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Analytical consideration of the selectivity of oligonucleotide hybridization

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ABSTRACT

Systematic analysis of factors determining efficiency in discrimination of a point substitution (SNP) within specific DNA sequences was carried out in the context of hybridization approach. There are two types of selectivity that are critical for the rational design of highly specific oligonucleotides probes. The first type is the real selectivity of hybridization (f_α) that is the ratio of association degrees of targets with an oligonucleotide probe upon the perfect and imperfect complex formation. This type of selectivity reflects the level of discrimination between matched and mismatched signals, which is determined both by experimental conditions and the thermodynamics of oligonucleotide hybridization. The second parameter characterizeing the efficiency of SNP discrimination is the limit selectivity of hybridization, which determines the utmost value of f_α at a given temperature. This value can be calculated as the ratio of corresponding equilibrium association constants of perfect and imperfect complex formation determined purely by thermodynamics. We have shown that the f_α function is the most reliable characteristic describing the hybridization selectivity. For the analytical system designed to reveal any type of perturbation in DNA (e.g. SNP or modification), there is usually a temperature at which f_α has its maximum value. The dependency of the f_α maximum on different experimental parameters as well as the structural characteristics of a probe are described in details. The results allowed us to postulate points of principle to rationally design the most selective probes on the basis of oligonucleotides or their derivatives.

Keywords: Allele Specific Hybridization; Duplex Stability; Oligonucleotide Probes; SNP Discrimination; Specificity; Thermodynamics

1. INTRODUCTION

The method of molecular hybridization of oligonucleotide probes with nucleic acids in order to reveal specific sequences has been widely utilized in molecular biology [1,2]. The hybridization allows for discrimination of minimal perturbations in the nucleic acid structure e.g., SNP, deletion, insertion (allele-specific hybridization). The high selectivity of interactions between nucleic acids and oligonucleotides or their analogs and derivatives is important for physico-chemical biology and, in particular, for DNA diagnostics [3-6]. To date, there are several strategies for enhancing selectivity of hybridization between oligonucleotides and nucleic acids (NA), including variation of hybridization conditions (temperature, the probe concentration, and the buffer composition) [7-9] and competitive inhibition through the use of stringency clamping [10] or molecular beacons [11,12]. Selectivity is also affected by the difference in kinetics of complex formation for complementary and mismatched duplexes [13-16]. Additionally, changing the probe length [17] or using tandem short probes [18-21] has been shown to influence selectivity. Another strategy involves the use of nanoparticles bearing immobilized oligonucleotide probes [22-24]. The use of modified oligonucleotides is the promising method for the enhancement of hybridization selectivity. Moreover oligonucleotide derivatives have unique properties, e.g., greater resistance to nuclease digestion and stronger affinity for NA. Modifications can be conventionally divided in two groups: (1) those that increase the thermostability of the DNA-probe complex (PNA [25]; LNA [4]; cyclic, cross-linked, and bicyclic oligo-nucleotides [26,27]; 3'-minor groove binder-DNA probes [28]; HNA and ANA [29], etc.) and (2) those that decrease the thermostability of the DNA-probe complex due to an artificial mismatch [30,31], a non-nucleotide insert [32,33] and others.

The main parameter currently used for comparison of

the probe selectivity is the difference between melting temperatures (ΔT_m) of complementary and mismatched complexes [12,28,30,31,34-37]. Other parameters used include the ratio of equilibrium association constants for the perfect (N) and mismatched (M) complexes K_N/K_M [25], the change of Gibbs energy $\Delta\Delta G^{O}$ [10,26-38, 38-41], and the difference $\alpha_N - \alpha_M$ [12,42] or the ratio α_N/α_M [43,44] of the association degrees.

To date, however, there is no generally accepted parameter for the quantitative evaluation of the hybridization selectivity. There are, furthermore, no analytical expressions for the influence of different parameters on the selectivity. The relationship between these parameters and the experimental ratio of the specific and nonspecific signals is still questionable. Some of these parameters, e.g., K_N/K_M and $\Delta\Delta G^{O}$, do not depend on certain experimental conditions like the concentrations of interacting components and buffer content, that do affect the real selectivity of hybridization.

In this work, we used the methodology of allele-specific hybridization to perform a systematic analysis of the factors determining efficiency of discrimination of a point substitution in specific DNA sequences. A number of the being theses such as "the shorter the probe, the higher its selectivity", "the rise of ΔT_m means the increase of selectivity", "molecular beacons are more selective than linear probes" etc., were revised. The results allowed us to reveal points of principle for the rational design of the most selective probes based on oligonu-cleotides or their derivatives.

2. MODELS AND METHODS

2.1. Model for Evaluation of Selectivity

Consider the variant of hybridization of an oligonucleotide probe (p) with the mixture of two templates, one of which is completely complementary (t_N) to the probe, and the other contains a single nucleotide substitution or any other modification of the primary structure (t_M), which leads to the decreased efficiency of complex formation:

$$t_N + p \underset{}{\overset{K_N}{\rightleftarrows}} t_N \cdot p \quad \text{and} \quad t_M + p \underset{}{\overset{K_M}{\rightleftarrows}} t_M \cdot p$$

The probe has, obviously, the maximal discrimination ability when the equilibrium concentration of the perfect complex [$t_N \cdot p$] maximally differs from that of the imperfect complex [$t_M \cdot p$]. The selectivity function f_α is the ratio of the concentrations of perfect and imperfect complexes:

$$f_\alpha = [t_N \cdot p]/[t_M \cdot p] = \alpha_N c_N / \alpha_M c_M$$

where α_N and α_M are the association degrees of the perfect and imperfect complexes, respectively, and c_N and c_M are the initial concentrations of the corresponding DNA templates. Association degree is the ratio of the duplex concentration [$t_{N,M} \cdot p$] in the equilibrium system to the total concentration of interacting components, e.g. $[t_{N,M}]_0$. If c_N is equal to c_M, selectivity function f_α is rep- resented as:

$$f_\alpha = \alpha_N / \alpha_M \tag{1}$$

Thus, f_α indicates the ratio of the degrees of complex formation for perfect and mismatched complexes. Conditions providing the maximum f_α value are, apparently, the same for the maximal selectivity of interaction of the probe with the template.

The association degrees of the corresponding complexes can be expressed in terms of the equilibrium constants of association of the probe with the templates. When concentration of the probe (c_p) sufficiently exceeds that of the templates (c_t) (that is usual for DNA analysis), the association degrees are simplified to:

$$\alpha_N \approx c_p / (1/K_N + c_p) \text{ and } \alpha_M \approx c_p / (1/K_M + c_p) \tag{2}$$

Eq.2 shows that the association degree depends on the probe concentration and does not depend on the template concentration. In this case, when a high excess of the probe is used, templates do not compete for binding to the probe. It does not matter, therefore, if either one or two templates are to be analyzed. Starting from (2), f_α can be presented as:

$$f_\alpha = a_N / a_M \approx (1/K_M + c_p)/(1/K_N + c_p) \tag{3}$$

where $K_i = \exp\left[\left(-\Delta H_i^{O} + T\Delta S_i^{O}\right)/RT\right]$, ΔH_i^{O} and ΔS_i^{O} are enthalpy and entropy of complex formation, respectively, i = N or M, R is the gas constant (1.987 cal/K·mol).

2.2. Calculation of Thermodynamic Parameters for DNA Complexes

Thermodynamic characteristics ΔH^{O} and ΔS^{O} for complementary and single mismatched complexes were calculated under standard conditions (1 M NaCl, p) using unified nearest neighbor parameters [45-50]. The parameters $\Delta\Delta H^{O}$ and $\Delta\Delta S^{O}$ were calculated as the difference between the corresponding characteristics:

$$\Delta\Delta H^{O} = \Delta H_N^{O} - \Delta H_M^{O} \quad \text{and} \quad \Delta\Delta S^{O} = \Delta S_N^{O} - \Delta S_M^{O}$$

Thermodynamic parameters of complex formation for averaged oligomer of the (l_p+ 1) length were calculated using the following equations:

$$\Delta H_N^{O} \approx l_p \Delta \overline{H}_N^{O} \tag{4}$$

$$\Delta S_N^{O} \approx l_p \Delta \overline{S}_N^{O} \tag{5}$$

where $\Delta \overline{H}_N^{O} = -8.36$ k·cal/mol and $\Delta \overline{S}_N^{O} = -22.4$ cal/(mol·K) are enthalpy and entropy, respectively, for

the formation of an averaged dinucleotide of the DNA helix, calculated by averaging of the nearest neighbors parameters. For simplicity, the terminal effects and initiation penalty were not taken into account in these calculations.

The $\Delta\Delta\bar{H}^{O}$ and $\Delta\Delta\bar{S}^{O}$ values for each *X*/*Y* mismatch averaged over all possible combinations of nearest neighbors were calculated according to:

$$\Delta\Delta\bar{H}\,(X/Y)=\left[\sum\Delta\Delta H\left(\frac{NXN}{N'YN'}\right)\right]\Big/n$$

and

$$\Delta\Delta\bar{S}^{O}(X/Y)=\left[\sum_{i=1}^{n}\Delta\Delta S_{i}^{O}\left(\frac{NXN}{N'YN'}\right)\right]\Big/n$$

where *N*/*N*' is complementary bp. The average statistical effect of a single internal mismatch in the duplex is characterized by values $\Delta\Delta\bar{H}^{O}=-16.9$ kcal/mol and $\Delta\Delta\bar{S}^{O}=-42.6$ cal/(mol·K). The ratio of the averaged enthalpy values for complex formation and destabilization requires, therefore, a value of approximately 2:

$$\Delta\Delta\bar{H}_{N}^{O}/\Delta\bar{H}_{N}^{O}\approx 2 \qquad (6)$$

2.3. Oligonucleotide System

The main thermodynamic data were obtained for DNA probes and the corresponding perfect and imperfect duplexes are listed in **Table 1**. Thermodynamic parameters were calculated in accordance with [45-50].

