A New Approach to Determine the Effect of Mismatches on Kinetic Parameters in DNA Hybridization Using an Optical Biosensor

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(Received 20 November 1995)

Abstract

We have demonstrated a simple yet direct method for determining the kinetic parameters in DNA-DNA interactions using biosensor technology based on the surface plasmon resonance phenomenon; a technique that does not require complex DNA labeling. To determine the effect of mismatches on the kinetics involved in DNA-DNA interactions, DNA hybridization kinetics were monitored in real time using synthetic oligonucleotides less than 20 bases in length which contained either a complementary sequence or mismatched bases. Upon analysis of the kinetic parameters obtained in oligonucleotide hybridization, we found that they were significantly affected by the presence of mismatches as well as by their number and location in a DNA duplex. In addition, the presented biosensor method is sensitive enough to detect kinetic effects caused by the presence of a single-mismatched base pair. Our findings strongly suggest that analysis of kinetic parameters involved in DNA-DNA interactions is advantageous for detecting the presence of mismatch base pairs in a DNA duplex.

Key words: kinetic parameter; surface plasmon resonance; hybridization

1. Introduction

The development of the polymerase chain reaction (PCR) technique has led to a rapid progression of improvements in the detection methods used to identify DNA structural changes, especially following the advancement of allele-specific oligonucleotide hybridization using a DNA probe, which has enabled the detection of structural changes in genes associated with genetic diseases. Thus, the specificity of the hybridization rate in oligonucleotide hybridization has provided the potential for new technology developments in molecular biology. The effect of the presence of mismatch formations on kinetics involved in DNA hybridization, yet, has not been clearly determined, therefore a straightforward and fast method is needed to analyze DNA-DNA interactions. Achieving this goal is not so easily accomplished though, because the molecular basis of such interactions occurring in a DNA hybridization reaction remains unclear, and also since limited research has been directed at investigating the effect of mismatched base pairs (non-Watson-Crick base pairs) in a DNA duplex. The above facts led to the present study in which we analyzed DNA-DNA interactions by measuring in real time the kinetic parameters in DNA hybridization, thereby enabling us to identify the effects of mismatch formations on kinetic parameters. To measure these parameters, we employed the BIAcore system that is based on technology using surface plasmon resonance (SPR), being specifically developed to perform real-time analysis of the kinetics in biomolecule interactions without the use of labeling molecules. Briefly, SPR is an optical phenomenon in which light is focused through a prism onto a thin gold film on a glass support, where at a particular angle of incidence, the intensity of the reflected light is decreased due to the energy transfer from the light beam to the surface electrons on the film. The angle of the reflected light is dependent on the reflective index near the surface of the thin gold film. Such changes are proportional to changes in the adsorbed biomolecules present on the surface of a “sensor chip,” which is comprised of a glass slide covered by the thin gold film coated with carboxymethylated dextran. A ligand is immobilized on the surface of the sensor chip and interactions between it and a target molecule can be monitored in real time via changes occurring in the refractive index expressed in resonance units (RU).

To study DNA-DNA interactions, we immobilized 5'-biotinylated 20-mer oligonucleotide onto streptavidin bound to the chip's dextran matrix. Next, the target DNA, which had either a complementary sequence or mismatched bases in its sequence, was injected into the instrument. The association and dissociation kinetics of DNA molecules were then monitored in order to evau-
ate the kinetic rate and affinity constants. Here, we describe a relatively simple, direct, biosensor technology-based method which can determine the kinetic parameters in a DNA-DNA interaction, with results showing that these parameters are affected by the presence of mismatches, as well as their number and location in a DNA duplex, and also by the presence of a single mismatched base pair. This finding leads to the possibility to develop a novel method for mutation detection in the diagnosis of genetic disease and various forms of cancer.

2. Materials and Methods

2.1. Instrumentation

The BIAcore system is comprised of an optical detector system (Fig. 1), a removable sensor chip, a microfluidic cartridge which controls the injection of the sample onto the sensor chip surface, and an autosampler for injecting the sample. The instrument is controlled by a personal computer with dedicated software and a data evaluation program. The microfluidic cartridge holds the sensor chip in contact with the prism which is part of the optical system. The sample in its buffer solution flows over the sensor chip consisting of a glass slide coated on one side with a thin gold film. The prism focuses polarized light into a transverse wedge which hits the glass-gold interface, and the reflected light is detected by a two-dimensional array of photodiodes which measure its change in intensity, e.g., at \( t_1 \) no target DNA is present in buffer flow, while at \( t_2 \) the target DNA is loaded and reacts with the probe (Fig. 2). The shift in angle \( \Delta R \) is continuously monitored and expressed as a sensorgram.

