Kinetics of binding of Antp homeodomain to DNA analyzed by measurements of surface plasmon resonance

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Abstract The kinetics of binding of the Antp homeodomain to three kinds of DNA fragment were analyzed by measurements of surface plasmon resonance at various temperatures. Non-specific and specific binding of the homeodomain to DNA was examined. In the case of non-specific binding, the association rate constant (k_{ass}) was estimated to be $1.41–2.62\times10^5$ M^{-1} s^{-1} and the dissociation rate constant (k_{diss}) was $1.36–3.10\times10^{-2}$ s^{-1}, thus, the dissociation constant (K_D) was $0.847–1.72\times10^{-7}$ M. The association seemed to be driven by entropy. In the case of specific binding, by contrast, the enthalpy term seemed to contribute more to the binding than did the entropy term. The k_{ass} was $2.04–2.59\times10^5$ M^{-1} s^{-1} and the k_{diss} was $0.759–1.16\times10^{-3}$ s^{-1}, thus, the K_D was $2.93–5.69\times10^{-9}$ M. These values were measured under the condition of 150 mM NaCl. Both interactions were strongly dependent on the concentration of NaCl. The K_D at 50 mM NaCl became several tens of times smaller than those at 150 mM. Possible reasons for the differences between non-specific and specific interactions are discussed.

Key words: Homeodomain; Surface plasmon resonance; Entropy; Enthalpy; Non-electrostatic force

1. Introduction

Homeobox-containing genes that encode transcription factors play major roles in determining cell fate [1]. Each homeodomain that is encoded by a homeobox can bind specifically to a region of double-stranded (ds) DNA with a specific recognition sequence [2–6]. The interactions between homeodomains and DNA have been extensively analyzed by foot-printing methods and gel shift assays [6–14]. Direct structural analysis of the complexes has also been performed with NMR and X-ray [15–19]. The results indicated that a homeodomain binds to a specific sequence of less than 10 bp. The dissociation constants (K_D) of complexes formed between the homeodomains and their specific recognition sequences are very small, ranging from 1×10^{-9} to 1×10^{-10} M. The K_D values for complexes between homeodomains and non-specific DNA sequences are two orders of magnitude higher than those for specific binding [20].

The BIAcore (biospecific interaction analysis) system, developed recently by Pharmacia Biosensor to measure surface plasmon resonance (SPR), allows interactions between two macromolecules to be monitored in real time [21,22]. This system has been used for analysis of interactions between the repressor of the *lac* operon and the relevant DNA sequence as well as for analysis of the action of the co-repressor in the methionine repressor-operator complex in *E. coli* [22,23]. In the present study, we analyzed the kinetic parameters of interactions between the Antp homeodomain and fragments of ds DNA under various different conditions with respect to salt concentration and temperature.

2. Materials and methods

2.1. Preparation of the Antp homeodomain and oligonucleotides

The Antp homeodomain used in the present study was described in detail by Otting et al. [15]. It is a polypeptide of 68 amino acids that includes the entire Antp homeodomain, with a serine residue at the 39th position replacing a cysteine residue. It was produced in *E. coli* and purified as described by Müller et al. [8]. Three kinds of 26 bp ds oligonucleotide, with one strand biotinylated at its terminus were synthesized chemically for this study. The sequences of these fragments are shown in Fig. 1. One fragment, the A element, contained an authentic binding sequence for the Antp homeodomain [8]. The other two sequences, the N and M elements, contained an irrelevant sequence and a mutated sequence, respectively. The molecular mass of the Antp homeodomain was 8.5 kDa and that of the biotinylated 26 bp DNA was 17.4 kDa.

