by arrow on the Scheme, the structure expanded in the squares) included:

1. Altered position of the phosphate group: 3'- or 5'-terminal (duplexes IIa and IIb).
2. Replacement of the 3'-terminal thymidine with ribouridine (duplexes IC, IIg) or a nucleotide with reversed configuration at C-2' and/or C-3' atoms of furanose, such a modified units being either the phosphate acceptor (duplexes Ib, Id–f, IIc and IIe) or donor (duplexes II d and II f).
3. Insertion of an 'extra' purine or pyrimidine residue at the donor or the acceptor end (duplexes Ig–k).
4. Replacement of the dG·dC pair one base step away from the ligation junction with a dG·dG mismatch (duplex H).

Analysis of the kinetic parameters of chemical ligation allowed a suggestion about the local conformation of the region adjacent to the nick.

**MATERIALS AND METHODS**

**General**

The following reagents were used: 1-ethyl-3-(3'-dimethylamino- propyl)-carbodiimidine, hydrochloride, CDI 1 (Merck), the rest of the water-soluble carbodiimides (CDI 2-8) were synthesized as described previously [3], γ-32P ATP (Amersham), T4 polynucleotide kinase, T4 DNA ligase (USSR), PNase A (Reanal), PNase T2 (Sankyo).

Buffer solution: A (for CDI-induced ligation)—50 mM MES [4], pH 6.0, 20 mM MgCl2; B (for PNase A)—0.1 M Tris-HCl, pH 7.2, 2 mM EDTA; C (for RNase T2)—0.04 M ammonium acetate, pH 4.5, 2 mM EDTA.

**Preparation of synthetic oligomers**

Oligodeoxynucleotides employed to form DNA duplexes I and II were synthesized by the phosphoramidite method on a Cyclon (Biosearch) DNA synthesizer. Nucleosides with reversed configuration at C2’ and (or) C3’ atoms were obtained from corresponding natural nucleosides via the anhydroderivatives [5]. The introduction of protecting groups was effected by the standard procedure. To phosphitylate MeOTtdxT and MeOTtrU the reaction time was increased to 5 and 24 hours, respectively. Modified hexanucleotides with the 3’-terminal phosphate group were obtained from heptanucleotides d(ACGGA)MrU, where M is dxT or aU, by periodate oxidation of the 2’,3’-cis-hydroxyl system followed by β-elimination [6]; oligonucleotides d(AAAA) and d(AACCTACCTp) were prepared in the same way. Synthesized oligomers were finally purified by HPLC on a Lichrocor C-18 column (4.6x250 mm, Merck) in an Altex chromatograph.

**Optical measurements**

Nucleotide concentration was determined on a Spectronic 2000 spectrophotometer (Bouch and Lomb). The molar extinction coefficients, ε260, of anomalous nucleotide residues were taken equal to ε260 of natural analogs. The melting curves of duplexes I and II were measured on a Cary 219 spectrophotometer (Varian) in thermostatted quartz cells of 1 mm path length.

**Hydration of water-soluble carbodiimides**

Initial carbodiimide concentration was 0.2 M. Hydration was studied in buffer A or in buffer A containing 5·10^-4 M (per monomer) dPA, d(AAAA) or d(AAAA)P. Carbodiimide conversion was assayed spectrophotometrically using a quantitative color reaction for the -N=C=N- group [3,7].

**Chemical and enzymatic ligation of oligonucleotides**

Nucleotide concentration of duplexes I and II was about 10^-3 M (per monomer); 32P labelled oligonucleotides were added in a 1.5-fold deficiency as compared to the unlabelled ones. The concentration of CDI 1—8 and incubation temperature were varied in different experiments (see footnotes to Tables 1 and 3). The changes in the concentrations of compounds were followed by withdrawing samples; carbodiimide conversion was assayed spectrophotometrically as described above, oligonucleotide conversion by electrophoresis in denaturing 20% polyacrylamide gel. The nucleotide fraction was precipitated with 2% LiClO4 in acetone beforehand.

Enzymatic ligation in duplexes IC, IIg under the action of T4 DNA ligase was carried out as in [8].

**Analysis of ligation product**

32P labelled oligonucleotide was treated with 0.3 N NaOH at 37°C for 3 hours. Then the reaction mixture was neutralized by 40% aqueous solution of acetic acid, oligonucleotide fraction was precipitated and analyzed by 20% polyacrylamide gel electrophoresis.