3. RESULTS

At first, the terminology for the following analytical consideration should be stated. According to IUPAC, "selectivity" is the extent, to which a particular method can be used to determine analytes under given conditions in the presence of other components of the similar behavior. Unlike, "specificity" is considered as an absolute term, and thus cannot be graded [51]. Therefore, it is necessary to use "selectivity" as the obligatory term if we consider the quantitative parameters characterizing the ability of an oligonucleotide probe to distinguish one sequence from the other one. It should be kept in mind, however, that there are different levels of the contextual usage of both terms, specificity and selectivity [52]. We considered the variant implying that the analyzed site was unique, *i.e.*, occurred once in both native and mutated form of the analyzed target. We used the term "se-

Table 1. Thermodynamic parameters for complex formation of probes with perfect and mismatched (C/A) templates.

Probe	Sequence 5'→3'	Complex type*	Duplexes	$-H°$, kcal/mol	$-S°$, cal/(mol·K)	$-H°$, kcal/mol	$-S°$ cal/(mol·K)
I	CTAA**C**TAACG	*N*	CTAA**C**TAACG GATT**G**ATTGC	73.0	206.6		
		M	CTAA**C**TAACG GATT**A**ATTGC	49.2	144.0		
II	CTAA**C**TAACGACATC	*N*	CTAA**C**TAACGACATC GATT**G**ATTGCTGTAG	113.5	316.5	23.8	62.6
		M	CTAA**C**TAACGACATC GATT**A**ATTGCTGTAG	89.7	253.9		
III	CTAA**C**TAA	*N*	CTAA**C**TAA GATT**G**ATT	51.8	150.1		
		M	CTAA**C**TAA GATT**A**ATT	28.0	87.5		
IV	CTAA**C**TAACG	*N*	CTAA**C**TAACG GATT**G**ATTGC	73.0	206.6	15.7	37.4
		M	CTAA**C**TAACG GATT**T**ATTGC	57.3	169.2		
V	CTAT**C**CAACG	*N*	CTAT**C**CAACG GATA**G**GTTGC	73.6	204.9	29.0	76.5
		M	CTAT**C**CAACG GATA**A**GTTGC	44.6	128.4		
VI	CTAC**C**GAACG	*N*	CTAC**C**GAACG GATG**G**CTTGC	76.9	211.4	21.1	50.2
		M	CTAC**C**GAACG GATG**A**CTTGC	55.8	161.2		

**N*—complementary complex; *M*—mismatched complex.

lectivity" as the parameter characterizing the quantitative level of discrimination between the perfect and imperfect (containing point substitution) nucleic acid analytes upon its hybridization with the sequence specific probe having the unique binding site within the DNA template.

3.1. Analysis of the Selectivity Function

One of the main experimental parameters often used upon optimization is the temperature of hybridization. Consider the dependence of the function f_α (3) on temperature using the example of probe I, which is used for discrimination of the substitution of A for G (**Table 1**). **Figure 1(a)** demonstrates the temperature dependence of function f_α and the association degrees of the complementary (α_N) and mismatched (α_M) complexes. The temperature dependence of function f_α is the bell-shaped curve. The temperature providing the maximum value of f_α is designated as $T_{\max}$.

The selectivity function is described by simple expressions within the defined temperature regions (**Table 2**).

1) When $T \le T_{c/10}^M$, $f_\alpha \to 1$ because the degree of association of the probe with either of the two templates is approximated to 1 (**Table 2**, p. 1) (S1.1).

2) When $T = T_m^M$, $f_\alpha \to 2$, provided that expression $T_m^M \le T_{c/10}^N$ is true (**Table 2**, p. 2) (S1.2).

3) Function f_α tends to $f_\alpha^{K_M}$ in the range of temperatures $T_{10c}^M \le T \le T_{c/10}^N$, where

$$f_\alpha^{K_M} = 1/c_p K_M \qquad (7)$$

Indeed, f_α at these temperatures corresponds to the $f_\alpha^{K_M}$, which is pseudolinear in log chart (S1.3) (**Figure 1(b)**). The $f_\alpha^{K_M}$ values depend on the hybridization properties of the mismatch complex and probe concentration c_p (**Table 2**, p. 3).

4) In the range of high temperatures $T \ge T_{10c}^N$, $f_\alpha \to K_N/K_M = \exp\left[\left(-\Delta\Delta H^{\mathrm{O}} + T\Delta\Delta S^{\mathrm{O}}\right)/RT\right]$, *i.e.* function f_α is determined by the ratio of equilibrium association constants of complementary and imperfect duplexes (S1.4). At these temperatures the f_α values fall into the "linear" region (log chart) and are described by a new function (**Figure 1(b)**) designated as the function of the high limit selectivity (**Table 2**, p. 5):

$$f_\alpha^{\lim} = \exp\left[\left(-\Delta\Delta H^{\mathrm{O}} + T\Delta\Delta S^{\mathrm{O}}\right)/RT\right] \qquad (8)$$

Function $f_\alpha^{\lim}$ depends only on temperature and the type of mismatch ($\Delta\Delta H^{\mathrm{O}}$ and $\Delta\Delta S^{\mathrm{O}}$); it shows the upper limit value of f_α at the given temperature.

5) When $T \ge T_\Delta$, where $T_\Delta = \Delta\Delta H°/\Delta\Delta S°$ is the temperature of inversion of selectivity, $f_\alpha \le 1$. It should be taken into account that T_Δ is the characteristic value for the given pair of match/mismatch. In these temperatures the values of association degrees α_N and α_M are extremely low for commonly used values of probe concentration. In the case of probe I, as an example, complex formation is extremely low ($\alpha_N < 10^{-7}$) at $T_\Delta > 100$˚C.

6) The most important temperature for any hybridization analysis is $T_{\max}$, corresponding to the real maxima of the selectivity function f_α. It is not feasible to solve the differential equation $f_\alpha'(T) = 0$ in a strong analytical way and, thus, to find a rigorous solution for the calculation of $T_{\max}$. The $T_{\max}$ value was, therefore, found numerically using the given probe concentration and

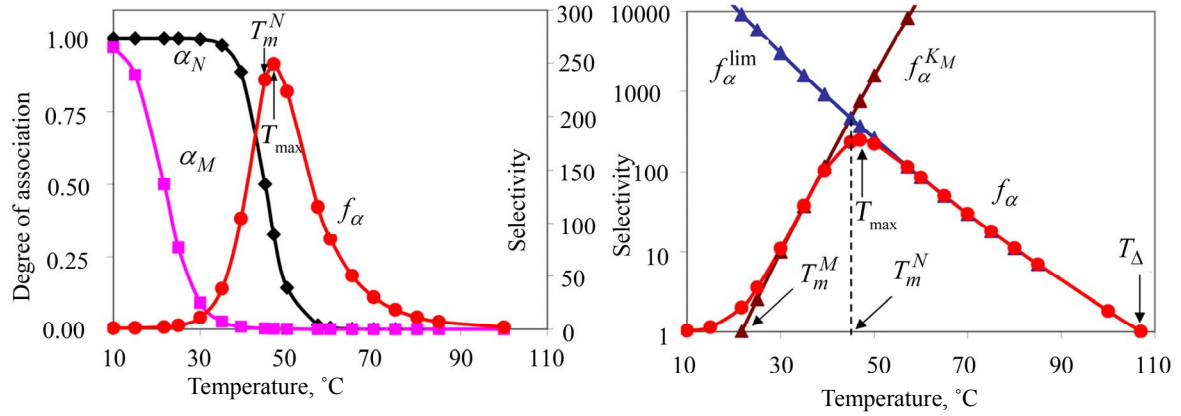

Figure 1. (a) Temperature dependence of the association degrees of the perfect (α_N) and imperfect (α_M) complexes (left axis) and of the selectivity function f_α (right axis) for probe I at c_p=10^{-5} M. (b) Temperature dependence of f_α, $f_\alpha^{\lim}$ and $f_\alpha^{K_M}$ (log scale). $T_{\max}$ is the temperature of the selectivity maximum, T_n^M and T_m^M are melting temperatures of the perfect and mismatched complexes respectively, T_Δ is temperature of inversion of selectivity.

Table 2. The selectivity function for probe I upon revealing C/A mismatch at different temperatures. Indexes N and M correspond to the complementary and imperfect complexes, respectively. See derivation of formula in supplementary material (S1.1 - S1.5).