2.2. Kinetic analysis with the BIAcore system

The BIAcore system was used for analysis of interactions between the homeodomain and DNAs. The procedures for the analysis were essentially the same as described by the developer [24]. In brief, the refractive index within the dextran matrix in the sensor chip was continuously monitored, plotted against time, and presented as a sensorgram. The SPR response was measured in resonance units (RU). For proteins and DNA, we assumed that 1000 RU corresponded to a surface concentration of 1 ng/mm^2 by reference to the reported data [24]. Streptavidin was covalently bound to a sensor chip. The molecular mass of streptavidin is 67 kDa. The amounts of streptavidin bound to a chip was estimated to be 0.1 pmol/mm^2. After inactivation of excess reactive groups on the sensor chip, the biotinylated DNA fragment (1 μg/ml in 100 mM Tris-HCl pH 7.5, 0.3 M NaCl, and 1 mM EDTA) was injected into the sensor chip. The Antp homeodomain, dissolved at various concentrations in HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.05% Tween 20), was injected over the immobilized DNA at a flow rate of 2 μl/min. The SPR response was monitored continuously, and then the chip was washed with HBS buffer. Finally, the surface with bound DNA was regenerated with a pulse of 4 μl of 0.05% SDS solution.

3. Results and discussion

3.1. Interactions between the Antp homeodomain and three kinds of DNA fragment

The kinetics of bindings of the Antp homeodomain to three kinds of DNA fragment were analyzed with the BIAcore system at various temperatures. The purified Antp homeodomain was injected into the chip at various concentrations (part of the data is shown in Fig. 2A,B). The nature of both the association and the dissociation of the homeodomain with

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Abbreviations: ds, double stranded; K_D, dissociation constant; BIA, biospecific interaction analysis; SPR, surface plasmon resonance; RU, resonance unit(s); k_{ass}, association rate constant; k_{diss}, dissociation rate constant; K_A, association constant

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A (Antp)	5'biotin-AGCTGAGAAAAAGCCATTAGAGAAGC-3' 3' TCGACTCTTTTTCGGTAATCTCTTCG-5'
N (nonspecific)	5'biotin-AGCTGAGAAAGCTGAGAAAGCTGAGA-3' 3' TCGACTCTTTCGACTCTTTCGACTCT-5'
M (mutation)	5'biotin-AGCTGAGAAAAAGCCGGGGGAGAAGC-3' 3' TCGACTCTTTTTCGGCCCCCTCTTCG-5'

Fig. 1. Nucleotide sequences of the oligonucleotides used in the present study. The A element contains an authentic binding sequence for the Antp homeodomain, which is boxed.

DNA appeared to be quite different with the A element as compared to the N and M elements. (1) In the case of the N and M elements, association occurred rapidly and reached a steady state at both high and low concentrations of the protein. Dissociation occurred rapidly and essentially monophasically. The N and M elements gave essentially the same results. (2) With the A element, the association occurred rapidly and reached a steady state at higher concentrations of the protein. At low concentrations of the protein, by contrast, the response increased gradually and did not reach equilibrium within the time frame of the experiment. Dissociation seemed to occur biphasically. This tendency towards biphasic dissociation became more evident with higher concentrations of the protein in solution. Changes in temperature did not seem to have any effect on the overall patterns of the sensorgrams in each case.

The biphasic dissociation suggested that two kinds of complex might be formed between the Antp homeodomain and the A element. We suspected that the results might reflect the specific and the non-specific binding of the homeodomain to the DNA. The amount of the homeodomain bound to DNA at a steady state at a concentration of free homeodomain of 200 nM in solution was estimated to be roughly 4 molecules per DNA fragment for the A element, 5 for the N element and 7 for the M element. The apparent differences among the three elements might have been due to differences in the amount of DNA bound to the sensor chip. When more DNA fragments were bound to the sensor chip, the ability of the protein to gain access to the DNA could have been reduced by steric hindrance. It is known that the homeodomain binds to DNA in a monomeric form [8] (in this earlier study, the authors showed that, even if dimerization were to occur, the dissociation constant must be $> 10^{-4}$ M). It is also known that there is only one recognition sequence in the A element. Thus, the results for the A element, when there was a high concentration of the homeodomain in solution, reflected both the specific and the non-specific binding. At low concen-