Hydrolysis of the ligation product with RNases A or T2 was carried out in buffer B or C, respectively, for 1.5 hours at 37°C. Statistical treatment of the data was carried out by the least squares method.

**RESULTS AND DISCUSSION**

**Kinetics of chemical ligation**

The general kinetic scheme of chemical ligation induced with a water-soluble carbodiimide as applied to homooligomer condensation in a triple-helical complex was proposed by Popov...
et al. [9]. The heterogeneous single-nicked DNA duplexes considered here offer a much better model allowing quantitative interpretation of the experimental data. Our theoretical consideration is based on Popov's kinetic scheme:

\[
\begin{align*}
S + X & \xrightarrow{k_1} SX \xrightarrow{k_2} S + Y \\
X & \xrightarrow{k_4} Y \\
& \xrightarrow{k_3} P + Y
\end{align*}
\]

where \(S\) is the nicked duplex,
\(P\) is the condensation product (ligated duplex),
\(X\) is the activating agent,
\(Y\) is the hydration product of the activating agent,
\(SX\) is the activated adduct.

The scheme was constructed with the following assumptions:
1. In the reaction conditions the duplex is regarded as a perfect helix.
2. Reactions II (hydration of the activated adduct) and IV (hydration of the activating agent) are regarded as pseudofirst-order reactions.
3. Modification of bases in the oligonucleotides forming the duplex is disregarded.
4. The internucleotide bond has no catalytic influence on hydration of the activating agent.

This scheme is described by a system of five equations which cannot be completely solved in analytical form without additional assumptions. Let us consider a possible case when steps I and IV i.e. reactions with nucleophiles, are rate-limiting. Besides, let us assume that \(k_1[S] \ll k_4\), Transformation of the kinetic equations taking into account these assumptions yielded the following equation [9]:

\[
\ln \left( \frac{[S]_0}{[S]} \right) = \frac{k_1[X]_0}{k_4} \frac{k_3}{k_2 + k_3} \cdot (1 - e^{-k_4t})
\]

The rate of accumulation of the ligation product is determined both by the relation between \(k_1[S]\) and \(k_4\) (showing what portion of the reagent is used to activate the phosphate group in the nick, as related to the amount undergoing hydration) and by the relation between constants \(k_2\) and \(k_3\) (showing the portion of the activated adduct \(SX\) decomposing by the productive pathway).

Let us consider the possibility of determining the rate constants for individual chemical ligation steps. The constant \(k_4\) is obtained in a separate experiment. The rate constant for formation of the activated adduct, \(k_1\), can be determined with the use of model systems, e.g. a mononucleotide or a phosphorylated oligonucleotide, whereby the general reaction scheme is simplified:

\[
\begin{align*}
S + X & \xrightarrow{k_1} SX \\
X & \xrightarrow{k_4} Y
\end{align*}
\]

where \(S\) is the mono- or oligonucleotide with phosphomonoester group,
\(X\) is the activating agent,
\(Y\) is the hydration product of the activating agent,
\(SX\) is the activated adduct.

Solution of the equations corresponding to this scheme, assuming a limiting step of adduct formation, leads to the following expressions:

\[
k_1[S]_0 + k_4 = \frac{1}{t} \cdot \ln \left( \frac{[X]_0}{[X]} \right)
\]

Thus, knowing the slope \(\alpha\) of the straight line in the plot \(\ln[X]\) vs \(t\), the rate constant for activated adduct formation can be obtained from the equation:

\[
k_1 = \frac{k^* - k_4}{[S]_0}
\]

Within the assumptions made (SX formation is the limiting step) the absolute values of the decomposition rate constants of the activated adduct cannot be determined. However, with the use of the transformed equation (1) the slope of the straight line in the plot

\[
\ln \left( \frac{[S]_0 - [P]}{[S]_0} \right) \text{ vs } \frac{1}{k_4} \cdot [X]_0 \cdot (e^{-k_4t} - 1)
\]

yields the apparent rate constant of the coupling reaction, \(k_3\cdot k_1/(k_2 + k_3)\). Knowing \(k_1\), it is easy to determine the ratio \(k_3/(k_2 + k_3)\) reflecting the portion of the activated adduct decomposing to give the chemical ligation product.

Let us analyze the factors affecting this ratio. In the course of breakdown of SX (Scheme 2) the activated phosphate can participate in two competing reactions — with the hydroxyl of the neighboring nucleotide unit and with a water molecule. Obviously, the \(k_2\) and \(k_3\) values are proportional to the partial positive charge on the phosphorus atom and nucleophilicity of the reacting groups. When different condensing agents are used the absolute values of the constants would change, but if the reactivity of the attacking groups is the same and there are no substantial distortions in the structure of the ligation site, then the \(k_2/(k_2 + k_3)\) ratio for a given complex will remain constant.