	Temperature	Equation	t,˚C	$f_\alpha(T)$	Value of selectivity
1	$\le T^M_{c/10}$	$T^M_{c/10}=\Delta H^{\circ}_M/\left(\Delta S^{\circ}_M+R\ln c_p/10\right)$	≤ 13.8	$f_\alpha(T)=1/1$	1
2	T^M_m	$T^M_m=\Delta H^{\circ}_M/\left(\Delta S^{\circ}_M+R\ln c_p\right)$	21.7	$f_\alpha\left(T^M_m\right)=2/1$	2
3	$\ge T^M_{10c}$	$T^M_{10c}=\Delta H^{\circ}_M/\left(\Delta S^{\circ}_M+R\ln 10c_p\right)$	≥ 30.0	$f^{K_M}_\alpha(T)=\dfrac{1}{c_p K_M(T)}$	≥ 11
	$\le T^N_{c/10}$	$T^N_{c/10}=\Delta H^{\circ}_N/\left(\Delta S^{\circ}_N+R\ln c_p/10\right)$	≤ 38.7		≤ 91
4	T^N_m	$T^N_m=\Delta H^{\circ}_N/\left(\Delta S^{\circ}_N+R\ln c_p\right)$	45.0	$f^{T_m}_\alpha=f^{\lim}_\alpha\left(T^M_m\right)/2$	234
	$T_{\max}$	$f'_a(T_{\max})=0$	47.0	$f^{\max}_\alpha=f_\alpha(T_{\max})$	249
5	$\ge T^N_{10c}$	$T^N_{10c}=\Delta H^{\circ}_N/\left(\Delta S^{\circ}_N+R\ln 10c_p\right)$	≥ 51.4	$f^{\lim}_\alpha(T)$	≤ 201
6	$\ge T_\Delta$	$T_\Delta=\Delta\Delta H^{\circ}/\Delta\Delta S^{\circ}$	≥ 107		≤ 1

thermodynamic parameters of hybridization. Aside from numerical calculation, the approximate evaluation of $T_{\max}$ is shown to be also possible. For this purpose, one should pay attention to the fact that functions $f^{K_M}_\alpha$ (7) and $f^{\lim}_\alpha$ (8) are pseudo linear in the log chart (**Figure 1(b)**), confining f_α at its left and right, and describing it well within certain temperature regions. The temperature where $f^{K_M}_\alpha$ and $f^{\lim}_\alpha$ intersect is the melting temperature of the complementary complex T^N_m, which is close to $T_{\max}$ (S1.4). At the same time, these functions intersect the abscissa axis at temperatures T^M_m and T_Δ. One can expect that the T^N_m values are as close to $T_{\max}$ as possible when $f^{K_M}_\alpha(T)$, $f^{\lim}_\alpha(T)$, and the abscissa axis form an isosceles triangle, *i.e.* $T^N_m-T^M_m=T_\Delta-T^N_m$. For probe I, these values are $T^N_m-T^M_m<T_\Delta-T^N_m$, which results in a shifting of $T_{\max}$ to higher temperatures.

Numerical calculations showed that the $T_{\max}$ values are close to T^N_m, *i.e.* to the melting temperature of the perfect complex for all probes (**Tables 3-5**). The difference ($T_{\max}-T^N_m$) in these examples is always positive and varies in the range from 0.6˚C to 4.9˚C although theoretically it should be negative in some cases when destabilization of the mismatched duplex is very high (unpublished data). This difference decreases for short probes. So, we can expect in most cases:

$$T_{\max}\approx T^N_m \tag{9}$$

It was shown using (9) that $f^{\max}_\alpha=f^{T_m}_\alpha$, where $f^{\max}_\alpha=f_\alpha(T_{\max})$ and $f^{T_m}_\alpha=f_\alpha\left(T^N_m\right)$. Comparison of these two parameters for all probes under investigation was carried out. We determined that $f^{T_m}_\alpha$ was 11% less on average than $f^{\max}_\alpha$, and deviation of $f^{T_m}_\alpha$ from $f^{\max}_\alpha$ was in the range of 0.3% - 23.4% (**Tables 3-5**). On the other hand, the selectivity function at melting temperature T^N_m is equal to the half of the limit selectivity function (S1.5).

$$f^{T_m}_\alpha=f^{\lim}_\alpha\left(T^N_m\right)/2 \tag{10}$$

The use of this equation simplifies the quantitative evaluation of the probe selectivity sufficiently upon revealing point mutations, because it is necessary to know only melting temperature of the perfect complex T^N_m and the influence of the mismatch on thermodynamic parameters, *i.e.*, $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$. Substitution of the T^N_m value into (10) gives (11) and allows one to determine parameters that influence the selectivity function close to its maximum.

$$\begin{aligned} f^{T_m}_\alpha &= \frac{1}{2}\exp\left(-\frac{\Delta\Delta H^{\circ}}{R}\left[\frac{1}{T^M_m}-\frac{1}{T_\Delta}\right]\right) \\ &= \frac{c_p^{-\frac{\Delta\Delta H^{\circ}}{\Delta H^{\circ}_N}}}{2}\exp\left(\frac{\Delta\Delta S^{\circ}}{R}-\frac{\Delta S^{\circ}_N}{R}\frac{\Delta\Delta H^{\circ}}{\Delta H^{\circ}_N}\right) \end{aligned} \tag{11}$$

These parameters are the length and the structure of the probe ($\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$). Below we will consider in detail the influence of each parameter on the maximum of the selectivity function.

3.2. Influence of the Probe Concentration on the Selectivity Function

The influence of probe I concentration on the selectivity function f_a is presented in **Figure 2** and **Table 3**. The

Table 3. Dependence of melting temperature and selectivity for probe I on its concentration upon revealing C/A mismatch.

c_p, M	T^N_m, ˚C	ΔT_m, ˚C	$T_{\max}$, ˚C	$f^{T_m}_\alpha$	$f^{\max}_\alpha$
10^{-5}	45.0	23.3	47.0	234	249
10^{-6}	38.7	24.9	40.7	496	527
10^{-7}	32.8	26.4	34.6	1050	1117
10^{-8}	27.0	27.7	28.8	2224	2365

Table 4. Thermodynamic and selectivity data characterizing the discrimination of the C/A mismatch using probes I-III of different length either at the same concentration or the same T_{max} values.

		c_p=10^{-5} M					T_{max} = 47.0°C								
Probe	Length	T_m, °C	ΔT_m, °C	T_{max}, °C	$f_\alpha^{T_m}$	f_α^{max}	c_p, M	T_m, °C	ΔT_m, °C	$T_{-1/2}$	$T_{+1/2}$	$f_\alpha^{T_m}$	f_α^{max}	$\int_{T_{max}-5}^{T_{max}+5} f_\alpha dT$	$\int_{20}^{80} f_\alpha dT$
II	15	61.3 50.9	10.3	63.9	37.7	44.8	1.3·10^{-9}	44.6 31.3	13.3	41.5	57.1	244.4	292	2416	4550
I	10	45.0 21.7	23.3	47.0	234.3	249.1	10^{-5}	45.0 21.7	23.3	39.4	57.1	234.3	249.1	2237	4813
III	8	26.3 –19.5	45.8	26.9	2441	2449	2.3·10^{-3}	46.3 8.2	38.2	36.6	57.1	199.1	199.8	1876	4857

Table 5. Pairs of "complementary" mismatches in order decreasing of limit selectivity. Mismatch X/Y, where X and Y are nucleotides in template and in probe, respectively.

	Mismatch	$f_\alpha^{lim}(T)$		"Complementary" mismatch	$f_\alpha^{lim}(T)$	
		20°C	80°C		20°C	80°C
1	C/C	50970	134	G/G	692	18
2	A/C	42619	48	T/G	1142	16
3	T/C	23686	64	A/G	2100	16
4	C/A	6437	10	G/T	199	4
5	C/T	3297	14	G/A	281	3
6	A/A	954	9	T/T	488	19

decrease of the typical probe concentration leads to an increase of the maximal value of f_α (T), and to its shift to lower temperatures, with the function values tending to f_α^{lim} at temperatures higher than T_{max}. This demonstrates the well known fact that a probe has higher discriminating ability when it is used at lower concentrations [7-9].

Dependence of $f_\alpha^{T_m}$ (10) on temperature in logarithmic scale is the "straight" line, which passes the f_α curves obtained for different concentrations of probe I at the temperatures corresponding to T_m^N values. The straight line intersects the curves of the selectivity functions of probe I close to their maxima (**Figure 2**). Thus the $f_\alpha^{T_m}$ function allows for the accurate evaluation of the f_α^{max} value at the known melting temperature of the perfect complex.

Using (11), we can evaluate the dependence of the maximal value of the selectivity function $f_\alpha^{T_m}$ upon varying probe concentration. Assume that probe concentration is changed by a factor of x. The change of the maximal probe selectivity (γ_c) can be determined from the ratio $f_\alpha^{T_m}(xc_p)$ to $f_\alpha^{T_m}(c_p)$ (S2):

$$\gamma_c = f_\alpha^{T_m}(xc_p) \Big/ f_\alpha^{T_m}(c_p) = x^{-\frac{\Delta\Delta H^o}{\Delta H_N^o}} \qquad (12)$$

The behaviour of change in $f_\alpha^{T_m}$ is exponential and depends not only on the concentration change ($\boldsymbol{x}$) but also on the relative enthalpic contribution of mismatch (perturbation) $\Delta\Delta H^O / \Delta H_N^O$.

The simplest way to demonstrate the behavior of γ_c is the use of an averaged probe (4) upon revealing an averaged mismatch (6). Equation (12) can be simplified and presented as the dependence of the change of selectivity on the length of a statistically averaged oligonucleotide probe.

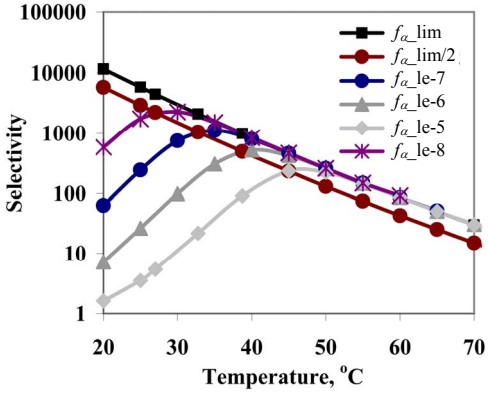

Figure 2. Temperature dependence of the selectivity functions for probe I upon revealing C/A mismatch when varying its concentration from 10^{-8} to 10^{-5} M.

$$\gamma_c = x^{-\frac{\Delta\Delta\bar{H}^o}{\Delta H_N^o}} \approx x^{-\frac{\Delta\Delta\bar{H}^o}{l_p \Delta\bar{H}_N^o}} \approx x^{-\frac{2}{l_p}} \qquad (13)$$

The equation shows that the greater the length of the probe, the less impact its concentration has on its selectivity.