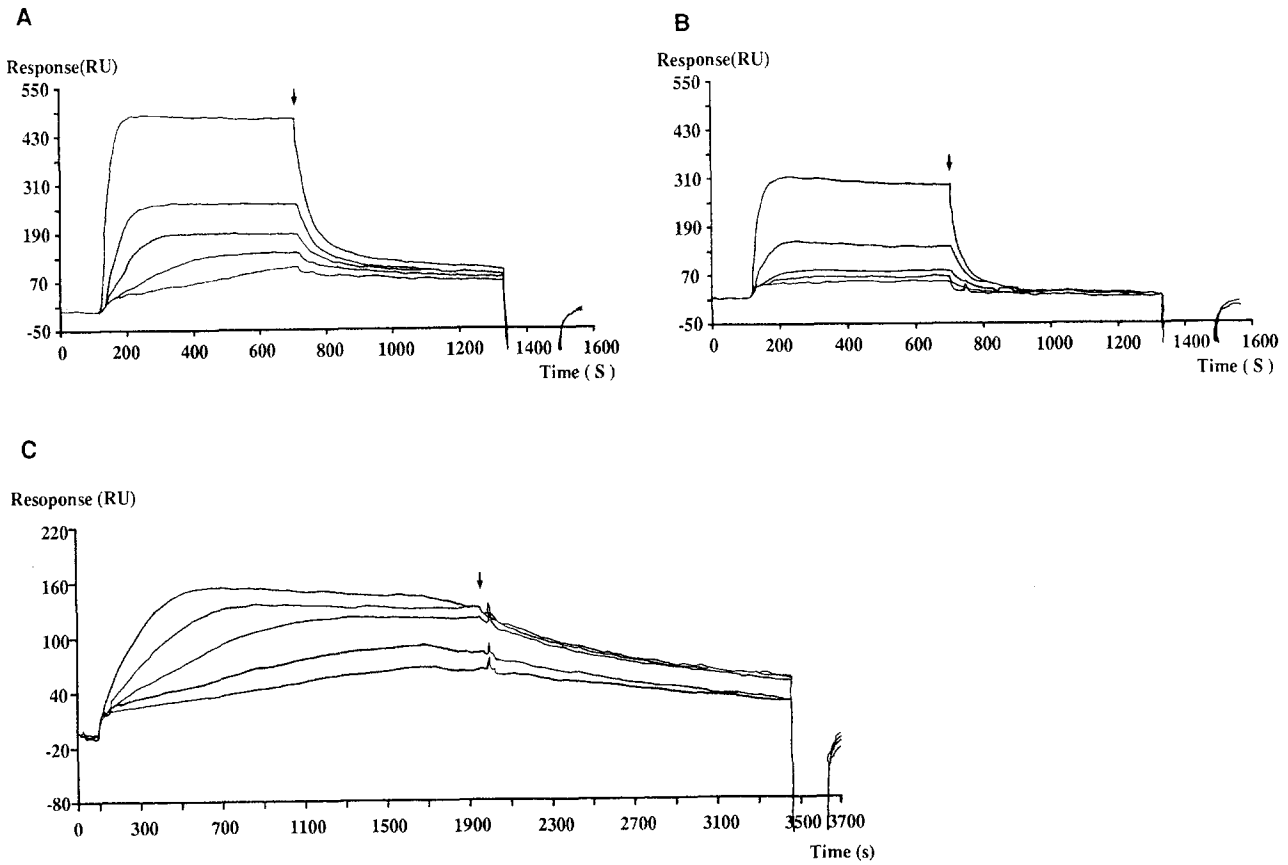

Fig. 2. Sensorgrams generated by the BIAcore system showing the interactions between the Antp homeodomain and three kinds of DNA fragment at various temperatures. A, B: The amounts of the DNA elements that were immobilized on the surface of the sensor differed among the elements. They were estimated to be 0.015 pmol/mm^2 for the A element, 0.007 pmol/mm^2 for the N element and 0.004 pmol/mm^2 for the M element. A 35 μl aliquot of a solution of the Antp homeodomain at concentrations of 12.5, 25, 50, 100, or 200 nM in HBS for the A and N elements and at concentrations of 50, 100, 175, 200, or 300 nM for the M element was injected, with subsequent washing with HBS buffer. Arrows indicate the times at which solutions were changed. Sensorgrams are indicated. Two examples are indicated: (A) the A element at 25°C; (B) the N element at 25°C. C: Sensorgrams for interactions between the free homeodomain at low concentrations in solution and the A element. A 60 μl aliquot of a 6.25, 10, 12.5, 20 or 25 nM solution of the Antp homeodomain in HBS buffer was injected at 25°C with subsequent washing with HBS buffer only. An arrow indicates the time at which the solutions were changed.

