Kinetic Analysis of Specificity of Duplex DNA Targeting by Homopyrimidine Peptide Nucleic Acids

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ABSTRACT A simple theoretical analysis shows that specificity of double-stranded DNA (dsDNA) targeting by homopyrimidine peptide nucleic acids (hpyPNAs) is a kinetically controlled phenomenon. Our computations give the optimum conditions for sequence-specific targeting of dsDNA by hpyPNAs. The analysis shows that, in agreement with the available experimental data, kinetic factors play a crucial role in the selective targeting of dsDNA by hpyPNAs. The selectivity may be completely lost if PNA concentration is too high and/or during prolonged incubation of dsDNA with PNA. However, quantitative estimations show that the experimentally observed differences in the kinetic constants for hpyPNA binding with the correct and mismatched DNA sites are sufficient for sequence-specific targeting of long genomic DNA by hpyPNAs with a high yield under appropriate experimental conditions. Differential dissociation of hpyPNA/dsDNA complexes is shown to enhance the selectivity of DNA targeting by PNA.

INTRODUCTION

Homopyrimidine peptide nucleic acids (hpyPNAs) are synthetic oligomers consisting of a polyamide backbone linked to pyrimidine nucleobases (Nielsen et al., 1991, 1994a; Hanvey et al., 1992; De Mesmaeker et al., 1995; Frank-Kamenetskii and Mirkin, 1995; Nielsen and Ørum, 1995; Noble et al., 1995; Demidov et al., 1996). They are very promising DNA-binding ligands: hpyPNAs are not digested by cellular enzymes (Demidov et al., 1994b), and they form highly selective and extraordinary stable complexes with double-stranded DNA (dsDNA) (Nielsen et al., 1991, 1993a; Hanvey et al., 1992; Peffer et al., 1993; Cherny et al., 1993). In this unusual structure, called the P-loop, two PNA oligomers invade the DNA duplex with the formation of triplex with the homopurine strand of the DNA target site, leaving the homopyrimidine strand displaced (Nielsen et al., 1993a,b, 1994b; Peffer et al., 1993; Cherny et al., 1993; Egholm et al., 1995; Griffith et al., 1995; Demidov et al., 1995, 1996; Wittung et al., 1996). In so doing, hpyPNA oligomers behave quite differently from triplex-forming oligonucleotides and their analogs, which occupy the major groove of the DNA double helix (reviewed by Frank-Kamenetskii and Mirkin, 1995). The exceptional stability and specificity of hpyPNA/dsDNA complexes make it possible to create artificial transcription promoters (Møllegaard et al., 1994), to map short targets on duplex DNA by electron microscopy (Demidov et al., 1994a), to convert single-strand-specific nucleases into sequence-selective cutters (Demidov et al., 1993), and to use PNAs as rare genome-cutters (Veselkov et al., 1996a,b). PNA oligomers compete very efficiently with proteins for binding sites on dsDNA (Hanvey et al., 1992; Nielsen et al., 1993a, 1994c; Larsen and Nielsen, 1996; Veselkov et al., 1996a,b).

Although the problem of specificity of interaction between a ligand and its binding site on DNA has been considered earlier (von Hippel and Berg, 1986; Perelroyzen and Vologodskii, 1988; Herschlag, 1991; Eaton et al., 1995), the case of hpyPNA/dsDNA interaction requires special treatment. Previously, this problem was treated under the assumption about equilibrium binding of a ligand to correct and incorrect binding sites. The assumption is not valid for the case of complex formation between hpyPNA and dsDNA. Our experimental studies of hpyPNA interaction with duplex DNA have led us to the conclusion that under real experimental conditions the PNA binding process is essentially irreversible (Cherny et al., 1993; Demidov et al., 1995, 1996, and manuscript in preparation). This is the consequence of the exceptional stability of the $(PNA)_2$/DNA triplex, which is additionally stabilized, as compared with canonical $(DNA)_3$ triplexes, because of the lack of the electrostatic repulsion between the participating DNA and PNA strands and by the additional H-bonds, water bridges, and van der Waals interactions between the DNA backbone and the PNA backbones (Betts et al., 1995).

We have recently demonstrated that prolonged incubation leads to virtually full and irreversible occupation, not only of the correct but also of mismatched binding sites on dsDNA by hpyPNAs (Demidov et al., manuscript in preparation). At least it is true for some DNA sites with one and two mismatches, which form incorrect but quite stable hpyPNA/dsDNA complexes. The number of such mismatched sites is much greater than the number of fully matched PNA targets in sufficiently large DNA molecules. These findings emphasize that selective targeting of correct sites can be reached in the case of hpyPNA/dsDNA interaction only on the basis of an optimum choice of the reaction time. In other words, specificity of targeting is an

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essentially kinetically controlled process. In the present paper we treat theoretically the problem of kinetic discrimination between binding sites in the case of irreversible targeting. We also consider the kinetic discrimination between PNA/DNA complexes under conditions favoring their dissociation.