Using (13), we now evaluate the change of selectivity for an averaged probe of 10 nt length (l_p + 1) when its concentration is changed by one order of magnitude. Calculations show the increase of the maximum of the selectivity function $f_\alpha^{T_m}$ for a statistically average probe and an average statistical mismatch by a factor of two ($\gamma_c \approx 0.1^{-2/9} \approx 1.7$) when the probe concentration decreases by one order of magnitude (x = 0.1), and the decrease of this function by a factor of 1.5 ($\gamma_c \approx 10^{-2/9} \approx 0.6$) when concentration increases by one order of magnitude (x = 10).

3.3. The Change of Selectivity upon Variation of the Probe Structure

Here we consider changes in the probe structure,

which do not influence the thermodynamic characteristics of mismatch discrimination ($\Delta\Delta H^{\circ}$, $\Delta\Delta S^{\circ}$). One can hypothesize that such a situation is possible when a change of any type is located far from the mismatch in the duplex, and there is virtually no cross-interaction between these elements.

Let us consider one particular variation in the probe structure, namely, the change of its length. Using (11), one can demonstrate that in this case, the γ_l value characterizes the change of $f_{\alpha}^{T_m}$ and depends on the melting temperatures of complementary complexes of probes of different lengths (S3).

$$\gamma_l = f_{\alpha 2}^{T_m} / f_{\alpha 1}^{T_m} = \exp\left[\Delta\Delta H^{\circ}\left(1/T_{m1}^{N} - 1/T_{m2}^{N}\right)/R\right] \quad (14)$$

When the probe is long enough, the terminal effects can be neglected [53], and (4) and (5) can be used for calculation of hybridization properties of an average probe. In this case γ_l can be expressed in terms of the probe lengths $l_1 + 1$ and $l_2 + 1$ (S3):

$$\gamma_l \sim c_p^{\frac{\Delta\Delta\bar{H}^{\circ}}{\Delta\bar{H}_N^{\circ}}\frac{(l_2 - l_1)}{l_1 l_2}} \quad (15)$$

In case of discrimination of an averaged mismatch (6), this equation is transformed to:

$$\gamma_l \sim c_p^{\frac{2(l_2 - l_1)}{l_1 l_2}} \quad (16)$$

Thus, the dependence of the maximum of the selectivity function on the probe length is a rough approximation described by the power function at the given concentration. The longer the probe, the weaker the dependence of selectivity on the change of its length.

Let us evaluate the change of selectivity of a statistically average probe with a length of $l_1 + 1 = 10$ at a concentration $c_p = 10^{-5}$ M using (16). The shortening ($l_1 + 1 = 6$) or lengthening ($l_2 + 1 = 14$) of the probe by four nucleotides results in the increase of $f_{\alpha}^{T_m}$ approximately by a factor of 7.7 (by one order of magnitude), or a decrease by a factor of 2, respectively.

The detailed analysis of the behavior of the selectivity function upon discrimination of the same mismatch C/A, using the probes of different lengths, is represented in **Table 4**. **Figure 3(a)** shows the dependence of f_{α} on temperature for probes I-III, with the lengths of 8, 10, and 15 nucleotides (**Table 4**) at 10^{-5} M concentration of each probe. As expected, the shortening of an oligonucleotide results in the shift of $T_{\max}$ to lower temperatures and in the increase of the $f_{\alpha}^{\max}$ values.

Interesting results were obtained when considering the behavior of $f_{\alpha}(T)$ in the case of a set of the probes of different length, but with the same $T_{\max}$. For this purpose, concentrations for the probes with lengths of 8 and 15 nucleotides were numerically found using (3) (**Table 4**). The behavior of f_{α} for these two probes differs markedly from each other (**Figure 3(b)**). The maximal selectivity value $f_{\alpha}^{\max}$ increases with the increase of the probe length. An elongated probe at temperatures $T < T_{\max}$ flattens the function f_{α}, as compared to shorter probes, while at a temperature close to $T_{\max}$ this function increases more dramatically for the longer probe. At temperatures $T \geq T_{\max}$, the function f_{α} tends to $f_{\alpha}^{\lim}$ for all probes, with f_{α} for the longer probe tending to $f_{\alpha}^{\lim}$ at lower temperatures. This can be expressed as $T_{10c}^{N} \to T_{\max}$ (**Figure 3(b)**). Thus, one can hypothesize that $f_{\alpha} \to 1$ at $T < T_{\max}$ and $f_{\alpha} \to f_{\alpha}^{\lim}$ at $T \geq T_{\max}$ when the lengthening of the probe is pronounced.

These results open a question about what criteria should be used for evaluating the discrimination ability of the probe at a definite temperature, or at some range of temperatures. We should still consider the temperature

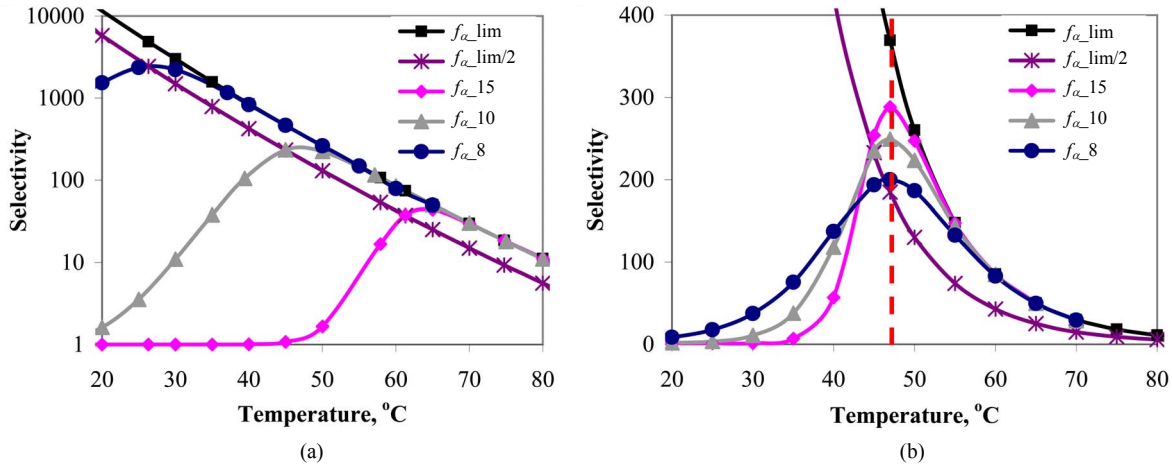

Figure 3. (*a*) Temperature dependence of the selectivity functions for probes with the length of 15(II), 10 (I), and 8 (III) nt ($c_p = 10^{-5}$ M). (*b*) Temperature dependence of the selectivity functions for probes with the length of 15 (II), 10 (I) and 8 (III) nt having the same $T_{\max}$ at concentrations $2.3 \cdot 10^{-3}$, $1 \cdot 10^{-5}$, and $1.3 \cdot 10^{-9}$ M, respectively.

range in which $f_\alpha^{\max}$ is found, taking into account that the precision calculation of $T_{\max}$ is restricted by the accuracy of thermodynamic parameters. Then the integral value of f_α within the given temperature range determines the probe selectivity. Consider probes I-III (**Table 4**) under conditions where their $T_{\max}$ are the same (**Figure 3(b)**), provided that the accuracy of $T_{\max}$ is ±5˚C, and find the values $\int_{T_{\max}-5}^{T_{\max}+5} f_\alpha(T)\mathrm{d}T$. The results show the higher integral selectivity of probe II (15-mer) within this temperature range as compared to the shorter oligonucleotides. On the contrary, short probe III has the highest integral selectivity within the whole experimental temperature range chosen from 20˚C to 80˚C. Nevertheless, there is no evident preference of one probe over another because of the slight dependence of the integral selectivity on the probe length (**Table 4**).

Let $T_{-1/2}$ and $T_{+1/2}$ are temperatures where selectivity function f_α^{Tm} is reduced by half.

$$T_{-1/2} = \Delta H_M^{\mathrm{O}} \Big/ \left(\Delta S_M^{\mathrm{O}} + R\ln\left(c_p f_\alpha^{T_m}/2\right)\right) \quad (17)$$

$$T_{+1/2} = \Delta\Delta H^{\mathrm{O}} \Big/ \left(\Delta\Delta S^{\mathrm{O}} - R\ln\left(f_\alpha^{T_m}/2\right)\right) \quad (18)$$

Although the T_m^N values for the probes differ insignificantly from each other at the same $T_{\max}$, the $T_{+1/2}$ values are 57.1˚C for all probes and the $T_{-1/2}$ values are 41.5˚C, 39.4˚C and 36.6˚C for the 15-mer, 10-mer, and 8-mer, respectively (**Table 4**). Thus $T_{-1/2}$ and $T_{+1/2}$ characterize the temperature range where selectivity function f_α has significantly high values.