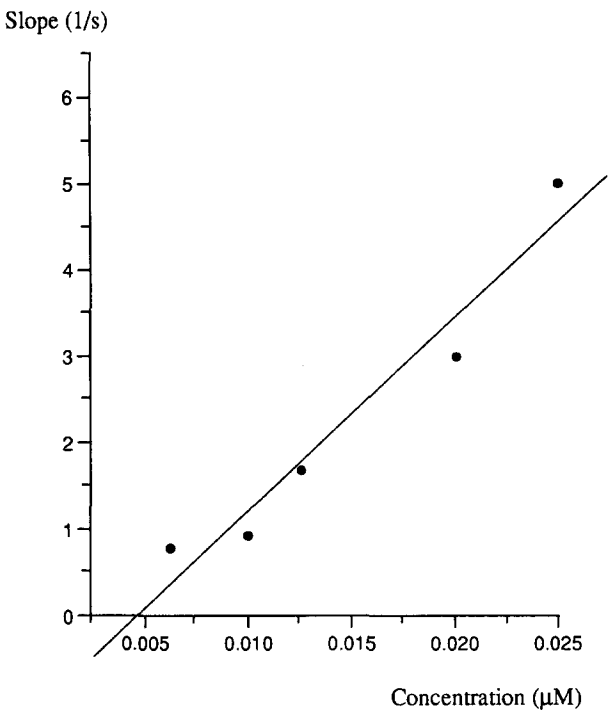

Fig. 3. Determination of k_{ass} for specific binding of the homeodomain to DNA. The principle for calculation of k_{ass} was as described in the legend to Table 1. Each point is derived from the data in Fig. 2C.

trations of the homeodomain in solution, the dissociation curve seemed to be monophasic. Therefore, we further performed analysis with the BIAcore system with the homeodomain at low concentrations to generate the sensorgrams that reflected specific binding exclusively (Fig. 2C). In this way, we were able to examine non-specific and specific binding, separately, as discussed below.

3.2. Association and dissociation rates for non-specific binding of the Antp homeodomain to DNA

According to the theory behind the BIAcore system, both association and dissociation rate constants (k_{ass} and k_{diss}) can be extrapolated from the data in sensorgrams [22]. The summary of the calculated data is given in Table 1. The k_{ass} for the complex between the Antp homeodomain and the N and M elements ranged from 1.41 to 2.62×10^5 M^{-1} s^{-1} and the k_{diss} ranged from 1.36 to 3.10×10^{-2} s^{-1}. Therefore, the K_D values were estimated to be $0.847–1.72\times10^{-7}$ M.

According to the BIAcore theory, the following equations hold at equilibrium [24]

$$k_{ass}\ C\ (R_{max}-R_{eq}) = k_{diss}R_{eq}$$

$$R_{eq}/C = K_A R_{max} - K_A R_{eq}$$

where R_{max} is the total number of binding sites of the immobilized ligand expressed in terms of the SPR response in RU, and R_{eq} is the equilibrium response. If these equations are valid, by plotting R_{eq}/C against R_{eq}, we can also calculate K_A from the slope of curves. However, this principle cannot be applied to cases of non-specific binding. When the concentration of the homeodomain in the solution was doubled, the response was more than doubled, suggesting that non-specific binding of the homeodomain to the DNA occurred cooperatively. Thus, once one homeodomain had bound non-specifically to DNA, the second molecule could bind more rapidly to the DNA than the first molecule. Cooperative binding of a homeodomain to DNA has been reported previously [25]. Therefore, the values cited for k_{ass} above should be considered the average values for mixed reactions.