2.2. Reagents

The following reagents were used: Sensor Chip CM5, N-hydroxsuccinimide (NHS), N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC), and ethanolamine hydrochloride (Pharmacia Biosensor AB); and streptavidin (Pierce Co.). Oligonucleotides were automatically synthesized using a Gene Assembler Special DNA synthesizer and commercially available amidites (Pharmacia Biotech AB).

2.3. Synthesis of oligonucleotides

The immobilized and target oligonucleotides, the sequences of which are summarized in Table 1, were synthesized using phosphoramidite chemistry. The sequences for oligonucleotides 613T, 615T, 620T, 696C and 697C are derived from the Phenylalanine Hydroxylase gene. The immobilized 5'-biotinylated 20-mer oligonucleotide of 400T, designed with a 50% GC content to avoid forming a secondary structure, 613T, 615T and 620T were synthesized using biotinylating amidite (Biodite, Pharmacia Biotech AB). All of the resultant oligonucleotides were purified with anion-exchange chromatography on a high-performance liquid chromatography system (FPLC System, Pharmacia Biotech AB). As a purification buffer, we used eluent A containing 0.01 M NaOH (pH 12.0) and eluent B containing 0.01 M NaOH and 1.5 M NaCl (pH 12.0). Purification and analysis of the oligonucleotide...
were performed on an anion-exchange chromatography column (Mono Q HR5/5, Pharmacia Biotech AB) with a 15–65% eluent B linear gradient at a flow rate of 1.0 ml/min. Purified oligonucleotide solutions were desalted, evaporated, resuspended in sterile water, and their concentration determined by an optical density measurement at 260 nm.

2.4. Biotinylated oligonucleotide immobilization

A biotinylated oligonucleotide probe was immobilized on the surface of the sensor chip by binding with streptavidin as previously described. Immobilization of streptavidin on the carboxymethylated dextran matrix covering the surface of the sensor chip was performed at 37°C as follows. The sensor chip was first washed for 5 min with HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA) at a flow rate of 5 µl/min, with the activation of the dextran matrix being carried out by injecting a mixture (35 µl) of 100 mM NHS and 400 mM EDC of equal volumes. In order to bind streptavidin to the dextran matrix covalently, 35 µl of streptavidin was injected at 400 µg/ml in 10 mM sodium acetate (pH 4.5), followed by reaction of excess activated groups with 1 M ethanolamine hydrochloride (pH 8.5) to give a reproducible response of about 13000 RU. The biotinylated oligonucleotide at 1 µM in TES buffer (10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1 mM EDTA) was then injected and consequently immobilized on the bound streptavidin. The SPR response of the immobilized oligonucleotide probe was about 3000 RU; a value indicating that about 3.0 ng/mm² of the oligonucleotide had been bound, i.e., 1000 RU corresponds approximately to 1.0 ng/mm² of biomolecules on the sensor chip surface.

2.5. DNA hybridization

To determine the kinetic parameters involved in the interactions between target and immobilized oligonucleotides, the target oligonucleotides dissolved in hybridization buffer (6×SSC: 0.9 M NaCl, 0.09 M sodium citrate, pH 7.2) were injected into the system and allowed to flow over the immobilized probe at different concentrations. This was performed at 37°C at a 2 µl/min flow rate for 5 min. After hybridization, the DNA-DNA duplex was washed with 6×SSC buffer alone at a flow rate of 2 µl/min for 5 min to initiate dissociation. The resultant SPR signal corresponded to the changes of the refractive index near the surface of the sensor chip due to the binding of the target oligonucleotide to the immobilized probe, and a decreasing SPR signal was observed during the dissociation of the target oligonucleotide from the formed oligonucleotide duplex on the sensor chip surface in the hybridization buffer only.

2.6. Measurement of association/dissociation rate constants

The theoretical background and basic method for determining the kinetic parameters using the BIAcore system were previously reported. The estimation of association and dissociation rate constants, k_{ass} and k_{dis}, was carried out with linear analysis software. Briefly, the rate of change in the amount of target DNA bound to the immobilized probe expressed in resonance units is described by

\[
\frac{dR}{dt} = k_{ass}(R_{max} - R)C - k_{dis}R
\]

where \(R_{max}\) is the maximum binding capacity of the immobilized DNA probe, \(R\) is the amount of target DNA bound to the immobilized probe, and \(C\) is the concentration of the flowed target DNA. In principle, \(k_{ass}\) can be obtained by plotting the slope of \(dR/dt\) versus \(R\) (expressed in RU) against the concentration of target DNA, i.e.,
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3. Results and Discussion