3.4. The Influence of the Mismatch Type on Hybridization Selectivity

The efficiency of mismatch discrimination for a probe depends on the value of its destabilization effect in the probe/template complex. The change of selectivity examined above while varying concentration and length of the probe do not involve the parameters $\Delta\Delta H^{\mathrm{O}}$ and $\Delta\Delta S^{\mathrm{O}}$, which determine the $f_\alpha^{\lim}$ values (8). One way to change the limit selectivity is to vary the type of mismatch.

For example, let us consider the situation when the same probe is used for revealing two different mismatches C/A and C/T (**Table 1**, I and IV). In this case, the difference in the efficiency of discrimination results only from the variation of the $\Delta\Delta H^{\mathrm{O}}$ and $\Delta\Delta S^{\mathrm{O}}$ values due to the nature of the imperfect base pair. Temperature dependence of functions f_α and $f_\alpha^{\lim}$ for the same probe ($c_p = 10^{-5}$ M and 10^{-8} M, ΔH_N^{O} and ΔS_N^{O} are constants) are depicted in **Figure 4**. The coincidence of the maxima of functions f_α(C/A) and f_α(C/T) characterizing the discrimination of these mismatches at $T_{\max} \approx$ 45˚C ($c_p = 10^{-5}$M) can be seen. The decrease of probe concentration ($c_p = 10^{-8}$M) leads to the lowering of $T_{\max}$ to 30˚C and to the difference between the $f_\alpha^{\max}$ values by a factor of more than three. The efficiency of discrimination of mismatches can thus sufficiently change when varying the $T_{\max}$ values. So, mismatches with the same selectivity at a definite temperature become markedly distinguishable at some other temperature.

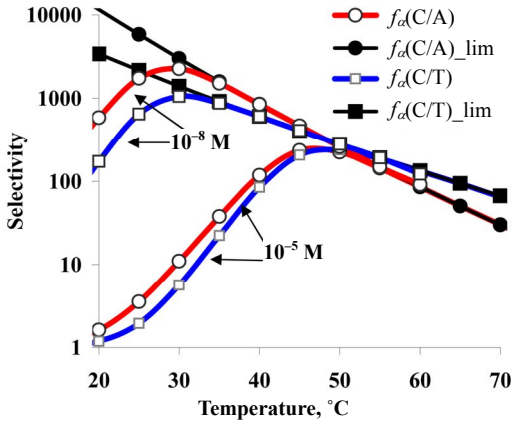

Figure 4. Temperature dependence of the selectivity functions when revealing two different mismatches C/A (I) and C/T (IV) using the same probe at 10^{-5} M and 10^{-8} M concentrations.

The range of the changes of f_α^{Tm} upon variation of a mismatch type and, consequently $\Delta\Delta H^{\mathrm{O}}$ and $\Delta\Delta S^{\mathrm{O}}$ parameters, was calculated using (10). If the $\Delta\Delta H^{\mathrm{O}}$ and $\Delta\Delta S^{\mathrm{O}}$ values change by h and s, respectively, the change of the selectivity function (γ_{pert}) near its maximum becomes the following (S4):

$$\gamma_{pert} = \frac{f_\alpha^{T_m}\left(\Delta\Delta H^{\mathrm{O}} + h, \Delta\Delta S^{\mathrm{O}} + s\right)}{f_\alpha^{T_m}\left(\Delta\Delta H^{\mathrm{O}}, \Delta\Delta S^{\mathrm{O}}\right)} = K_{pert}\left(T_m^N\right) \quad (19)$$

where $K_{pert}(T) = \exp[(-h + Ts)/RT]$ is the "perturbation" constant. The change of the maximal selectivity function eventually depends on parameters h and s. The expression $\gamma_{pert} > 1$ is true when the ratio h/s is higher than the melting temperature of the complementary complex ($h/s > T_m^N$).

There is no need to determine the dependence of f_α on temperature for each particular case in order to evaluate the maximal probe selectivity. Instead, the expression $f_\alpha^{\lim}(T)/2$ can be used, allowing preliminary evaluation of $f_\alpha^{\lim}$ at a given hybridization temperature according to (10).

We compared the efficiency of discrimination of all types of internal single mismatches in DNA/DNA duplexes using the thermodynamic parameters $\Delta\Delta H^{\mathrm{O}}$ and $\Delta\Delta S^{\mathrm{O}}$ [53]. There are 12 types of internal mis-

matches as a result of orientational asymmetry. For example, mismatches A/G and G/A are not equivalent because they correspond to A/T and G/C complementary pairs in a perfect complex, respectively. The values of $f_{\alpha}^{\lim}(T)$ for 12 types of mismatches averaged over nearest neighbors are presented in **Table 5**. The maximal discrimination ability of probes is shown for the imperfect pairs C/C, A/C, and T/C. The efficiency of discrimination of all other mismatches is sufficiently lower and depends significantly on the chosen temperature conditions.

A mutation can be revealed in either of two strands of dsDNA that allows one to say about "complementary" related mismatches. The selectivity of revealing mismatches follows the trend: C/C > G/G, A/C > T/G, T/C > A/G, C/A > G/T, C/T > G/A at any temperatures over the range of 20˚C to 80˚C. In the case of the pair of "complementary" mismatches A/A and T/T the former is better discriminated at temperatures < 45˚C and the latter at temperatures > 45˚C.

Using the dependence $f_{\alpha}^{\lim}(T)/2$, we can choose the type of a mismatch, temperature, and oligonucleotide probe that provide the maximal efficiency of discrimination, and therefore, the highest selectivity for revealing point mutations.

3.5. The Change of Selectivity Conferred by Using Modified Probes

Most often, there is a necessity to determine how modification of a native probe influences its selectivity. To date, there is no systematic information about thermodynamic characteristics describing in detail the imperfect complexes formed by modified oligonucleotides, e.g., PNA, LNA, etc.

When the same type of a mismatch has to be discriminated, variation of the nearest neighbors of the polymorphic site can be a model of the modification resulting in the change of $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$ Let us examine the identification of a C/A mismatch using probes I, V, and VI (**Table 1**), which are distinguished from each other only by the nucleotide sequences around the mismatch. **Figure 5** demonstrates the temperature

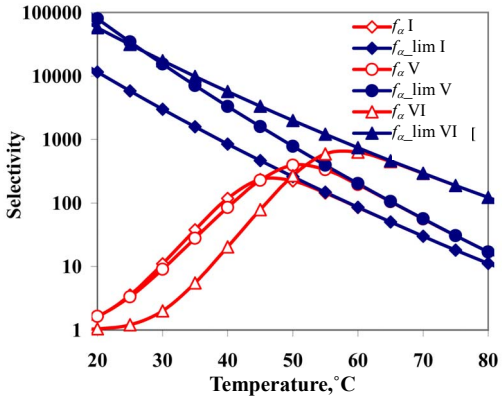

Figure 5. Temperature dependence of the selectivity functions when revealing C/A mismatch using 10^{-5} M probes I, V, or VI.

dependence of the selectivity function and limit selectivity function for these probes. At the defined concentration, the temperature of the selectivity maxima varies in its dependence on hybridization properties of the probes. The selectivity of each probe is always higher at the melting temperature of its complementary complex ($T_m^{N(\mathrm{I})}$, $T_m^{N(\mathrm{V})}$ or $T_m^{N(\mathrm{VI})}$) than at the other temperature (**Table 6**). However, the straight lines $f_{\alpha}^{\lim}(T)$ are not intersected in the range from 30 to 80˚C; we can therefore say that the attainable selectivity of the probes increases in a row I < V < VI (**Figure 5**). The same ratio is true for the selectivity maxima of these probes $f_{\alpha}^{T_m}$ at a definite temperature.

One of interesting question that can be asked while searching the new types of modification (perturbation) of the probe, is what values of the $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$ can provide the high level of limit selectivity. As can be seen from (8), the function $\ln f_{\alpha}^{\lim}(T)$ is a hyperbola, and can be fitted with a high accuracy ($R^2 > 0.98$) by the linear dependence in the temperature range from 0˚C to 100˚C:

$$\ln f_{\alpha}^{\lim}(T) \approx \frac{c_1 \Delta\Delta H^{\circ}}{R} T - c_2 \frac{\Delta\Delta H^{\circ}}{R} + \frac{\Delta\Delta S^{\circ}}{R} \quad (20)$$

where c_1 and c_2 are positive constants (S5). Thus, the slope of the function depends only on $\Delta\Delta H^{\circ}$, while the constant term is determined by both types of thermody

Table 6. Thermodynamic and selectivity data obtained upon discrimination of C/A mismatch using 10^{-5} M probes I, V, or VI (**Table 1**), which differ from each other by neighbors nearest to the mutation site.

Probe	$f_{\alpha}(T)$			ΔT_m, ˚C	$f_{\alpha}^{\max}$	$T_{\max}$, ˚C
	$T_m^{N1} = 45$	$T_m^{N5} = 50$	$T_m^{N6} = 55$			
I	$f_{\alpha}^{T_m} = 234$	223	144	23.3	249	47.0
V	228	$f_{\alpha}^{T_m} = 394$	336	28.3	403	51.2
VI	78	269	$f_{\alpha}^{T_m} = 598$	25.1	665	57.8

namic characteristics.

The discrimination of mismatches is characterized by the decrease of selectivity upon increasing the temperature if unmodified probes are used. One can expect, in general, that some modifications of the probe structure can change such a relationship. There are three possible cases when $f_\alpha^{lim}(T) > 1$ in the temperature range from 0 to 100˚C:

1) $\Delta\Delta H^O < 0$ and $T_\Delta > 0$˚C:, $f_\alpha^{\lim}(T)$ decreases with temperature increase;

2) $\Delta\Delta H^O = 0$ and $\Delta\Delta S^O > 0$, $f_\alpha^{\lim}(T)$ is independent on temperature and the perfect complex is more favorable in entropy than the mismatched one $\Delta S_N^O > \Delta S_M^O$;

3) $\Delta\Delta H^O > 0$ and $T_\Delta < 0$˚C:, $f_\alpha^{\lim}(T)$ increases with temperature increase.