3.3. Association and dissociation rates for specific binding of the homeodomain to DNA

As mentioned above, the results for interactions between the Antp homeodomain and the A element might have reflected both specific and non-specific binding. Differences between the A element and the N and M elements became clearer when the concentration of the homeodomain in the solution injected into the chip was low. Therefore, we calculated k_{ass} and k_{diss} from the data shown in Fig. 2C. As shown in Figs. 3 and 4A,B, the k_{ass} at 25°C was estimated to be 2.26×10^5 M^{-1} s^{-1}; the k_{diss} was estimated to be 6.63×10^{-4} s^{-1} and, therefore, K_D was estimated to be 2.93×10^{-9} M. Although the data for the A element shown in Fig. 2A reflected both specific and non-specific binding, the dissociation rate could be interpreted with relative ease, as shown in Fig. 4C,D. Non-specifically bound molecules were dissociated much more rapidly than specifically bound molecules. When the dissociation curves at an early stage of the reaction were used for calculations of k_{diss}, the value (2.51×10^{-2} s^{-1} at 37°C) was similar to those for the N and M elements

Table 1
The values of k_{ass}, k_{diss} and K_D for the Antp homeodomain and the N and M elements at various temperatures

		k_{ass}[a] ($\times10^5$ M^{-1} s^{-1})	k_{diss}[b] ($\times10^{-2}$ s^{-1})	K_D[c] ($\times10^{-7}$ M)
N element	20°C	1.85	2.32	1.25
	25°C	1.94	2.78	1.43
	30°C	2.62	2.22	0.847
	37°C	1.41	1.36	0.964
M element	20°C	1.68	2.62	1.56
	25°C	1.73	2.98	1.72
	30°C	1.99	3.10	1.56
	37°C	1.83	2.37	1.30

[a] k_{ass} values were calculated from part of the data shown in Fig. 2, based on the equation: slope (dR/dt vs. R) = k_{ass} C + k_{diss} (R is the amount of bound homeodomain in RU and C is the concentration (M) of the homeodomain in the solution).
[b] k_{diss} values were calculated based on the equation: $\ln Rt_1/Rt_n = k_{diss}(t_n - t_1)$. The data for the dissociation curve at an early stage were used.
[c] K_D was calculated from the equation: $K_D = \frac{k_{diss}}{k_{ass}}$.

Table 2
The values of k_{ass}, k_{diss} and K_D for complexes specifically formed between the Antp homeodomain and the A element

	k_{ass} ($\times10^5$ M^{-1} s^{-1})	k_{diss} ($\times10^{-3}$ s^{-1})	K_D ($\times10^{-9}$ M)
20°C	2.59	0.759	2.93
25°C	2.43	0.828	3.41
	(2.26)[a]	(0.663)[a]	(2.93)[a]
30°C	2.36	1.01	4.28
37°C	2.04	1.16	5.69

[a] These values were estimated from the data in Fig. 2C. The other values were estimated from the data in Fig. 2A.