The rightfulness of the assumptions made in composing the chemical ligation kinetics is confirmed by the following data.

1. Analysis of the melting curves of duplexes Ia and Ia’ shows that at the temperature of 10°C the amount of unpaired molecules can be neglected. The \(T_m\) of duplexes Ia and Ia’ are 35.0 and 34.0°C, respectively, and the water-soluble carbodiimide lowers the \(T_m\) of nucleotide complexes only by a few degrees.

2. The kinetic curves of hydration of CDI 1—8 are linearized well in the plot \(\ln[X]\) vs \(t\) [3], which confirms the pseudofirst order of the reaction.

3. During chemical ligation under conditions when the double helix is stable, modification of the nucleotide material by CDI is insignificant [2].
4. In contrast to reactions in organic solvents where carbodiimide is also consumed in the interaction with the internucleotide phosphate [10], in the aqueous solution we could observe no appreciable influence of phosphodiester groups on carbodiimide hydration. Thus, in the presence of 5 \times 10^{-4} \text{ M} \) (per monomer) of d(AAAA) the CDI 1 hydration curve practically coincides with the control one obtained in buffer A. The rates of interaction of CDI 1 with the 3'- and the 5'-terminal phosphate groups are approximately the same. This is evidenced by close \( k_1 \) values obtained in buffer A containing dpA or d(AAAAp): 3.2 and 3.3 h^{-1}M^{-1}, respectively.

**Template-directed ligation of oligomers with different water-soluble carbodiimides**

When designing the set of CDI 1 to 8, the rationale was as follows. CDI 1 is known to undergo tautomeric cyclization in aqueous solutions [11]. The structural alterations introduced by us allow elucidation of the roles of both tautomeric forms in coupling reactions. The propensity of CDI 1–8 to ring-chain tautomerism and the hydration constants in the absence and in the presence of a phosphate donor—mononucleotide—are presented elsewhere [3]. It turned out that carbodiimides containing two methylene links instead of three and a terminal tertiary amino group are prone to form a stable five-membered ring hindering their interaction with nucleophiles. Carbodiimides with a quaternary ammonium group can exist in an aqueous buffer solution exclusively in the linear form which is the reactive one.

For compounds that are short-lived in buffer A, such as CDI 5, 6 and 8, \( k_1 \) cannot be determined exactly, since the component corresponding to carbodiimide interaction with the phosphononoester group is hard to discern on the background of its own rapid hydration. The \( k_1 \) and \( k_4 \) values for some carbodiimides that are most promising as ligation agents are presented in Table 1.

The activity of carbodiimides was first assessed by their ability to produce a pyrophosphate bond in duplex 1a'. This duplex was chosen as a test system owing to the high reaction rate, which speeds up and facilitates 'selection' of carbodiimides by their activity. As can be seen in Fig. 1, CDI 3 and 4 forming a stable five-membered ring proved ineffective in the chemical ligation reactions. With the other carbodiimides kinetic curves were obtained for accumulation of the ligation product with a pyrophosphate bond (Fig. 2). It should be noted that in the standard reaction time, 6 h, only 20–40% of even the most active condensing agents undergoes hydration (Fig. 2).

### Table 1. Kinetic characteristics of water-soluble carbodiimides*

<table>
<thead>
<tr>
<th>Number of CDI</th>
<th>Buffer A, 0°C</th>
<th>Buffer A, 10°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_1 ) M^{-1}h^{-1}</td>
<td>( k_1 ) M^{-1}h^{-1}</td>
</tr>
<tr>
<td>1</td>
<td>0.19 ± 0.03</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>0.12 ± 0.02</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>4.8 ± 0.02</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>8.6 ± 1.0</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>1.3 ± 6.1</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>8</td>
<td>2.0 ± 0.3</td>
<td>0.06 ± 0.06</td>
</tr>
</tbody>
</table>

* The \( k_1 \) and \( k_4 \) values taken from [3].

** Pyrophosphate bond synthesis in duplex 1a'; carbodiimide concentration 0.1 M; the ratios of constants are presented per one phosphomonoester group in the reaction site.

*** Phosphodiester bond synthesis in duplex 1a'; carbodiimide concentration 0.1 M; the ratios of constants are presented per one phosphomonoester group in the reaction site.