The first case ($\Delta\Delta H^O < 0$) is the main subject of the analysis in this work. The other cases are obviously uncharacteristic for the obtained thermodynamic effects caused by mismatches in duplexes formed by unmodified oligonucleotides. The third case, when $\Delta\Delta H^O > 0$, however, is very attractive for the application of allele specific hybridization at high temperatures. Function $f_\alpha(T)$ increases with the temperature rise without reaching a maximum. The hypothetical situation corresponding to this condition is depicted in **Figure 6**. In this case, the lengthening of the probe, the increase of its concentration, and the increase of hybridization temperature are favorable for the selectivity of the interaction. It would be promising if a type of modification that results in a positive change in enthalpy was found.

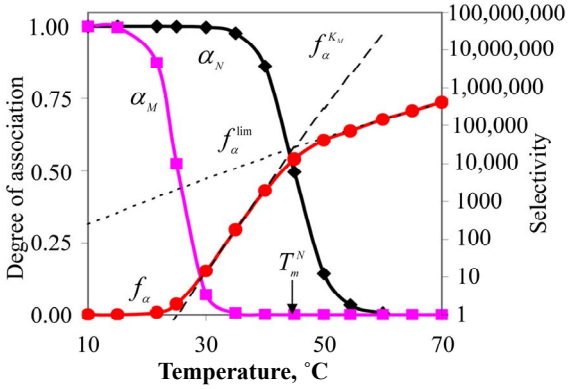

Figure 6. Temperature dependence of the association degrees of the perfect (α_N) and imperfect (α_M) complexes (left axis) and of the selectivity functions f_α, $f_\alpha^{\lim}$ and $f_\alpha^{K_M}$ (right axis, log scale) for some modified probe at $c_p = 10^{-5}$ M. T_m^N is melting temperature of the perfect complex. The values $\Delta H^O = 73$, $\Delta\Delta H^O = 23.8$ kcal/mol, $\Delta S^O = -206.6$, $\Delta\Delta S^O = -95$ cal/(mol·K) are calculated for the hypothetic case.

4. DISCUSSION

4.1. Parameters of Selectivity Used in the Literature

The identification of point mutations in nucleic acids using the allele-specific hybridization method implies, as a rule, that nucleotide sequences of NA, and the type and location of a probable nucleotide substitution are known. The aim of the analysis of the polymorphic site is to distinguish mutated and normal states with the highest reliability. The selectivity of the analysis is determined by the prevalence of perfect complex formation of a probe in comparison with imperfect one.

There are several approaches in the literature to the evaluation of hybridization selectivity based on the comparison of the association degrees of the perfect and mismatched complexes. Some authors have used the difference between them $\alpha_N - \alpha_M$ [12,42]. We suppose that the use of the ratio of these parameters is more correct since, according to IUPAC, selectivity in analytical chemistry is the ratio of specific and nonspecific signals [51]. Here, we used the selectivity function f_α, which is the ratio α_N/α_M, for the evaluation of the ability of probes to discriminate point mutations.

One of the first attempts in quantitative evaluation of selectivity of hybridization between an extended oligonucleotide probe and genomic DNA was performed in the work of Hearst J.E., who proposed the function $\lg(f_\alpha)$ [43]. In this case, the selectivity was calculated using the empirical formula: $\lg[f_\alpha(T_m)] = (3.8 - \lg c_p)\Delta l/l$, where $l + 1$ is the probe length, Δl is the length of the non-complementary region of the probe-DNA complex, and c_p is the probe concentration. Graphic analysis of this function showed that the maximal selectivity of interaction between a probe and a template is achieved at a melting temperature of the perfect complex. This formula was not, however, widely adopted in practice. Function f_α was also used as one of the criteria for the choice of "structure-free" oligonucleotides as elements of combinatorial DNA word sets [44]. The ratio of specific to nonspecific signals, that is, in general, f_α, is used in hybridization analysis for the evaluation of the selectivity of a given probe [17,54]. The detailed analysis of the influence of different parameters on this function was not, however, carried out.

Evaluations of selectivity are more often based on other parameters such as K_N/K_M [25], $\Delta\Delta G^O$ [10, 26-28,38-41], and ΔT_m [12,28,30,31,34-37]. The ratio of equilibrium constants of perfect and imperfect complex formation K_{N^O}/K_M is equal to $f_\alpha^{\lim}$ (8) per se. This ratio can be used for evaluation of real hybridization selectivity in two cases: when hybridization temperature

is more or equal to T_{10c}^{N}, *i.e.*, when $f_\alpha \to f_\alpha^{\lim}$ (**Table 2**, p.5) and at the melting temperature of complementary complex where $f_\alpha = f_\alpha^{\lim}\left(T_m^N\right)/2$. So, the ratio K_N/K_M describes the limit selectivity values; it corresponds to the real selectivity f_α in particular cases. This argumentation is fully applicable to the characteristic of selectivity $\Delta\Delta G^{\mathrm{O}}$ since $\Delta\Delta G^{\mathrm{O}} = -RT\ln(f_\alpha^{\lim})$. In contrast to the ratio of K_N/K_M, the $\Delta\Delta G^{\mathrm{O}}$ does not directly describe the reachable ratio of specific to nonspecific signals because this parameter is the logarithm of $f_\alpha^{\lim}$ normalized by RT value, which is monotonously increased with temperature rising. This can lead to the erroneous interpretation about the selectivity criterion based on $\Delta\Delta G^{\mathrm{O}}$ upon increasing the hybridization temperature. For example, in the hypothetic case, when the function $f_\alpha^{\lim}(T)$ seems to be constant ($\Delta\Delta H^{\mathrm{O}} \to 0$), the selectivity criterion referred to $\Delta\Delta G^{\mathrm{O}}$ changes shows a slight improvement of the mismatch discrimination. But $\Delta\Delta G^{\mathrm{O}}$ is, nevertheless, a useful criterion, which can be used for the comparison of the general selectivity of a set of probes at a given temperature.

To date, one of the most utilizing parameters for comparison of probe selectivity is $\Delta T_m = T_m^N - T_m^M$. Conclusions concerning the enhancement of hybridization selectivity caused by the modification of a probe are often based upon increasing the ΔT_m value. As it can be seen from the expression $f_\alpha^{T_m} = \exp\left[-\Delta T_m \Delta H_M^{\mathrm{O}}/\left(T_m^N T_m^M\right)\right]$, there is no direct relation between $f_\alpha^{T_m}$ and ΔT_m, so it is difficult to evaluate the hybridization selectivity just from the ΔT_m value.

The additional analysis allows us to estimate how the change of ΔT_m affects the maximal hybridization selectivity. It was shown above that $f_\alpha^{T_m} = f_\alpha^{K_M}\left(T_m^N\right)/2 = f_\alpha^{\lim}\left(T_m^N\right)/2$ (10). Using the approximation $\ln f_\alpha^{K_M}(T) \approx k\left(T - T_m^M\right)$, where k is proportional to ΔH_M^{O} (analogous to (20)), we can express $\ln\left(2f_\alpha^{T_m}\right) \approx k\Delta T_m$. The following equation can be used to analyze the change of $f_\alpha^{T_m}$ caused by variation of any parameters affecting the relative thermostabilities of complexes: $\gamma = f_{\alpha 2}^{T_m}/f_{\alpha 1}^{T_m} = \exp\left(k_2\Delta T_{m2} - k_1\Delta T_{m1}\right)$.

Thus, the comparison of ΔT_m values can be used as a certain characteristic of the selectivity change only when $k_1 = k_2$. This is true when $\Delta H_{M1}^{\mathrm{O}} = \Delta H_{M2}^{\mathrm{O}}$. If the ΔH_M^{O} values are not equal and unknown, the difference $\Delta T_{m1} - \Delta T_{m2}$ cannot show changes of hybridization selectivity upon the variation of the system parameters (length and modification of a probe, type of a mismatch, and buffer composition).

4.2. Selectivity of Hybridization

The maximal selectivity of hybridization can be provided by changing the thermodynamics of duplex formation (e.g. the structure of a probe), probe concentration, and hybridization temperature. It should be noted that all of these parameters influence the hybridization efficiency of the probe and, consequently, are included in the analyzed selectivity function f_α.

When examining the discrimination ability of two different probes intended to reveal the same substitution in an analyzed template, it is necessary to clearly distinguish the notions of the probe selectivity $f_\alpha(3)$ for the specific hybridization conditions and the limit probe selectivity $f_\alpha^{\lim}$ as a general parameter (8). Thus, to determine what probe has the maximal selectivity, it is necessary to know the dependence of the $f_\alpha^{\lim}$ values on temperature. A given probe is, in general, more selective than another probe if the corresponding functions $f_\alpha^{\lim}(T)$ are greater in the range of used hybridization temperatures.

The results allow us to conclude that the thesis "affinity and specificity are anticorrelated" [55] become rather questionable. Actually, the increase in affinity (equilibrium binding constant) of a probe due, for example, to its lengthening or changing temperature, can both decrease and increase the real selectivity f_α. There are condition ranges where these functions are correlated (for example, in temperature range $T_{\max} - T_\Delta$), anticorrelated ($T_N^M - T_{\max}$) or practically independent on each other ($T < T_m^M$). In general, in case of hybridization native oligonucleotide probes, the sign and the value of $\Delta\Delta H°$ results in correlation of the limit selectivity function $f_\alpha^{\lim}(T)$ with affinities of a probe ($K_N(T)$ or $K_M(T)$).