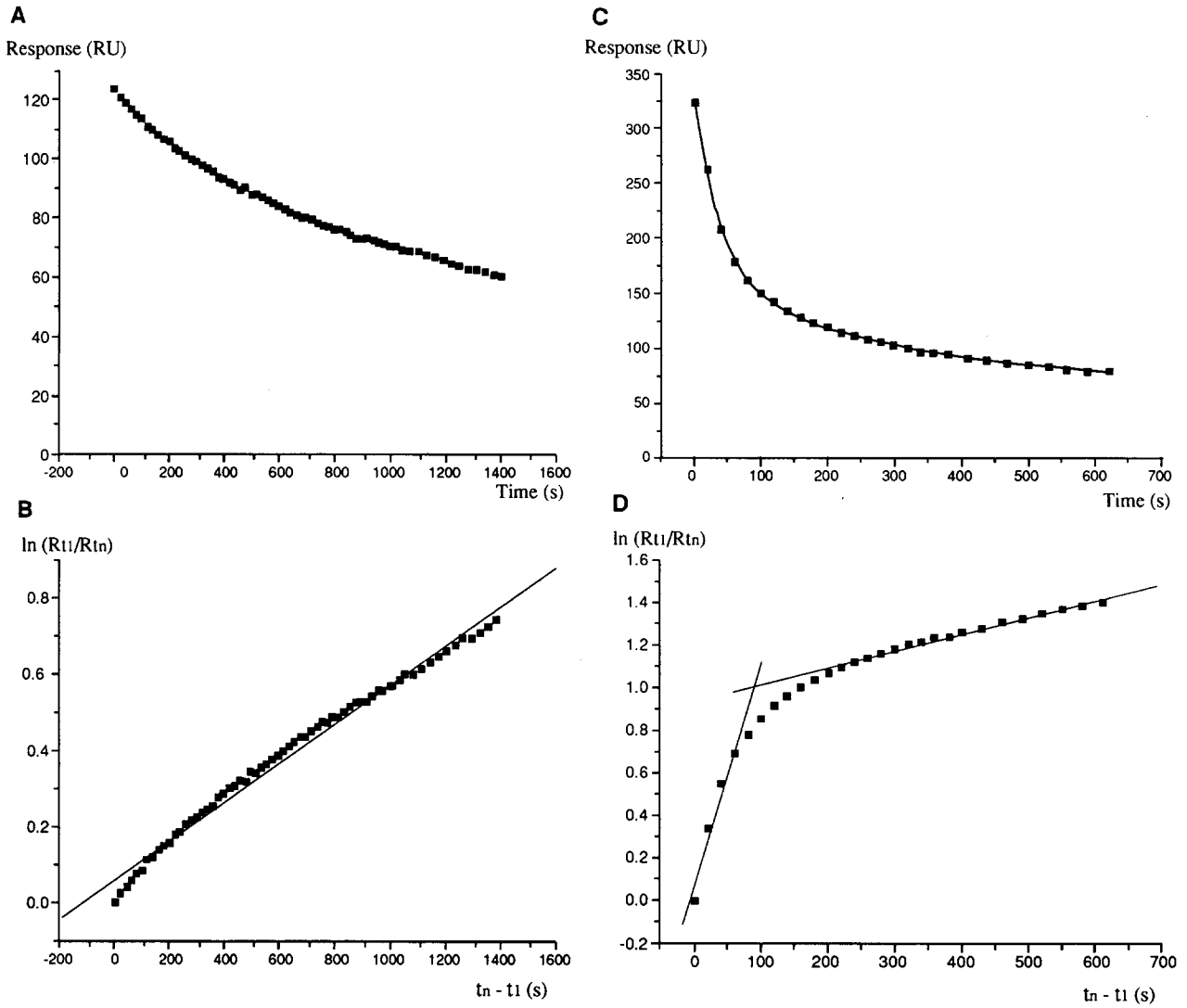

Fig. 4. Determination of k_{diss} for complexes formed between the homeodomain and the specific sequence. The principle for calculation of k_{diss} was essentially as described in the legend to Table 1. In the case of low concentrations of homeodomain in solution, dissociation occurred monophasically (A). Therefore, plots of Rt_1/Rt_n versus $(t_n - t_1)$ yielded a straight line (B). The calculations were based on the data in Fig. 2C. At high concentrations of the homeodomain (in the indicated example, the concentration of the homeodomain was 200 nM and the temperature was 37°C), the dissociation seemed to occur biphasically (C). This dissociation curve had two components, as shown in (D). The rapid dissociation component accounted for 63% of the total and k_{diss} was estimated to be 2.51×10^{-2} s^{-1}. The slowly dissociating component accounted for 37% of the total and k_{diss} was estimated to be 1.16×10^{-3} s^{-1}.

(1.36×10^{-2} and 2.37×10^{-2} s^{-1}, respectively, at 37°C). When the curves for dissociation at a later stage were used, each calculated value of k_{diss} was assumed to correspond to that of the specific interaction. The k_{diss} values obtained from the results shown in Fig. 2C were in good agreement with the values obtained from the data derived from Fig. 2A, as indicated in Table 2. Thus, we calculated the value of k_{ass} from the data in Fig. 2A, and the value of k_{diss} from the data at a later stage in Fig. 2A. The differences in binding between the specific and non-specific interactions were mainly due to differences in dissociation rates.

K_D values for the complexes between the Antp homeodomain and the A element have been obtained by equilibrium studies and by kinetic binding studies using the gel shift method, and they have been reported to be 1.6×10^{-9} M and 1.8×10^{-10} M, respectively [10]. These values are even smaller than those obtained in the present study. Although this discrepancy could derive from differences in methodologies, the reported data were based on observations in a solution of 75 mM NaCl. We used a concentration of NaCl of 150 mM in these experiments. Therefore, we examined the effects of the concentration of NaCl on the binding.