![Figure 1. Electrophoretic analysis (20% denaturing PAGE) of reaction mixtures containing duplex 1a', after 24 h of incubation in buffer A at 0°C with various carbodiimides (initial CDI concentration is 0.1 M). The 32P label was incorporated in hexanucleotide d(pGGTGGT). The lane number corresponds to that of carbodiimide. The chain length of oligonucleotides is indicated to the right. The arrows indicate the positions of markers: bromophenol blue (BPB) and xylene cyanol (XC).](image1)

![Figure 2. Accumulation of the duplex 1a' ligation product under the action of various carbodiimides: (1) CDI 1, (2) CDI 5, (3) CDI 7, (4) CDI 2, (5) CDI 6, (6) CDI 8; and hydration of the corresponding carbodiimides at 0°C in buffer A: (1') CDI 1, (2') CDI 5, (3') CDI 7, (4') CDI 2, (5') CDI 6, (6') CDI 8. For reaction conditions and assay techniques see Materials and Methods and footnotes to Table 1.](image2)
The same group of carbodiimides were used as condensing agents to synthesize a phosphodiester bond in duplex la (Fig. 3). As expected, the efficiency of internucleotide bond formation with linear CDI 5–8 was low because of their rapid hydration under reacting conditions. The most efficient phosphodiester bond formation was achieved with the less reactive but ‘longer living’ CDI 1 and 2.

The kinetic data on the accumulation of the ligation product in duplexes la and la’ under the action of CDI 1, 2 and 5–8 allowed the apparent reaction constants $k_1/k_2/(k_2+k_3)$ to be determined (Table 1). Evaluation of $k_3/(k_2+k_3)$ was only possible for CDI 1, 2 and 7 for which the $k_j$ is reliably known (Table 1). In the case of duplex la’ (pyrophosphate bond formation), taking into account that there are two phosphate groups in the reaction site, the $k_3/(k_2+k_3)$ value was divided by two.

Analysis of the data obtained allows the following conclusions to be made.

1. There is an interrelationship between the composition of carbodiimides and the kinetic parameters of their reactions, the hydration rate constants and the apparent constants of chemical ligation.

2. The $k_3/(k_2+k_3)$ values for a given duplex are close for different carbodiimides (taking into account the significant inaccuracy in determining the $k_1$ for CDI 7): ca. 0.004 with duplex I (phosphodiester bond formation) (Fig. 4, Table 1) and ca. 0.13 for duplex II (pyrophosphate bond formation) (Table 1).

Consequently, this parameter can serve as an invariant of the duplex. Obviously this is only true when the active phosphate group derivative of the oligonucleotide does not distort the structure of the reactive site.

3. Only a minor portion of the water-soluble carbodiimide is spent to activate the phosphomonoester groups: ca. 7% of the carbodiimides capable of cyclization (CDI 1 and 2) and ca. 1.3% of noncyclizing ones (CDI 7). In the course of pyrophosphate bond formation ca. 13% of the activated adduct decomposes via the productive pathway, whereas with phosphodiester bond formation this portion decreases to 0.4%. The results obtained provide an assessment of the relative nucleophilicity of the hydroxyl and the phosphomonoester groups attacking the activated phosphate (there is a 30-fold difference, which does not contradict the published data [9]).

Interconnection between kinetic parameters and DNA local conformation at the ligation junction

Ligation in duplexes I and II was carried out using CDI 1 as the condensing agent. Among the set tested, it was this carbodiimide that proved the most suitable for the formation of a phosphodiester bond.

A preliminary study was made of thermal stability of the duplexes I and II. It turned out that none of the modifications used except the G•G mismatch (which lowered the $T_m$ of the
double helix by 10°C had an appreciable effect on duplex stability (the $T_m$ of duplexes I was about 33°C, duplexes II about 47°C).

Under optimal conditions (buffer A, temperature 10°C, 0.2—0.3 M CDI) coupling reaction proceeded in all duplexes tested but with different efficiency. Figures 5 and 6 exemplify the results of electrophoretic analysis of some reaction mixtures.

Before analyzing the kinetic patterns, we shall present data confirming the structure of ligation products. The primary structure of 15—17-membered oligomers containing no anomalous nucleotide units was confirmed by Maxam-Gilbert procedure. The nature of the bond formed with the participation of rU as well as aU, Iu, or xU was proved by alkaline and enzymic hydrolysis (with RNases A and T2) of the corresponding oligomers. The conditions of hydrolysis were chosen so as to provide complete hydrolysis of a control hexanucleotide (see, for instance, Fig. 7, lanes 5 and 6), whereas oligomers and d(AACCTACCxTGGTGGT) proved to be more slowly cleaved by snake venom phosphodiesterase with maximal retardation observed for the dxT-containing oligomer (data not shown).