THEORETICAL ANALYSIS

Kinetic consideration of hpyPNAs binding to dsDNA

We consider the hpyPNAs binding as an irreversible process and assume that the formation of the PNA/DNA complex with the correct site occurs with the kinetic constant k_c, whereas the formation of the corresponding complex with an incorrect (mismatched) site occurs with the kinetic constant k_i. Usually the number of PNA molecules in kinetic experiments is much larger than the number of DNA target sites, and the process of the binding of hpyPNA to dsDNA sites obeys pseudo-first-order kinetics (Demidov et al., 1995, 1996). Therefore, the fraction α of DNA molecules that form the correct complex with PNA by the time moment t is equal to

$$\alpha = 1 - \exp(-k_c t) \tag{1}$$

The fraction α_i of DNA molecules that form an incorrect (mismatched) complex with PNA by the same time is equal to

$$\alpha_i = 1 - \exp(-k_i t) = 1 - (1 - \alpha)^{k_i/k_c} = 1 - (1 - \alpha)^{f_i} \tag{2}$$

where the $f_i = k_i/k_c$ value determines the site selectivity of the dsDNA recognition by hpyPNA. This means that the probability β_i that a particular mismatched site will not bind to PNA by this moment is

$$\beta_i = (1 - \alpha)^{f_i} \tag{3}$$

For a DNA molecule with multiple mismatched sites, the probability β of not binding PNA at any mismatched site will be

$$\beta = \Pi\beta_i = \Pi(1 - \alpha)^{f_i} = (1 - \alpha)^{\Sigma f_i} = (1 - \alpha)^F \tag{4}$$

where the product and the summation are taken over all nonspecific binding sites and $F = \Sigma f_i = \Sigma k_i/k_c$ characterizes the integral selectivity of dsDNA recognition by a certain hpyPNA oligomer. The β quantity monotonously decreases with time and determines the specificity of DNA targeting during PNA binding.

Let us define now the yield Y of DNA molecules uniquely targeted by PNA at the correct site without any mismatched binding. Because different sites on DNA are occupied independently, the Y value can be presented as

$$Y = \alpha\beta = \alpha(1 - \alpha)^F \tag{5}$$

or, as a function of t:

$$Y = \exp(-Fk_c t)[1 - \exp(-k_c t)] \tag{6}$$

At the initial stage of PNA binding, the probability of forming incorrect hpyPNA/dsDNA complexes is small and the correct complex is preferentially formed. However, the number of mismatched complexes increases with time, thus

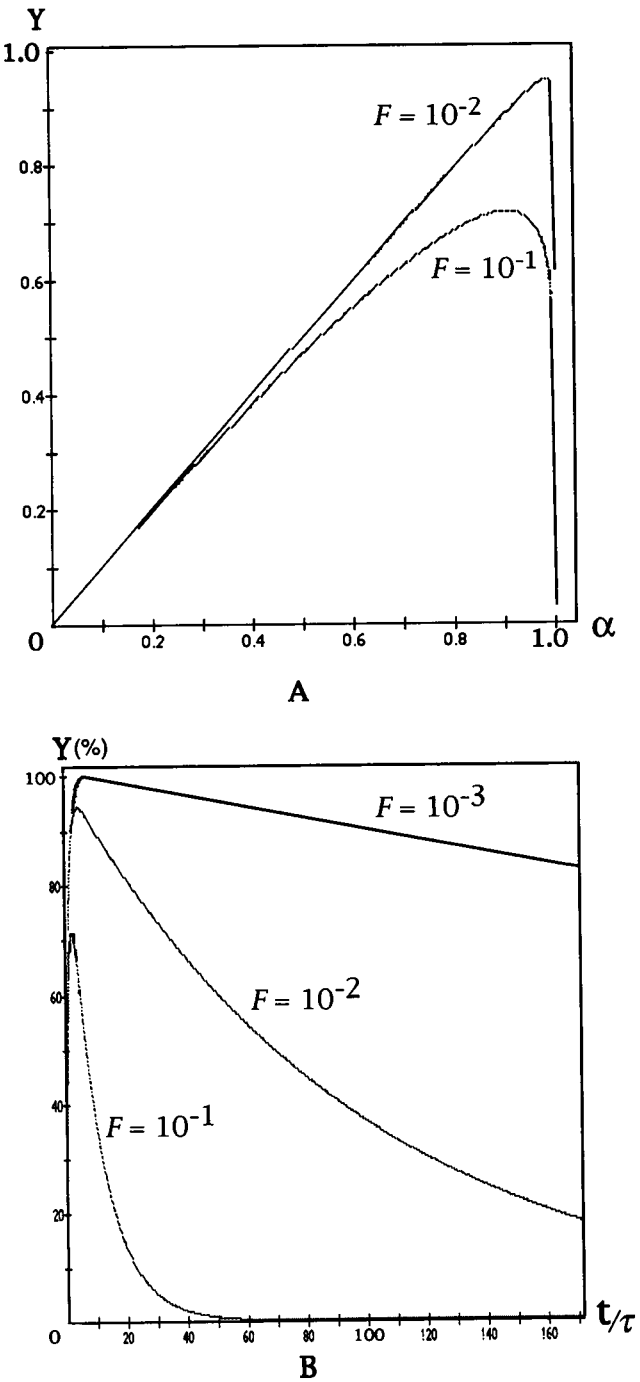

FIGURE 1 Functions $Y(\alpha)$ and $Y(t)$, which illustrate time dependence of the yield of DNA molecules uniquely targeted by hpyPNA at the correct site, without any mismatched binding for different values of the integral selectivity, F.