The $f_\alpha^{\lim}$ value reflects the potentially attainable selectivity; for native oligonucleotide probes, it usually increases with the decrease of hybridization temperature. One of the approaches to varying the limit probe selectivity when revealing a certain mismatch is, therefore, the modification of the probe structure, which leads to the change of the $\Delta\Delta H^{\mathrm{O}}$ and $\Delta\Delta S^{\mathrm{O}}$ parameters. In this context, it is very important to determine the thermodynamic characteristics of new oligonucleotide probes designed from their derivatives or analogues in detail. The absence of the appropriate information in most cases makes the understanding of the real effect of modification on changes of hybridization selectivity difficult. Only a few examples of well-grounded statements concerning the enhancement of selectivity of modified probes in comparison with native ones are described in the literature. Oligonucleotides containing a number of C5-propynyl-modified pyrimidines displayed enhanced specificity due to the long-range cooperativity of interaction between modified bases [56]. Many derivatives of natural oligonucleotides and their analogues are proposed for enhancing the selectivity of nucleic acid

recognition. The most promising compounds are PNA [25]; LNA [4], cyclic, cross-linked, and bicyclic oligonucleotides [26,27], oligonucleotides bearing minor groove binders [28], and probes containing analogues of nitrogen bases [30,57].

The selectivity function was shown to reach two upper values, which were determined by the distinctive behavior of this function in two cases: temperature was varied, or it was not. If temperature is the variable parameter, the unique case is fulfilled with a maximum of the selectivity function. This is the most probable case for SNP identification systems when unmodified oligonucleotides with $\Delta\Delta H^{O} < 0$ are used. In this case, there is the temperature (T_{max}) close to the melting temperature of the perfect complex (T_m^N) where the selectivity reaches maximum. For systems with $\Delta\Delta H^{O} > 0$, f_α is a monotonically increasing function independently of all possible types of experimentally controlled variables. In this case, the maximal selectivity at any given temperature is the limit selectivity $f_\alpha^{lim}(T)$. However, this leads to the necessity of decreasing efficiency of formation of the perfect duplex as much as possible for reliable detection at the given sensitivity of the method. This is the general rule to reach the maximal selectivity of hybridization independently of the thermodynamic features of the system, *i.e.*, for any $\Delta\Delta H^{O}$. Therefore, it is necessary to find a compromise between the observable selectivity level and the recorded signal to noise ratio. In this way, the highest level of selectivity can be achieved when the efficiency of hybridization is extremely low. Therefore, the corresponding experimental conditions should be used only when the sensitivity of detection of the hybridization signal is high enough.

The comparative analysis of the dependence of hybridization selectivity on the length of a native oligonucleotide revealed a quite unexpected fact (**Figure 3**). The longer the oligonucleotide probe, the higher the maximum value of selectivity observed at the same T_{max}. The differences are, however, not sufficient: for example, the value of the maximal selectivity at a given temperature for an 8-mer is 1.5 times lower than for a 15-mer probe (**Table 4**). Therefore, the choice of probe length should be determined by other parameters. The minimal length of the probe is determined by probe concentration and the unique binding site in the DNA to be analyzed. The upper limit of the probe length is conditioned by a possibility of obtaining the detectable hybridization signal at low probe concentration under a given experimental condition. In our example, to maintain the same T_{max} for a 10-mer and a 15-mer, the concentration of the longer oligonucleotide should be decreased by four orders of magnitude in comparison with the shorter one (**Table 4**). Thus, the choice of the probe length for a given temperature is a complex problem concerned with the sensitivity of detection, the sequence specificity of the probe, and, to a lesser degree, with the maximal selectivity of hybridization.

Identical conclusions can be reached when no changes of probe structure influence the $\Delta\Delta H^{O}$ and $\Delta\Delta S^{O}$ values. One example of this is the variation of the mismatch position in a probe binding site, which can change the GC content of the probe. Another example is some modification of the probe, which does not affect the duplex structure close to the mismatched base pair.

These conclusions allow us to suppose that molecular beacons are not more selective than linear probes [11]. Because of the formation of hairpin, the concentration of non-structured form of molecular beacon interacting with a target reduces that can increase f_α. Since the $\Delta\Delta H^{O}$ and $\Delta\Delta S^{O}$ values do not change, the same selectivity can be reached when concentration of the ordinary linear probe is reduced. Advantage of molecular beacons consists in their ability to generate the specific signal but not in enhancing the hybridization selectivity.

The most readily available way to increase the probe selectivity is modifications of the probe structure, which resulted in the change of $\Delta\Delta H^{O}$ and $\Delta\Delta S^{O}$ parameters. The increase or decrease of the thermostability of the probe complexes, and the commonly used ΔT_m parameter do not reflect a change of selectivity. Only the knowledge of thermodynamics of complexation allows one to describe a change of attainable selectivity. Unfortunately, it is rather difficult to predict the exact effects of any modification on entropy and enthalpy of the probe complexation. Therefore, to choose a suitable modification it is necessary to screen a large number of variants. This is a difficult task because many of the detailed thermodynamic data should be obtained and analyzed in accordance with analytical considerations represented here.

4.3. Conclusions

There are two principal ways of increasing the hybridization selectivity. The first way is to search modified oligonucleotide probes for those that provide an enhanced level of discrimination of any perturbation in the specific sequence in comparison with native oligonucleotides. The second way is the optimization of hybridization conditions and minor variations in probe structure (for example, the length change). In both cases a successful result can be obtained if there is an accurate thermodynamic description of the analyzed system.

To evaluate the real effect caused by modification of the probe on the selectivity, it is necessary to analyze the changes of the $f_\alpha^{lim}(T)$ values. For this purpose, the corresponding thermodynamic characteristics ($\Delta\Delta H^{O}$

and $\Delta\Delta S^{O}$) should be determined. These thermodynamic parameters should depend not only on the modification of the probe, but also on buffer conditions (pH, ion composition, ionic strength, and, in some cases, on organic or inorganic additions having the preferable affinity either to matched or mismatched complexes). As a result, the enhancement of $f_{\alpha}^{\lim}(T)$ by itself does not guarantee the maximal selectivity of hybridization while using a given probe at given conditions. The choice of the optimal probe in any particular case is a multiparametric task that implies the optimization of the probe structure, its concentration and hybridization temperature on the basis of the analysis f_{α} function.

In this work, we considered the theoretical aspects of hybridization selectivity while using the oligonucleotide probe for revealing any perturbation in an analyzed NA duplex. The proposed analytical description allows one to evaluate the efficiency of discrimination of such a perturbation and can provide the basis for software for the rational design of the optimal probe structure.

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APPENDIX

S1. Analysis of the Selectivity Function

S1.1. Function f_α tends to 1 in the range of temperatures $T \le T_{c/10}^M$: $f_\alpha\left(T_{c/10}^M\right) = \dfrac{1/K_M + c_p}{1/K_N + c_p} \approx \dfrac{c_p}{c_p} = 1$

This is true if $1/K_M \ll c_p$ and $1/K_N \ll c_p$, since $K_N > K_M$, one can consider only $1/K_M \ll c_p \Rightarrow 1/K_M = c_p/10$, then

$$\frac{1}{K_M} = \frac{c_p}{10} \Rightarrow \exp\left(\frac{\Delta H_M^{\mathrm{O}} - T\Delta S_M^{\mathrm{O}}}{RT}\right) = \frac{c_p}{10}$$

$$\Rightarrow \frac{\Delta H_M^{\mathrm{O}} - T\Delta S_M^{\mathrm{O}}}{RT} = \ln\frac{c_p}{10}$$

$$\Rightarrow T\left(\Delta S_M^{\mathrm{O}} + R\ln\frac{c_p}{10}\right) > \Delta H_{\mathrm{M}}^{\mathrm{O}}$$

$$\Rightarrow T_{c/10}^M = \frac{\Delta H_M^{\mathrm{O}}}{\Delta S_M^{\mathrm{O}} + R\ln\left(c_p/10\right)}$$

S1.2. Function f_α tends to 2 when $T = T_m^M$: $f_\alpha\left(T_m^M\right) = \dfrac{1/K_M + c_p}{1/K_N + c_p} \approx \dfrac{c_p + c_p}{c_p} = 2$ due to $\dfrac{1}{K_M\left(T_m^M\right)} = c_p$

This is true if $1/K_N \ll c_p$, that is observed if $1/K_N = c_p/10$ and corresponds to temperature $T_{c/10}^N = \dfrac{\Delta H_N^{\mathrm{O}}}{\Delta S_N^{\mathrm{O}} + R\ln(c_p/10)}$. Thus, if $T_m^M \le T_{c/10}^N$, inequality is fulfilled.

S1.3. Function f_α tends to $f_\alpha^{K_M}$ in the range of temperatures $T_{10c}^M \le T \le T_{c/10}^N$:

$$f_\alpha = \frac{1/K_M + c_p}{1/K_N + c_p} \approx \frac{1/K_M}{c_p} = \frac{1}{c_p K_M} = f_\alpha^{K_M}$$

This is true if $1/K_M \gg c_p$ and $1/K_N \ll c_p$, that corresponds to the range $T_{10c}^M \le T \le T_{c/10}^N$.