Table 3
Thermodynamic parameters for interactions between the Antp homeodomain and DNA at 25°C

	ΔG^a ($\times 10^4$ J/mol)	ΔH^b ($\times 10^4$ J/mol)	$-T\Delta S^c$ ($\times 10^4$ J/mol)
A element	−4.83	−2.99	−1.85
N element	−3.90	1.83*	−5.73*
M element	−3.89	0.77*	−4.94*

[a]ΔH was estimated from the equation: $\Delta G = -RT \ln K_A$.
[b]ΔH was estimated from van't Hoff's equation.
[c]$-T\Delta S$ was estimated from the equation: $\Delta G = \Delta H - T\Delta S$.
*These values are rough estimates.

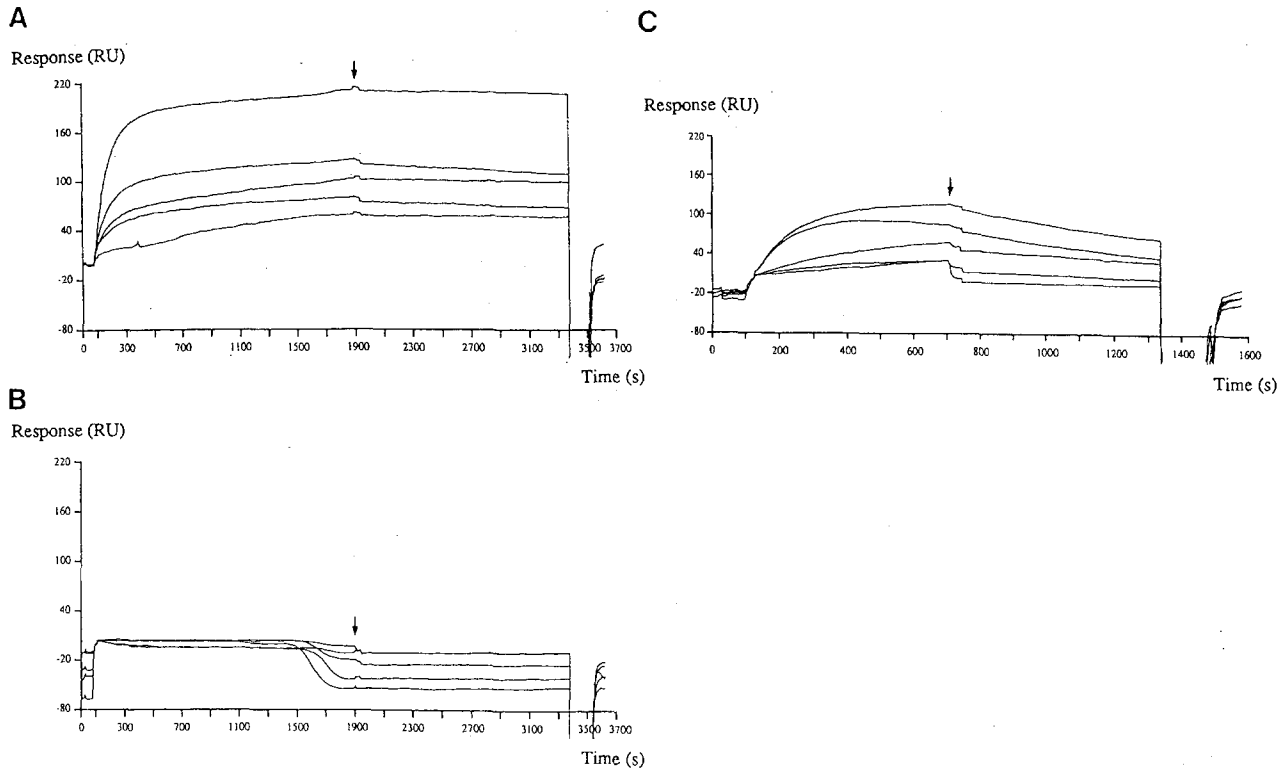

Fig. 5. Sensorgrams for the concentrations of NaCl of 50 and 300 mM. Aliquots of 12.5, 20, 25, 40 and 50 nM solution of the homeodomain with different concentrations of NaCl were injected into the chip. A: 50 mM NaCl for the A element. B: 300 mM NaCl for the A element. C: 50 mM NaCl for the N element. After injection of the solution of homeodomain, the chip was washed with the same solution without the homeodomain. Arrows indicate the times at which the solutions were changed.