compromising the specificity of PNA targeting. Therefore, it is reasonable to assume that the Y value reaches maximum at some degree of PNA binding. Fig. 1 demonstrates that this is really the case. The highest yield, Y_{max}, and the corresponding α_{max} value can be found from the condition

$$(dY/d\alpha)_{|\alpha=\alpha_{max}} = 0 \tag{7}$$

Equations 5 and 7 lead to the following equations:

$$\alpha_{max} = 1/(1 + F) \tag{8}$$

$$Y_{max} = (1/F)[F/(1 + F)]^{(1+F)} \tag{9}$$

From Eqs. 1 and 8 one can obtain the optimum time, t_{max}, of DNA incubation with PNA, at which the maximum yield of DNA molecules that are uniquely targeted by hpyPNA at the correct site is achieved:

$$t_{max} = -\tau \ln[F/(1 + F)] \cong -\tau \ln F \tag{10}$$

where $\tau = 1/k_c$ is the characteristic time of the correct complex formation. Note that at the times $t > t_{max}$ the Y value will be proportional to $\exp(-Fk_c t)$. Therefore, the decrease in this value with time will be steeper at larger F. Fig. 1 *B* illustrates this peculiarity of the time course of the Y value and shows that it decreases exponentially with the characteristic time $t \cong \tau/F$.

The degree of nonspecific binding may likewise be characterized by the yield of nonspecific complexes formed by mismatched binding. From Eqs. 1 and 4 one can obtain the time course of the fraction, $Y_{ns}(t)$, of nonspecifically targeted dsDNA molecules:

$$Y_{ns}(t) = 1 - \beta = 1 - \exp(-Fk_c t) \tag{11}$$

Therefore, at time $t \cong \tau/F$ the selectivity of PNA binding will be completely compromised by nonspecific complexes. The nonspecific binding, Y_{ns}, at the optimum time, t_{max}, will be (see Eqs. 4 and 8)

$$\begin{aligned} Y_{ns}(t_{max}) &= 1 - \beta(t_{max}) \\ &= 1 - (1 - \alpha_{max})^F = 1 - [F/(1 + F)]^F \end{aligned} \tag{12}$$

Fig. 2 shows that even as small a value of the integral selectivity as $F = 10^{-1}$ makes it possible to achieve decent specificity of hpyPNA targeting. In this case the Y_{max} value will be more than 70% and the $Y_{ns}(t_{max})$ value will be about 20%. Quantitative targeting, together with good specificity, can be achieved for values of $F \leq 10^{-2}$: less than several percent of DNA molecules will be targeted at nonspecific sites, whereas more than 95% of the DNA molecules will be uniquely targeted at the correct site without any mismatched binding. The Y_{max} value of the specific targeting will exceed the $Y_{ns}(t_{max})$ value of nonspecific binding by more than 10 times for $F \leq 10^{-2}$ and by more than 100 times for $F \leq 10^{-3}$.

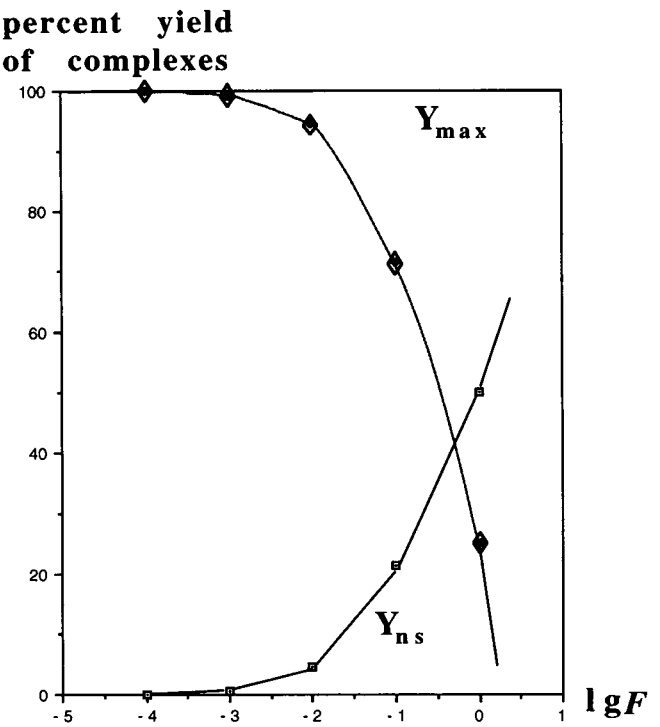

FIGURE 2 Dependence of the maximum yield, Y_{max}, of selective targeting (◇) and the yield, $Y_{ns}(t_{max})$, of nonspecific binding (□) of hpyPNA to DNA on the integral selectivity, F.

Kinetic consideration of dissociation of hpyPNA/dsDNA complexes

So far we have considered the specificity of dsDNA recognition, which can be achieved during the irreversible process of hpyPNA binding. Now we are going to analyze the process of dissociation of the preformed hpyPNA/dsDNA complexes. Such dissociation can be reached by a drastic change in the conditions, usually achieved by increasing incubation temperature or by alkali treatment (Cherny et al., 1993; Demidov et al., manuscript in preparation). We may neglect the PNA binding to DNA under conditions unfavorable for their complex formation, especially if the free PNA is supposed to be removed from the solution after formation of PNA/dsDNA complexes. The dissociation of the PNA/dsDNA complexes is apparently described by pseudo-first-order kinetics with kinetic constants k_c^- and k_i^- for correct and incorrect (mismatched) binding sites, respectively. Then the fraction α_- of DNA molecules keeping the correct site bound with PNA at the time t is equal to