3.1. Kinetic measurements using complementary oligonucleotides

Figure 3 shows a sensorgram of the interactions between the immobilized 20-mer oligonucleotide probe (400T) and a 20-mer oligonucleotide with either a complementary (401C) or unrelated sequence [(dG)20], in which the former sequence indicates a large increase in relative RU, whereas the latter sequence contrastingly indicates a small increase. These results demonstrate that the large increase in relative RU is due to a specific interaction taking place between the two oligonucleotides; hence a particular DNA-DNA interaction can be observed as previously described by monitoring the kinetic parameters of the interaction using the BIAcore system.16 With the resultant sensorgram data, \( k_{\text{ass}}, k_{\text{diss}}, \) and \( K_a \) for five different concentrations of 401C were easily calculated (Fig. 4A). \( \frac{dR}{dt} \) is the rate of formation of surface complex, i.e., the derivative of the observed sensorgram curve. Figure 4B shows \( \frac{dR}{dt} \) as a function of \( R \) for the various concentrations. \( k_{\text{ass}} \) for this interaction was obtained by plotting the slope of \( \frac{dR}{dt} \) vs. \( R \) for each concentration with respect to concentration (Fig. 4C), then determining the resultant slope and calculating \( k_{\text{ass}} \) using Eq. (1). The average value of \( k_{\text{ass}} \) for 401C was \( 2.9 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \), having a range of \( 2.3-3.1 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) in four separate experiments. The intercept on the ordinate theoretically represents \( k_{\text{diss}} \); however, by washing the DNA-DNA duplex in a target oligonucleotide-free buffer, the value of \( k_{\text{diss}} \) can be calculated using changes in the resonance signal when dissociation occurs after hybridization using Eq. (2). Consequently, sensorgrams were used from at least two different concentrations to calculate \( k_{\text{diss}} \) with the average value for 401C being \( 1.2 \times 10^{-4} \text{s}^{-1} \), ranging from 1.1 to 1.4 \( \times 10^{-4} \text{s}^{-1} \).
3.2. Interactions with target DNAs having mismatch formations

To study how the presence of mismatched base pairs in the target oligonucleotides affects the kinetic parameters, we analyzed the interactions between 401C and various target 20-mer oligonucleotides containing two (421C), four (441C, 443C), or six (462C) mismatched base pairs, but otherwise having the same sequence (Table 1). Figure 5A shows the effect of mismatches on the sensorgram for 401C, 421C and 462C. As shown in Fig. 5B, changes in \( k_{\text{ass}} \) were inversely proportional to the number of mismatched base pairs, with the most significant result being for 462C, since the value of the constants could not be determined. This occurred because 462C showed such a slight increase in relative RU (Fig. 5A). Since 462C had a mismatched base pair at every three or four base pairs, such a duplex of 20-mer oligonucleotides is believed to be too unstable to measure its kinetic parameters. As shown in Fig. 5B, \( k_{\text{diss}} \) was affected more than \( k_{\text{ass}} \) by the presence of mismatched base pairs, and therefore their presence also affected \( K_a \). Although 441C and 443C contained the same number of mismatched base pairs, their locations were different, and this may have been the reason why \( K_a \) for 441C was seven times smaller. To examine the effects of location, we analyzed the kinetic parameters of 20-mer oligonucleotide duplexes (425C–429C) having two mismatched base pairs of either purine-purine or pyrimidine-pyrimidine at different locations, and the results are shown in Fig. 6. The different values for the parameters may be due to the location of mismatched base pairs. It is noted that \( k_{\text{diss}} \) shows greater comparative differences than \( k_{\text{ass}} \). In addition, for 425C to 428C, \( K_a \) successively decreases, then markedly increases for 429C. It should be noted that 425C, 426C, 427C, and 429C have at least eight base pairs that consecutively match, whereas 428C has only six such matches, thereby indicating that the number of consecutively matched base pairs in their sequences affects kinetic parameters even though the number of mismatched base pairs are the same. Another factor that may affect the kinetic parameters, is which mismatched base pairs are present, i.e., G-A or G-T mismatches are stable whereas A-A, T-T, C-T or C-A mismatches are unstable. As shown in Fig. 6, 425C and 426C have C-T and A-G mismatches, 427C and 428C have two of C-T, and 429C has two of G-A.