Let us now analyze the kinetic parameters of coupling reaction in duplexes I and II. The linear anamorphoses of the kinetic curves are presented in Figs. 8 and 9. The $k_3/(k_2+k_3)$ ratios were obtained from their slopes (Table 3); the values of $k_1$ and $k_4$ are given in Table 1. It can be seen that the ratio of the constants varies greatly for different duplexes. It would be risky to attempt to derive conformational conclusions from these values alone. However, by analyzing the whole body of data for the series of duplexes and experimental facts from earlier publications, interesting information can be obtained on the influence of various modifications on the local structure of the double helix.

So, the $k_3/(k_2+k_3)$ values for duplexes Ia and II are practically the same (Table 3). This indicates that the structural perturbation caused by the dG•dG pair does not affect the orientation of the reacting groups of the neighbouring unit and is therefore only local. This conclusion does not contradict the data on the influence of dG•dG pairs on the physicochemical parameters of oligonucleotide duplexes [12]. On the other hand $^{31}$P NMR

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**Table 2.** Enzymatic and chemical hydrolysis of ligation products in duplexes Ic—f.

<table>
<thead>
<tr>
<th>No</th>
<th>Oligonucleotide structure</th>
<th>Hydrolysis extent, %</th>
<th>RNase A</th>
<th>RNase T2, 0.3 N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>d(AACCTACC)xUd(GGTGGT)</td>
<td>30</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>d(AACCTACC)Ud(GGTGGT)</td>
<td>0</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>d(AACCTACC)xUd(GGTGGT)</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>d(AACCTACC)Ud(GGTGGT)</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>d(AAAUUU)*</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Hexaribonucleotide was used as a control.
The ratio of kinetic constants calculated on the basis of data in [2].

studies indicate that the distortion unduced by dG·dG mismatch is still felt several base pairs away [13]. An ‘extra’ nucleotide residue decreases the efficiency and the rate of the chemical reaction; the \( k_3/(k_2+k_3) \) value for duplexes Ig-k falls by half as compared with the nonmodified duplex. According to the published data, an ‘extra’ adenine base [14] is integrated into the double helix, whereas thymine at low temperature most probably remains outside the helix [15]. It can be supposed that a single-strand break at the place of the defect increases the extent of freedom of the unpaired unit. If the ‘extra’ unit is the phosphate donor (duplex Ik), then there is an additional factor diminishing the efficiency of coupling reaction; the activated derivative proves less protected by the duplex from the attack by water.

An important and nontrivial observation is that the ligation parameters differ for the nonmodified duplexes Ia and IIa. This may be explained by the different nature of joined units: dT and dG in duplex Ia, dT and dC in duplex IIa (such a sequence dependence of the chemical ligation efficiency has been noted before [16]) as well as by the different secondary structure of duplexes I and II. Elements of the A form in the structure of duplexes I have been suggested on the basis of analysis of the CD spectra and the literature data [17 and its references]. Can one suggest that this is due to a drastically altered conformation of the 3'-terminal unit. Typical for B-DNA is the C2'-endo conformation of the furanose rings with pseudo-axial orientation of the 3'-hydroxyl group (Scheme 3). At the same time, in a hybrid duplex comprising covalently bound RNA and DNA blocks the ribonucleotide units, because of their structural conservatism, retain the C3'-endo conformation typical of the A form [19], with the 3'-hydroxyl group occupying the pseudo-equatorial orientation which sterically hinders its interaction with the phosphate group of the adjacent molecule. If this suggestion is true then chemical ligation in A-form duplexes would be much less efficient than in B-like helices. Indeed, low yields of ligation products were observed in our experiments with synthetic RNA fragments (next publication).

The structural inequality of duplexes I and II is reflected in the fact that the relative decline in coupling efficiency (as compared with nonmodified counterparts) is different for duplexes Ic and IIc (Table 3). Also different are the percentages of their 3'-5' - and 2'-5'-linked isomers: 87% of the natural bond in the ligated site of duplex IIg and 30–40% in duplex Ic. Obviously in these duplexes the activated phosphate is in dissimilar position with respect to the 3'- and 2'- hydroxyls.