$$\alpha_- = \alpha_-(0)\exp(-k_c^- t), \tag{13}$$

where $\alpha_i^-(0)$ is the fraction of the DNA molecules initially targeted by PNA at the correct site. At the same time, the fraction α_i^- of DNA molecules with a certain mismatched site keeping a PNA oligomer is

$$\begin{aligned} \alpha_i^- &= \alpha_i^-(0)\exp(-k_i^- t) \\ &= \alpha_i^-(0)(\alpha_-/\alpha_-(0))^{k_i/k_c} = A_i \alpha_- f_i^- \end{aligned} \tag{14}$$

where $\alpha_i^-(0)$ is the fraction of DNA molecules initially targeted by hpyPNA at the incorrect site; $f_{i-} = k_{i-}/k_{c-}$ is the ratio of kinetic constants that determines the differential discrimination of a correct PNA binding site versus the mismatched one due to dissociation of PNA/dsDNA complexes; $A_i = \alpha_i^-(0)/\alpha_-(0)f_{i-}$. Thus the probability β_{i-} that the particular mismatched site will be free from PNA at this stage of the process of dissociation of the PNA/dsDNA complex is

$$\beta_i^- = 1 - A_i\alpha_- f_{i-} \tag{15}$$

Therefore, for DNA molecule with N mismatched sites, the probability, β_-, of being free from PNA at any mismatched site will be

$$\beta_- = \Pi\beta_i^- = \Pi(1 - A_i\alpha_- f_{i-}) \tag{16}$$

where the product corresponds to all nonspecific binding sites. The β_- quantity monotonously increases with time and determines the specificity of DNA targeting in the process of PNA dissociation.

The following equation describes the fraction, Y_-, of DNA molecules without any mismatched binding that remain uniquely targeted by PNA at the correct site during PNA dissociation:

$$Y_- = \alpha_-\beta_- = \alpha_-\Pi(1 - A_i\alpha_- f_{i-}) \tag{17}$$

Functions $Y_-(\alpha_-)$ and $Y_-(t)$ behave qualitatively similarly to $Y(\alpha)$ and $Y(t)$ (see Fig. 3). The maximum yield, Y_{max-}, which can be achieved during the PNA dissociation, is observed at such α_{max-} that

$$(dY_-/d\alpha_-)_{|\alpha- = \alpha_{max-}} = 0 \tag{18}$$

From Eqs. 17 and 18 we obtain the result that α_{max-} obeys the relation

$$\Sigma A_i f_{i-}\alpha_{max-} f_{i-}/(1 - A_i\alpha_{max-} f_{i-}) = 1 \tag{19}$$

where the summation corresponds to all nonspecific binding sites.

Equation 19 is readily solved under the simplified assumption that for the DNA molecule with one correct and N mismatched sites initially targeted by specific PNA in a completely nonspecific manner ($\alpha_-(0) = \alpha_i^-(0) = A_i = 1$), the f_{i-} parameters are equal ($f_{i-} = f_-$):

$$\alpha_{max-} = 1/(1 + Nf_-)^{1/f-} \tag{20}$$

In this case Eq. 17 yields

$$Y_{max-} = (Nf_-)^N/(1 + Nf_-)^{(N+1/f-)} \tag{21}$$

From Eqs. 13 and 20 one can obtain the optimum time, t_{max-}, of PNA dissociation, at which the maximum yield Y_{max-} is achieved,

$$t_{max-} = (\tau_-/f_-)\ln(1 + Nf_-), \tag{22}$$

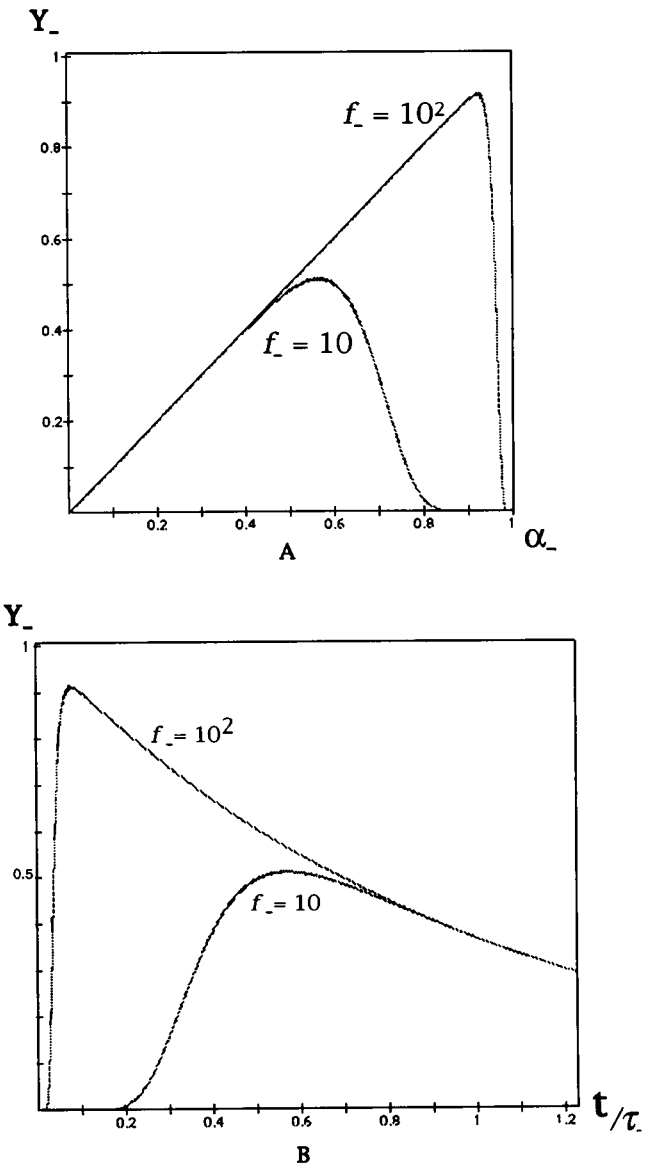

FIGURE 3 Functions $Y_-(\alpha_-)$ and $Y_-(t)$ for the case of $N = 30$; $\alpha_i^-(0) = \alpha_-(0) = 1$, which illustrate time dependence of the selectivity of megabase dsDNA targeting by a hpyPNA oligomer during its dissociation for different values of f_-.