3.3. Effect of the number of consecutive matches

The results shown in Fig. 6 indicate that the number of consecutively matched base pairs in these oligonucleotide sequences would affect the kinetic parameters of a DNA interaction. Moreover, since all the oligonucleotides used here are immobilized at one end (5'), the position of the mismatched base pairs with respect to the attachment point may affect the kinetic parameters. We analyzed 20-mer oligonucleotides having different numbers of consecutively matched base pairs at the 5' or 3' end. As shown in Fig. 7, as the number of consecutively matched base pairs decreases, \( k_{\text{ass}} \) decreases and \( k_{\text{diss}} \) increases, and therefore \( K_a \) decreases. Obviously, the kinetic parameters are almost identical using these two different types of target DNA in the attachment point on a sensor chip surface. This result suggests that the distance between the immobilized end of a probe and the location of sequence-specific interaction does not appear to affect the kinetic parameters under the present conditions. It should be noted that when comparing the \( K_a \) values for 723A versus 725A or 727A, the latter values are markedly smaller and their number of consecutively matched base pairs is less than 17. We suggest that an oligonucleotide of shorter than 17 bases is more severely affected by the presence of mismatched base pairs.

3.4. Effect of a single mismatch formation

The kinetic parameters of various target oligonucleotides having lengths shorter than 20 bases when interacting with 400T were measured and the effect of a single mismatched base pair was determined by comparing them to oligonucleotides with a perfectly matched sequence or a single mismatched base in the middle, and the results are shown in Fig. 8. The difference between the \( K_a \) values of these two sequences of the same length clearly increases as the sequence length is shortened. Also, \( K_a \) for the 20-mer 401C is 2.5 times larger than its identical sequence with a single mismatched base pair. On the other hand, the 15-mer 775C yielded a \( K_a \) of \( 1.8 \times 10^9 \text{M}^{-1} \), but its value for the 15-mer 755C was only \( 2.5 \times 10^8 \text{M}^{-1} \), i.e., the former oligonucleotide had a \( K_a \) value 7 times larger. For the 11-mer 759A, \( K_a \) could not be calculated due to a low \( K_{\text{ass}} \) value; a result which clearly indicates that the kinetics of a DNA-DNA interaction are significantly affected by the presence of a single mismatched base pair. In addition, the presence of a single mismatched base pair more strongly affects \( k_{\text{diss}} \) than \( k_{\text{ass}} \). This result is in agreement with those of Wallace et al. and Herning et al. additionally reported that a shorter oligonucleotide is a more effective probe although a minimum size is required to recognize a unique sequence in a DNA target. Since it has also been found that an oligonucleotide DNA probe with a length of at least 19 nucleotides has a high probability of recognizing a unique sequence in a human genome, the length of probe needed for recognizing a unique sequence probably varies with the sensitivity of the analysis system used. Furthermore, we analyzed the effect of location of a single mismatch on the kinetics using a 13-mer oligonucleotide having a single mismatch at different locations in its sequence, at the first, fifth or seventh base from its 5' end. The \( K_a \) for a complementary sequence was
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Figure 5. A. Sensorgrams of interactions between 400T and 0.35 mM of the target oligonucleotides 401C, 421C, and 462C. B. Effect of mismatched base pairs on the kinetic parameters for interactions between 400T and various target 20-mer oligonucleotides having mismatch formations. The mismatched bases in the sequence are underlined.

Figure 6. Effect of the position of two mismatched base pairs on the kinetic parameters in a 20-mer oligonucleotide duplex. The mismatched bases in the sequence are underlined.

9.4 \times 10^8 \text{ M}^{-1}, however, the values for sequences having a mismatch at the first, fifth or seventh base were 6.7 \times 10^8, 1.6 \times 10^8 and 1.9 \times 10^7 \text{ M}^{-1}, respectively. The variation in the \( K_a \) value between a complementary oligonucleotide and an oligonucleotide having a single mismatched base pair is largest when its location is at the midpoint. In the BIAcore system, the optimal size of oligonucleotide probe to detect a single mismatched base pair effectively whose location is in the middle of its sequence is between 11 and 15 nucleotides. We, therefore, measured the kinetic parameters of synthetic oligonucleotides having the sequence of the phenylalanine hydroxylase (PAH) gene, known to cause phenylketonuria by the presence of a single point mutation, when interacting with 20-mer, 15-mer or 13-mer oligonucleotides as an immobilized probe. The effect of a single mismatched base pair was determined by comparing kinetic parameters of oligonucleotides having either perfectly matched sequences or a single mismatched base pair at the midpoint of their sequences. It is interesting to note that the difference in \( K_a \) values for normal and mutant PAH genes also increases as the length of the probe is shortened, as shown in Fig. 9. When interacting with a 15-mer immobilized probe, the \( K_a \) for 696C was 8 times larger than its identical sequence having a single mismatched base pair, 697C. In addition, the \( K_a \) value for 696C was 1.1 \times 10^8 \text{ M}^{-1} when interacting with a 13-mer immobilized probe, whereas that of 697C was 31 times greater at 3.4 \times 10^6 \text{ M}^{-1}. These results, using the SPR technique, suggest that the optimal size of an oligonucleotide probe for effectively detecting the presence of a single mismatched base pair is between 11 and 15 nucleotides. Here, we determined the effect on kinetic parameters due to the presence of a single mismatched base pair located in the middle of a DNA duplex, and also found that the location of mismatched base pairs affects the kinetic parameters. Thus, to detect...
a single mismatched base pair in a target oligonucleotide effectively, the probe should contain a single mismatched base in the middle of its sequence.