Now we shall consider duplexes Ib, Id–f, Ile–f containing nucleotide residues with reversed configuration at the furanose C2' and/or C3' atoms. As can be seen in Table 3, the presence of an anomalous nucleoside residue (dT, dU, IU or xU) markedly attenuates the ligation efficiency. The most interesting data were obtained with arabinosyl-containing systems. The \( k_3/(k_2+k_3) \) value for duplex IIe with an aU unit in the acceptor end is one order of magnitude lower than that for the nonmodified duplex Ila (Table 3). At the same time, when the arabinose derivative is the phosphate donor (duplex III containing a -paUp fragment), the \( k_3/(k_2+k_3) \) value is comparable to that of the nonmodified duplex IIb (Table 3). Apparently the conformation of an anomalous unit adjacent to the nick differs depending on whether it contains a 3'-phosphate or not. It is known that, unlike natural monomers, the arabinonucleosides show a clear preference for one or the other conformation of the furanose ring depending on the position of the phosphate group: (C3'-endo) N-type for paA, and (C2'-endo) S-type for aAp [20]. This dependence is also maintained at the oligomeric level. Thus a dinucleoside phosphate aApA is a mixed S-N conformer (the strong bias toward S-type conformation of the aap-residue and preference for N-type in -paA residue), a study of a trimer aApApaA allowed it to be described as an S-S-N conformer [20]. Based on the results of chemical ligation and on the above data one can postulate with good probability that the -paU residue in duplexes Id and Ile has N-type conformation with pseudo-equatorial position of the 3'-hydroxyl group (Scheme 4). When the arabinose derivative contain the phosphate group at O3' (duplex IIb) the conformation of the anomalous furanose changes from N- to S-type, ensuring the pseudo-axial position of the reacting group which is optimal for interaction.
From these suggestions it follows that in a DNA duplex the arabinonucleotide residues retain their structural autonomy, i.e. persist in the conformation typical of them at the monomeric or oligomeric levels. Since our findings and the NMR spectroscopic data on nicked and non-nicked DNA duplexes [21] argue in favour of at least partially fixed conformation of the nucleotide residues at the nick, one can suppose that in the covalently continuous DNA the arabinonucleotide residues would also be in S-type conformation. This is in line with the data [22] on EcoRI cleavage of DNA duplexes containing AA in the cleavage site.

Different kinetic patterns are observed for dxT-containing duplexes IIc,d. The coupling yield is not appreciably affected by the position of the phosphate group in the ligation site, and in both cases it is significantly lower than with the nonmodified duplex Ia (Table 3). This may point either to retention of the same conformation of the 3'-terminal anomalous unit that is unfavorable for ligation, or to steric hindrances due to inversion of the 3'-hydroxyl group. Since there are no data on the conformation of dxT-derivatives, to check our suggestions we undertook an analysis of the molecular models of these compounds. This analysis demonstrates that for dxT and its 3'-phosphate the S-type conformation is the spatially unobstructed one (Scheme 5), whereas in the N-type the substituent at C3' situated above the sugar ring is in sterically unfavorable proximity to the hydroxyl at C5' and to the heterocyclic base. For -dxTp and -dxTp residues in the nick an S-type conformation determines the pseudo-equatorial position of O3' atom, which, as already mentioned, tells adversely on the efficiency of oligonucleotide ligation.

The chemical ligation technique used in the present work to elucidate the local structure of DNA is in a sense similar to the enzymic one [22], but unlike restriction endonucleases which cleave DNA in a strictly definite place, a condensing agent joins any nucleotide units at the single-strand break. Various conformational alterations at the nick, while affecting to a varying extent the reaction efficiency, nevertheless do not block it completely. This approach, supplementing the conventional physicochemical methods and theoretical conformational analysis, allows a more reliable assessment of the conformation of nucleotide residues adjacent to the nick and even 'within' an intact double helix.

**CONCLUSION**

Studying the relationship between the kinetic parameters of chemical ligation and the local structure of nucleotide duplexes is of key importance for further development of the method both in theoretical and applied aspects. First, this expands our knowledge of the peculiarities of reactions in spatially organized complexes stabilized by weak multipoint interactions, of the extent of restraint on the reacting groups therein, and helps to improve the kinetic description of such enzyme-like reactions etc. Second, such an approach allows evaluation of the prospects of introducing a certain modification in the DNA sugar-phosphate backbone by chemical ligation, provides an assessment of the selectivity of reactions involving polyfunctional units (e.g., ribose derivatives), and opens the way to chemical probing of the fine structure of nucleotide duplexes.