where $\tau_- = 1/k_c^-$ is the characteristic time of correct complex dissociation.

After fast dissociation of mismatched PNA/DNA complexes, the rate of decrease of the fraction of DNA molecules, which remain uniquely targeted by hpyPNA at the correct site, depends only on the dissociation of correct complexes. Thus, after t_{max-}, the rate of decrease of the Y_- value is independent of parameter f_- (see Fig. 3 *B*). By contrast, in the process of hpyPNA binding to dsDNA, the selectivity of sequence-specific targeting of DNA duplex after fast formation of correct complexes with PNA is compromised by a slower accumulation of mismatched complexes and therefore depends on the f value (see Fig. 1 *B*).

DISCUSSION

For the sake of clarity, we limit our discussion by consideration of only two types of hpyPNA/dsDNA complexes: one corresponding to the correct site and the other one corresponding to single-mismatched sites. The analysis of specificity allowing for the possible multiple mismatched sites can be done in a similar way by taking into account greater kinetic disadvantage of such cases. Let us start with the problem of PNA/DNA association. We consider a simplified situation assuming the same values of parameters for PNA binding with N mismatched sites: $f_i = f$, $F = Nf$. For hpyPNAs binding to dsDNA, significant discrimination of single-mismatched sites may be reached, so that $f \leq 10^{-2}$ (Demidov et al., 1995, 1996). For sufficiently short DNA fragments there will be only one site (or very few sites) for mismatched PNA binding. In the case of $f = 10^{-2}$ and $N = 1$, one can obtain, from Eqs. 9 and 10, $Y_{max} = 0.945$ for the optimum incubation time and $t_{max} = 4.6\tau$. This means that at this time as many as 94.5% of DNA molecules are uniquely targeted at the correct site without any mismatched binding, and only 4.5% of DNA molecules have a mismatched site occupied by PNA (Eq. 12). However, an increase in the time of incubation by 5–10 times ($t = 25 \div 50\tau$) leads to a proportion of DNA molecules with incorrect PNA binding of 20–40%, thus drastically deteriorating the specificity of the hpyPNA/dsDNA recognition (see Eq. 11).

The change in PNA concentration produces an even stronger effect on the specificity of hpyPNA/dsDNA recognition due to nonlinear (from quadratic to cubic) dependence of the rate of PNA binding to dsDNA on PNA concentration (Demidov et al., 1995, 1996; Wittung et al., 1996). Therefore, only a two- to threefold increase in PNA concentration may lead to acceleration of PNA binding by about an order of magnitude and to at least a 10-fold decrease in the τ value. As a result, in contrast to entirely specific binding of hpyPNA during the optimum time $t = 4.6\tau$ for a certain PNA concentration, the PNA binding at the same time but with increased concentration will occur at a nonoptimum relation, $t \geq 46\tau'$, where $\tau' = 1/10\ \tau$. This means that now from 30% to 50% of DNA molecules will carry mismatched complexes. Note that for PNA "clamps" (bis-PNAs), consisting of two hpyPNA oligomers linked together, the situation with the concentration differences is less dramatic because the dependence of the rate of bis-PNA binding to dsDNA on bis-PNA concentration is close to linear (Demidov et al., 1995, 1996; Bentin and Nielsen, 1996).

Generally, in the case of virtually complete binding of PNA to its correct site ($\alpha \geq 0.9$), the specificity of PNA targeting is mainly determined by the probability, $\beta = \exp(-ft/\tau)$, of nonformation of the incorrect complexes. Thus changes in the time or in the rate of PNA binding have to lead to a very rapid (exponential) decrease of the yield of the specific PNA targeting. Fig. 4 *A* illustrates these estimations and clearly demonstrates that under an appropriate choice of the kinetic parameters (mainly, time of the PNA