Thus, if

$$T_{10c}^M < T_{c/10}^N \Rightarrow \frac{\Delta H_M^{\mathrm{O}}}{\Delta S_M^{\mathrm{O}} + R\ln 10c_p} < \frac{\Delta H_N^{\mathrm{O}}}{\Delta S_N^{\mathrm{O}} + R\ln\left(c_p/10\right)}$$

inequality is fulfilled.

S1.4. Function f_α tends to $f_\alpha^{\lim}$ in the range of high temperatures $T \ge T_{10c}^N$:

$$f_\alpha = \frac{1/K_M + c_p}{1/K_N + c_p} \approx \frac{K_N}{K_M} = \exp\left(-\frac{\Delta\Delta H^{\mathrm{O}} - T\Delta\Delta S^{\mathrm{O}}}{RT}\right) = f_\alpha^{\lim}$$

This is true if $c_p \ll 1/K_M$ and $c_p \ll 1/K_N$, since $K_N > K_M$, one can consider only $c_p \ll 1/K_N$, Let $1/K_N = 10c_p$, then $T_{10c}^N = \dfrac{\Delta H_N^{\mathrm{O}}}{\Delta S_N^{\mathrm{O}} + R\ln 10c_p}$

S1.5. Quantitative Evaluation of Maximum Selectivity $f_\alpha^{T_m}$:

$$f_\alpha^{\lim}\left(T_m^N\right) = \frac{K_N}{K_M} = \frac{1}{c_p K_M\left(T_m^N\right)},$$

$$f_\alpha^{K_M}\left(T_m^N\right) = \frac{1}{c_p K_M\left(T_m^N\right)}$$

$$f_\alpha^{\lim} = f_\alpha^{K_M} \Rightarrow \frac{K_N}{K_M} \Rightarrow \frac{1}{c_p K_M} \Rightarrow K_N = \frac{1}{c_p} \Rightarrow T = T_m^N$$

$$f_\alpha^{T_m} = f_\alpha\left(T_m^N\right) = \frac{1/K_M + c_p}{1/K_N + c_p} \approx \frac{1/K_M}{c_p + c_p}$$

$$= \frac{1}{2c_p K_M\left(T_m^N\right)} = \frac{f_\alpha^{\lim}\left(T_m^N\right)}{2} = \frac{f_\alpha^{K_M}\left(T_m^N\right)}{2}$$

$$f_\alpha^{T_m} = f_\alpha\left(T_m^N\right) = \frac{f_\alpha^{\lim}\left(T_m^N\right)}{2} = \frac{1}{2}\exp\left(-\frac{\Delta\Delta H^{\mathrm{O}}}{RT_m^N} + \frac{\Delta\Delta S^{\mathrm{O}}}{R}\right)$$

$$= \frac{1}{2}\exp\left(-\frac{\Delta\Delta H^{\mathrm{O}}}{R\Delta H_N^{\mathrm{O}}/\Delta S_N^{\mathrm{O}} + R\ln c_p} + \frac{\Delta\Delta S^{\mathrm{O}}}{R}\right)$$

$$= \frac{1}{2}c_p^{-\frac{\Delta\Delta H}{\Delta H_N}}\exp\left(\frac{\Delta\Delta S^{\mathrm{O}}}{R} - \frac{\Delta S_N^{\mathrm{O}}}{R}\cdot\frac{\Delta\Delta H^{\mathrm{O}}}{\Delta H_N^{\mathrm{O}}}\right)$$

S2. Dependence of the Maximum of the Selectivity Function on the Probe Concentration

$$\gamma_c = \frac{f_\alpha^{T_m}\left(xc_p\right)}{f_\alpha^{T_m}\left(c_p\right)}$$

$$= \frac{\left(xc_p\right)^{-\frac{\Delta\Delta H^{\mathrm{O}}}{\Delta H_N^{\mathrm{O}}}}\exp\left(\frac{\Delta\Delta S^{\mathrm{O}}}{R} - \frac{\Delta S_N^{\mathrm{O}}}{R}\frac{\Delta\Delta H^{\mathrm{O}}}{\Delta H_{\mathrm{N}}^{\mathrm{O}}}\right)\Big/2}{c_p^{-\frac{\Delta\Delta H^{\mathrm{O}}}{\Delta H_N^{\mathrm{O}}}}\exp\left(\frac{\Delta\Delta S^{\mathrm{O}}}{R} - \frac{\Delta S_{\mathrm{N}}^{\mathrm{O}}}{R}\frac{\Delta\Delta H^{\mathrm{O}}}{\Delta H_{\mathrm{N}}^{\mathrm{O}}}\right)\Big/2}$$

$$= x^{-\frac{\Delta\Delta H^{\mathrm{O}}}{\Delta H_N^{\mathrm{O}}}}$$

S3. Dependence of the Maximum of the Selectivity Function on the Probe Length

$$\gamma_l = \frac{f_\alpha^{T_{m2}}}{f_\alpha^{T_{m1}}} = \frac{\exp\left[\Delta\Delta H^{\mathrm{O}}\left(1/T_\Delta - 1/T_{m2}^N\right)/R\right]}{\exp\left[\Delta\Delta H^{\mathrm{O}}\left(1/T_\Delta - 1/T_{m1}^N\right)/R\right]} = \exp\left[\frac{\Delta\Delta H^{\mathrm{O}}}{R}\left(\frac{1}{T_{m1}^N} - \frac{1}{T_{m2}^N}\right)\right]$$

$$= \exp\left[\frac{\Delta\Delta H^{\mathrm{O}}}{R}\left(\frac{\Delta S_{N1}^{\mathrm{O}} + R\ln c_p}{\Delta H_{N1}^{\mathrm{O}}} - \frac{\Delta S_{N2}^{\mathrm{O}} + R\ln c_p}{\Delta H_{N2}^{\mathrm{O}}}\right)\right] \sim \exp\left[\Delta\Delta H^{\mathrm{O}}\left(\frac{1}{\Delta H_{N1}^{\mathrm{O}}} - \frac{1}{\Delta H_{N2}^{\mathrm{O}}}\right)\ln c_p\right]$$

$$= c_p^{\Delta\Delta H^{\mathrm{O}}\frac{\left(\Delta H_{N2}^{\mathrm{O}} - \Delta H_{N1}^{\mathrm{O}}\right)}{\Delta H_{N1}^{\mathrm{O}}\Delta H_{N2}^{\mathrm{O}}}} \approx c_p^{\frac{\Delta\Delta H^{\mathrm{O}}}{\Delta\overline{H}_N^{\mathrm{O}}}\frac{(l_2 - l_1)}{l_1 l_2}}$$

S4. Dependence of the Maximum of the Selectivity Function on the Type of a Mismatch

$$\gamma_{pert} = \frac{f_\alpha^{T_m}\left(\Delta\Delta H^{\mathrm{O}} + h, \Delta\Delta S^{\mathrm{O}} + s\right)}{f_\alpha^{T_m}\left(\Delta\Delta H^{\mathrm{O}}, \Delta\Delta S^{\mathrm{O}}\right)} = \frac{f_\alpha^{\lim^*}\left(T_m^N\right)/2}{f_\alpha^{\lim}\left(T_m^N\right)/2} = \exp\left(-\frac{\Delta\Delta H^{\mathrm{O}} + h - T_m^N\left(\Delta\Delta S^{\mathrm{O}} + s\right)}{RT_m^N} + \frac{\Delta\Delta H^{\mathrm{O}} - T_m^N\Delta\Delta S^{\mathrm{O}}}{RT_m^N}\right)$$

$$= \exp\left(-\frac{h - T_m^N s}{RT_m^N}\right) = K_{pert}$$

S5. Linear Dependence Logarithm of Limit Selectivity Function

Within temperature range from 0˚C to 100˚C the function $\ln f_\alpha^{\lim}(T)$ can be described by linear dependence with good accuracy. So we can propose:

$$\ln f_\alpha^{\lim}(T) = -\Delta\Delta H^{\mathrm{O}}\left(1/T - 1/T_\Delta\right)/R \approx kT + b$$

$$\left.\begin{aligned} kT_0 + b &= -\Delta\Delta H^{\mathrm{O}}\left(1/T_0 - 1/T_\Delta\right)/R \\ kT_{100} + b &= -\Delta\Delta H^{\mathrm{O}}\left(1/T_{100} - 1/T_\Delta\right)/R \end{aligned}\right\}$$

$$\Rightarrow k\left(T_0 - T_{100}\right) = \frac{\Delta\Delta H^{\mathrm{O}}}{R}\left(\frac{1}{T_{100}} - \frac{1}{T_0}\right)$$

$$\Rightarrow k = \frac{\Delta\Delta H^{\mathrm{O}}}{RT_{100}T_0} = \frac{\Delta\Delta H^{\mathrm{O}}}{R}c_1$$

$$b = -\Delta\Delta H^{\mathrm{O}}\left(1/T_0 - 1/T_\Delta\right)/R - \Delta\Delta H^{\mathrm{O}}/RT_{100}$$

$$= -\Delta\Delta H^{\mathrm{O}}\left(1/T_0 + 1/T_0 - 1/T_\Delta\right)/R = -c_2\Delta\Delta H^{\mathrm{O}}/R + \Delta\Delta S^{\mathrm{O}}/R$$

$$\mathrm{In}\, f_\alpha^{\lim}(T) \approx (c_1\Delta\Delta H^{\mathrm{O}}/R)\cdot T - c_2\,\Delta\Delta H^{\mathrm{O}}/R + \Delta\Delta S^{\mathrm{O}}/R$$

where c_1=9.8·10^{-6} K^{-2} and c_2=6.3·10^{-3} K^{-1}