We have demonstrated a new approach to determine the effect of mismatch formations in a DNA duplex on the kinetic parameters involved in DNA-DNA interactions between an immobilized 20-mer oligonucleotide and target oligonucleotide. When using an oligonucleotide probe, optimization of the conditions needed to form stable hybrids with high specificity is important. To determine a stringent condition which enables more efficient analysis, the melting temperature (Tm) of a perfect hybrid and its predicted optimal hybridization temperature should be investigated, since it is generally known that there is a reduction of Tm by 1–1.5°C for every 1% mismatching of base pairs in double-stranded DNA.18 When performing hybridization using an oligonucleotide probe shorter than 20 nucleotides, however, the Tm decreases by approximately 5°C for every mismatched base pair; therefore, the effect of the presence of mismatched base pairs more strongly depends on their number. In the BIAcore system, a hybridization stringent condition of 37°C is present due to a limitation in its optical system, nevertheless, it was possible to detect the effects of a single mismatched base pair on the kinetic parameters reproducibly and effectively. In our study here, only syn-
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The 20-, 15- and 13-mer oligonucleotides were used as immobilized probes. The mismatched bases in the sequence are underlined.

\[ \text{Probe} \\
5' \text{GAGAAGGCCGAGGTATTGT} 620T \\
5' \text{AAGGGCCGAGGTATT} 615T \\
5' \text{AGGGCGAGGTAT} 613T \]

Figure 9. Effect of the presence of a single mismatched base pair in the PAH gene. The 20-, 15- and 13-mer oligonucleotides were used as immobilized probes. The mismatched bases in the sequence are underlined.

thetic oligonucleotides were employed as probe and target DNA. More stringent conditions, e.g., using a buffer containing detergents or chemicals which affect the hybridization reaction, may be required to analyze a mixed sample or longer lengths of DNA since the effect of intermolecular secondary structure formations should be considered together with the fact that the BIAcore system has a limitation of up to 37°C for the hybridization temperature.

Previous studies have examined the effects of a mismatched duplex in DNA using oligonucleotides labeled with \(^{32}\text{P}\) or fluorescent chemicals.\(^4\) This complex technique is not necessary when using the BIAcore system for measurements of kinetic parameters. In fact, without labeling, we observed a significant destabilizing effect due to a single mismatched base pair existing in a DNA duplex between an immobilized 20-mer oligonucleotide probe and 11-, 13- and 15-mer target oligonucleotides. Evidently, the presence of a single mismatched base pair in the 20-mer synthetic oligonucleotide for the PAH gene could be clearly detected using the immobilized 13- and 15-mer oligonucleotide probes.

In summary, our results clearly indicate that the kinetic parameters of a specific DNA hybrid formation are significantly affected by the presence of mismatched base pairs. Since the employed technique does not require DNA labeling to detect the effect of a single mismatched base pair on kinetic parameters, it is possible that a simple and rapid method can be developed for molecular level DNA structural analysis and diagnosis of genetic diseases and various cancers. Further research will be directed toward using this new biosensing approach to develop a fast and simple detection method for point mutations in oncogenes, which after development is expected to provide rapid progress in the detection of structural changes in genes and in understanding the molecular mechanisms of genetic diseases and various cancers caused by mutations.

Acknowledgements: Sincere gratitude is extended to Drs. Michio Oishi and Tadayoshi Kawasaki for valuable discussions, Drs. Christine Summers and Katsuyuki Saito for critical reading of the manuscript, and Mr. Tooru Negishi for beneficial technical assistance. This research was performed as a part of the Research and Development Projects of Innovative Technology for the Earth supported by the New Energy and Industrial Technology Development Organization (NEDO).

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