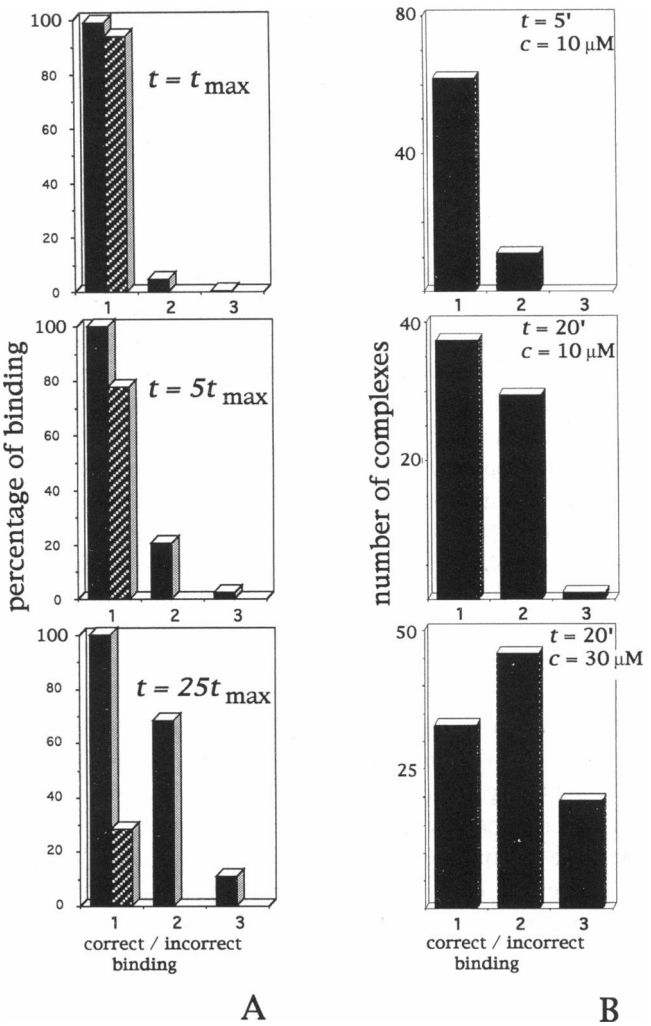

FIGURE 4 Diagrams illustrating the kinetically controlled specificity of dsDNA targeting by hpyPNAs. (*A*) Results of calculations of the time course of degree of PNA binding with complementary DNA site (no. 1) and with two incorrect sites having the selectivity parameters $f = 10^{-2}$ (no. 2) and $f = 10^{-3}$ (no. 3). Stippled columns show the percentage yield, Y, of DNA molecules uniquely targeted by a hpyPNA oligomer at the correct site without any mismatched binding ($t_{max} = 4.6\tau$ is the optimum time of DNA incubation with PNA at which the maximum yield, Y_{max}, is achieved for the case of $f = 10^{-2}$; see the text for details). (*B*) Experimental data by Demidov et al. (manuscript in preparation) on the kinetics of complex formation of PNA H-T_{10}-Lys(biotin)NH_2 with the different sites on linearized pT10 plasmid (pUC19 derivative with decaadenine insert into polylinker). The PNA/DNA complexes were obtained in the Tris-EDTA buffer (pH 7.5), with the desired PNA concentrations for the specific time as indicated, and were visualized by electron microscopy by A. V. Kurakin and D. I. Cherny using streptavidin as a marker (see Demidov et al., 1994a, for details). Column no. 1 corresponds to the formation of correct PNA/DNA complex with fully complementary target site A_{10}/T_{10} on pT10 plasmid. Column no. 2 represents the total of the PNA/DNA complexes formed with two mismatched sites on plasmid DNA that are inherent to the parental pUC19 vector: the single-mismatched site, T_7GT_3/A_3CA_7, and the double-mismatched site, AGA_7G/CT_7CT. These sites are located at 1407–1417 and 1438–1447 bp, respectively (GenBank data), and are too close to each other to be indistinguishable in the micrographs. Column no. 3 corresponds to the inherent pUC19 site, $AGA_5TA_3CA_3/T_3GT_3AT_5CT$, with two mismatches, which is located at 2564–2578 bp.

incubation with dsDNA for given PNA concentration), even the f value of 10^{-2} is sufficient to provide reasonable discrimination of the individual mismatched DNA site with virtually complete PNA/dsDNA binding to the correct site. However, specificity of dsDNA recognition by hpyPNA is kinetically controlled and may be completely lost during a prolonged incubation of DNA with PNA at a given PNA concentration (and/or because of the choice of too high a PNA concentration for the given incubation time). Fig. 4 *A* shows a gradual loss of specificity of PNA binding to the correct DNA site and the accumulation of nonspecific complexes due to binding of PNA with two incorrect sites having the selectivity parameters $f = 10^{-2}$ and $f = 10^{-3}$. These theoretical predictions qualitatively agree with observations by Demidov et al. (manuscript in preparation), who used electron-microscopic visualization of hpyPNA binding to plasmid DNA (see Fig. 4 *B*). With increasing time of binding or PNA concentration, the progressive occupation by hpyPNA of single- and double-mismatched DNA sites was observed, completely compromising the specificity of PNA binding.

Our analysis makes it possible to choose optimal conditions for targeting long genomic DNA molecules with PNA. Let us consider as an example the case of million-base-pair-long DNA targeted by a hpyPNA decamer. For such length of DNA fragment with a quasirandom sequence of nucleotides, the decapurine target site will be statistically unique, and there will be $N = 30$ sites with single mismatches. In this case the value $f = 10^{-2}$ is not sufficient for selectivity: at best, for $F = 0.3$, only half of the DNA molecules could be uniquely targeted at the correct site without any mismatched binding, whereas nearly one-third of them will carry incorrect PNA/DNA complexes (see Fig. 2). We therefore need a higher degree of discrimination between correct and mismatched sites in long genomic DNA molecules. The f value may depend not only on the type of mismatches (Demidov et al., 1995), but also on the kind of PNA molecules (e.g., on their length, sequence composition, type of construction, etc.) and on the conditions of PNA binding. This important question requires further study.