3.4. *Effects of the concentrations of NaCl*

The concentration of NaCl in the running buffer was varied from 0 to 300 mM. As shown in Fig. 5B, virtually no binding was observed at 300 mM NaCl.,At 0 mM NaCl, the sensorgram was irregular and reproducibility was very poor (data not shown). Fig. 5A,C shows the sensorgrams for the A and N elements, respectively, in the presence of 50 mM NaCl. In the case of the N element, k_{ass} was estimated to be 1.89×10^{5} M^{-1} s^{-1} and k_{diss} to be 1.07×10^{-3} s^{-1}. Thus, K_D was 5.66×10^{-9} M. Therefore, the complexes formed in solution with 50 mM NaCl were two orders of magnitude more stable than those in solution with 150 mM NaCl. This tendency became more apparent with the A element. Dissociation did not seem to occur during the time frame of the experiment. However, when the concentration of the homeodomain was 20 or 40 nM, k_{diss} was estimated to be around 5×10^{-6} s^{-1}. The values might be below the limits of sensitivity of the BIAcore system [24]. Therefore, we concluded that k_{diss} should be around 10^{-6} s^{-1}. Since the k_{ass} was calculated to be 1.20×10^{5} M^{-1} s^{-1}, K_D should be of the order of 10^{-11} M. Although we have not carried out the experiments at 75 mM NaCl, the K_D value extrapolated from those at 50 mM and 150 mM appeared to be in agreement with the values previously reported as mentioned above.

3.5. *Estimation of thermodynamic parameters by van't Hoff analysis*

Since, in present study, we measured the k_{ass} and k_{diss} at various temperatures, we were able to calculate the change in enthalpy associated with the formation of the complex by using van't Hoff's equation:

$$d\ln K_A/d(1/T) = -\Delta H/R$$

Since ΔH could be temperature-dependent, it is difficult to estimate accurately the correct values of these thermodynamic parameters from values obtained at only four different temperatures. In the case of the A element, however, the four points that corresponded to $d\ln K_A/d(1/T)$ yielded a straight line, indicating a negative value of ΔH for this association. In the case of the N and M elements, by contrast, the data seemed to fluctuate, but there was a tendency for K_A to have higher values at higher temperatures. Although values of ΔH for the N and M elements that are given in Table 3 are rough estimates, the values should be positive. Thus, we can conclude that the association of the homeodomain with the non-specific DNA was driven by entropy and that, in the case of specific binding, the enthalpy term contributed more to the binding than did the entropy term.

The above conclusion that the association of the homeodomain with the non-specific DNA was driven by enthalpy appeared to be a general phenomenon in many systems [26,27]. Long-range electrostatic forces bring protein and DNA into proximity, and charged groups of the protein displace cations and water molecules from DNA. This process is accompanied by a large positive entropy change [26,28–30]. Even in the case of the association of the homeodomain with the specific DNA, similar phenomena should happen, thus resulting in an increase in entropy. However, major differences observed between specific and non-specific complexes might be a tight complex versus a loose complex. Although some other

changes such as bending could be induced in the conformation of either DNA or the protein, the structural analyses of the complexes of the homeodomain and the specific DNA did not indicate such conformational changes [15–19]. Therefore, the reasons why the enthalpy term contributed more than the entropy term to the association of the homeodomain with the specific DNA could be that many contacts such as hydrogen bonds and van der Waals' contacts formed at the boundary should contribute favorably to such binding. Thus, the favorable enthalpy could overcome the unfavorable entropy derived from the formation of a tight complex, such as loss of bond configuration entropy and the decrease in molecular translational entropy.

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