However, the f value of 10^{-3}, which we also observed in our experiments (Demidov et al., 1995, 1996), already provides reasonable discrimination between correct and single-mismatched sites in the genomic-size, megabase DNA molecule. In this case $F = 3 \times 10^{-2}$, and using the optimum time of binding, we conclude that as many as 87% of megabase DNA molecules can be selectively targeted by PNA at the unique site, whereas 10% of DNA molecules will be targeted at nonspecific sites. Such high selectivity of recognition of short homopurine sites on genomic DNA allowed us to use bis-PNAs as rare genome-cutters (Veselkov et al., 1996a,b). Additional discrimination between correct and mismatched complexes in this case could be achieved because of spontaneous or methylase-induced dissociation of incorrect and therefore less stable complexes.

Although we treated association and dissociation kinetics within the framework of very similar assumptions, the obtained results are quite different. One reason for this is the different initial conditions for these two cases. The other stems from the fact that in the case of association the specificity of DNA targeting, determined by the β quantity (Eq. 4), is at maximum at the initial stage of the process and then monotonously decreases with time. By contrast, in the case of dissociation, the specificity of DNA targeting is determined by the β_- quantity (Eq. 16) and increases with time. As a result, it is possible to significantly improve discrimination between correct and mismatched PNA/dsDNA complexes by stimulating the dissociation of already formed complexes (e.g., at elevated temperatures). From Eqs. 21 and 22 one can conclude that for megabase DNA molecules, which were initially targeted by PNA in a completely nonspecific manner ($\alpha_-(0) = \alpha_i^-(0) = 1$; $N = 30$), even a 100-fold difference between the dissociation rates of correct and mismatched PNA/DNA complexes ($f_- = 10^2$) can lead to excellent selectivity. In this case a very fast and selective dissociation of incorrect PNA/dsDNA complexes ($t_{max-} = 0.08\tau_-$) yields 91.4% of DNA targeted by PNA only at the unique site. Note that to reach the same level of selectivity during the PNA association with megabase DNA, it is necessary to have more than a 1000-fold difference between the rates of PNA association with correct and mismatched sites. In the less discriminative case of $f_- = 10$, the dissociation of PNA/dsDNA complexes during the optimum time $t_{max-} = 0.57\tau_-$ still yields a reasonable selectivity of targeting: ~50% of megabase DNA molecules will have only the correct site targeted by PNA, and less than 10% of the DNA molecules will carry mismatched complexes. Thus, the differential dissociation of PNA/dsDNA complexes, when possible, can further enhance the selectivity of dsDNA targeting by hpyPNA. Our data on differential melting of PNA H-$T_2CT_2CT_4$-LysNH_2/λ DNA complexes (Demidov et al., manuscript in preparation) provide experimental support for this possibility.

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REFERENCES

Bentin, T., and P. E. Nielsen. 1996. Enhanced peptide nucleic acid binding to supercoiled DNA: possible implications for DNA "breathing" dynamics. *Biochemistry.* 35:8863–8869.

Betts, L., J. A. Josey, J. M. Veal, and S. R. Jordan. 1995. A nucleic acid triple helix formed by a peptide nucleic acid-DNA complex. *Science.* 270:1838–1841.

Cherny, D. Y., B. P. Belotserkovskii, M. D. Frank-Kamenetskii, M. Egholm, O. Buchardt, R. H. Berg, and P. E. Nielsen. 1993. DNA unwinding upon strand displacement of binding of PNA to double stranded DNA. *Proc. Natl. Acad. Sci. USA.* 90:1667–1670.

De Mesmaeker, A., K.-H. Altmann, A. Waldner, and S. Wendeborn. 1995. Backbone modifications in oligonucleotides and peptide nucleic acid systems. *Curr. Opin. Struct. Biol.* 5:343–355.

Demidov, V. V., D. I. Cherny, A. V. Kurakin, M. V. Yavnilovich, V. A. Malkov, M. D. Frank-Kamenetskii, S. H. Sönnichsen, and P. E. Nielsen. 1994a. Electron microscopy mapping of oligopurine tracts in duplex

DNA by peptide nucleic acid targeting. *Nucleic Acids Res.* 22: 5218–5222.

Demidov, V., M. D. Frank-Kamenetskii, M. Egholm, O. Buchardt, and P. E. Nielsen. 1993. Sequence selective double strand DNA cleavage by PNA targeting using nuclease S1. *Nucleic Acids Res.* 21:2103–2107.

Demidov, V. V., M. D. Frank-Kamenetskii, and P. E. Nielsen. 1996. Complexes of duplex DNA with homopyrimidine peptide nucleic acid (PNA): new principle of biomolecular recognition. *In* Biological Structure and Dynamics, Vol. 2. R. H. Sarma and M. H. Sarma, editors. Adenine Press, Schenectady, NY. 129–134.

Demidov, V. V., V. N. Potaman, M. D. Frank-Kamenetskii, M. Egholm, O. Buchardt, S. H. Sönnichsen, and P. E. Nielsen. 1994b. Stability of peptide nucleic acids in human serum and cellular extracts. *Biochem. Pharmacol.* 48:1310–1313.

Demidov, V. V., M. V. Yavnilovich, B. P. Belotserkovskii, M. D. Frank-Kamenetskii, and P. E. Nielsen. 1995. Kinetics and mechanism of polyamide ("peptide") nucleic acid binding to duplex DNA. *Proc. Natl. Acad. Sci. USA.* 92:2637–2641.

Eaton, B. E., L. Gold, and D. A. Zichi. 1995. Let's get specific: the relationship between specificity and affinity. *Chem. Biol.* 2:633–638.

Egholm, M., L. Christensen, K. L. Dueholm, O. Buchardt, J. Coull, and P. E. Nielsen. 1995. Efficient pH-independent sequence-specific DNA binding by pseudoisocytosine-containing bis-PNA. *Nucleic Acids Res.* 23:217–222.

Frank-Kamenetskii, M. D., and S. M. Mirkin. 1995. Triplex DNA structures. *Annu. Rev. Biochem.* 64:65–95.

Griffith, M. C., L. M. Risen, M. J. Greig, E. A. Lesnik, K. G. Sprankle, R. H. Griffey, J. S. Kiely, and S. M. Freier. 1995. Single and bis peptide nucleic acids as triplexing agents: binding and stoichiometry. *J. Am. Chem. Soc.* 117:831–832.

Hanvey, J. C., N. C. Peffer, J. E. Bisi, S. A. Thomson, R. Cadilla, J. A. Josey, D. J. Ricca, C. F. Hassman, M. A. Bonham, K. G. Au, S. G. Carter, D. A. Bruckenstein, A. L. Boyd, S. A. Noble, and L. E. Babiss. 1992. Antisense and antigene properties of peptide nucleic acids. *Science.* 258:1481–1485.

Herschlag, D. 1991. Implications of ribozyme kinetics for targeting the cleavage of specific RNA molecules in vivo: more isn't always better. *Proc. Natl. Acad. Sci. USA.* 88:6921–6925.

Larsen, J. H., and P. E. Nielsen. 1996. Transcription-mediated binding of peptide nucleic acid (PNA) to double-stranded DNA: sequence-specific suicide transcription. *Nucleic Acids Res.* 24:458–463.

Møllegaard, N. E., O. Buchardt, M. Egholm, and P. E. Nielsen. 1994. Peptide nucleic acid·DNA strand displacement loops as artificial transcription promoters. *Proc. Natl. Acad. Sci. USA.* 91:3892–3895.

Nielsen, P. E., M. Egholm, R. H. Berg, and O. Buchardt. 1991. Sequence selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science.* 254:1497–1500.

Nielsen, P. E., M. Egholm, R. H. Berg, and O. Buchardt. 1993a. Sequence specific inhibition of restriction enzyme cleavage by PNA. *Nucleic Acids Res.* 21:197–200.

Nielsen, P. E., M. Egholm, R. H. Berg, and O. Buchardt. 1993b. Peptide nucleic acids (PNAs). DNA analogues with a polyamide backbone. *In* Antisense Research and Application. S. Crook and B. Lebleu, editors. CRC Press, Boca Raton, FL. 363–373.

Nielsen, P. E., M. Egholm, and O. Buchardt. 1994a. Peptide nucleic acids (PNAs). A DNA mimic with a peptide backbone. *Bioconjug. Chem.* 5:3–7.

Nielsen, P. E., M. Egholm, and O. Buchardt. 1994b. Evidence for $(PNA)_2$/DNA triplex structure upon binding of PNA to dsDNA by strand displacement. *J. Mol. Recognit.* 7:165–170.

Nielsen, P. E., M. Egholm, and O. Buchardt. 1994c. Sequence-specific transcription arrest by peptide nucleic acid bound to the DNA template strand. *Gene.* 149:139–145.

Nielsen, P. E., and H. Ørum. 1995. Peptide nucleic acid (PNA), a new molecular tool. *In* Molecular Biology: Current Innovations and Future Trends, Part 2. Horizon Scientific Press, Wymondham, England. 73–89.

Noble, S. A., M. A. Bonham, J. E. Bisi, D. A. Bruckenstein, P. H. Brown, S. C. Brown, R. Cadilla, M. D. Gaul, J. C. Hanvey, C. F. Hassman, J. A. Josey, M. J. Luzzio, P. M. Myers, A. J. Pipe, D. J. Ricca, C. W. Su, C. L. Stevencon, S. A. Thomson, R. W. Wiethe, and L. E. Babiss. 1995. Impact of biophysical parameters on the biological assessment of peptide nucleic acids, antisense inhibitors of gene expression. *Drug Dev. Res.* 34:184–195.

Peffer, N. J., J. C. Hanvey, J. E. Bisi, S. A. Thomson, F. C. Hassman, S. A. Noble, and L. E. Babiss. 1993. Strand-invasion of duplex DNA by peptide nucleic acid oligomers. *Proc. Natl. Acad. Sci. USA.* 90: 10648–10652.

Perelroyzen, M. P., and A. V. Vologodskii. 1988. Theoretical analysis of "addressed" chemical modification of DNA. *Nucleic Acids Res.* 16: 4693–4704.

Veselkov, A. G., V. V. Demidov, M. D. Frank-Kamenetskii, and P. E. Nielsen. 1996a. PNA as a rare genome-cutter. *Nature.* 379:214.

Veselkov, A. G., V. V. Demidov, P. E. Nielsen, and M. D. Frank-Kamenetskii. 1996b. A new class of genome rare cutters. *Nucleic Acids Res.* 24:2483–2488.

von Hippel, P. H., and O. G. Berg. 1986. On the specificity of DNA-protein interactions. *Proc. Natl. Acad. Sci. USA.* 83:1608–1612.

Wittung, P., P. E. Nielsen, and B. Nordén. 1996. Direct observation of strand invasion by peptide nucleic acid (PNA) into double-stranded DNA. *J. Am. Chem. Soc.* 118:7049–7054.