## 1) Kinetic Scheme for DNA Duplex Formation and Dissociation in the Internal Cavity of αHL

Single-channel current analysis revealed two τ_{off} and P values for the dissociation of oligo-B (5'-GGTGAATG-3') from the tethered DNA strand oligo-A (5'-CATTCACC-3'): $\tau_{off-1} = 84 \pm 10$ ms, $P_1 = 11 \pm 2$ % of the events; $\tau_{off-2} = 821 \pm 113$ ms, $P_2 = 89 \pm 2$ %. Two different τ_{off} and probability values were also found for the dissociation of oligo-D (5'-TACGTGGA-3') from the tethered oligo-C (5'-TCCACGTA-3'): $\tau_{off-1} = 37 \pm 5$ ms, $P_1 = 10 \pm 3$%; $\tau_{off-2} = 290 \pm 40$ ms, $P_2 = 90 \pm 3$%. The following analysis of the findings for oligo-A and -B applies in principle also to oligo-C and oligo-D.

To account for the values, two simple kinetic models can be envisioned. Both are based on the assumptions that (*i*) oligo-B in the cis chamber and in the cavity of the αHL pore are in rapid equilibrium and (*ii*) dissociation of oligo-B from oligo-A is followed by the relatively rapid exit from the cavity proven by the spike at the end of the binding events.

Model I
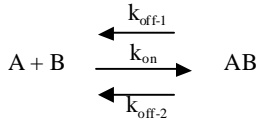
In this model, DNA duplex AB forms by the association of DNA strands A and B and is assumed to dissociate along two kinetically different routes characterized by the rate constants k_{off-1} and k_{off-2}. In accordance, the overall rate of duplex dissociation is:

$$v_{off} = (k_{off-1} + k_{off-2}) \cdot [AB]$$

The probability for duplex AB to dissociate along route 1 is given by:

$$P_1 = k_{off-1} / (k_{off-1} + k_{off-2}) = \tau_{off-2}/(\tau_{off-1} + \tau_{off-2})$$

The experimentally derived values of $\tau_{off-1} = 84$ ms and $\tau_{off-2} = 821$ ms yield $\tau_{off-2}/(\tau_{off-1} + \tau_{off-2}) = 0.91$. But the experimental value for P_1 is 0.11. Therefore, the observed kinetic parameters cannot be explained by kinetic Model I. More likely, hybridization follows kinetic Model II, characterized by two separate types of binding event:

Model II

$$A + B \xrightleftharpoons[k_{\text{off-1}}]{k_{\text{on-1}}} (AB)_1$$
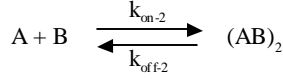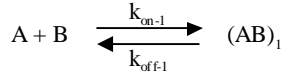
$$A + B \xrightleftharpoons[k_{\text{off-2}}]{k_{\text{on-2}}} (AB)_2$$

The overall rate of strand association in this model is:

$$v_{\text{on}} = k_{\text{on}} \bullet [A] \bullet [B] = (k_{\text{on-1}} + k_{\text{on-2}}) \bullet [A] \bullet [B]$$

The individual rate constants for duplex formation and dissociation, and the stability constants are:

$$k_{\text{on-1}} = P_1 \bullet k_{\text{on}} \qquad\qquad k_{\text{on-2}} = P_2 \bullet k_{\text{on}}$$
$$k_{\text{off-1}} = 1/\tau_{\text{off-1}} \qquad\qquad k_{\text{off-2}} = 1/\tau_{\text{off-2}}$$
$$K_{\text{d-1}} = k_{\text{off-1}}/k_{\text{on-1}} \qquad\qquad K_{\text{d-2}} = k_{\text{off-2}}/k_{\text{on-2}}$$

Inserting the values for $P_1 = 0.11$, $P_2 = 0.89$, $k_{\text{on}} = 1.5 \times 10^7\,\text{M}^{-1}\bullet\text{s}^{-1}$, $\tau_{\text{off-1}} = 84$ ms, and $\tau_{\text{off-2}} = 821$ ms gives

$$k_{\text{on-1}} = 1.6 \times 10^6\,\text{M}^{-1}\bullet\text{s}^{-1} \qquad\qquad k_{\text{on-2}} = 1.3 \times 10^7\,\text{M}^{-1}\bullet\text{s}^{-1}$$
$$k_{\text{off-1}} = 11.9\,\text{s}^{-1} \qquad\qquad k_{\text{off-2}} = 1.2\,\text{s}^{-1}$$
$$K_{\text{d-1}} = 7.4 \times 10^{-6}\,\text{M} \qquad\qquad K_{\text{d-2}} = 9.2 \times 10^{-8}\,\text{M}$$

The composite stability constant, $K_{\text{d-comp}}$, was obtained from the weighted mean lifetime, $\tau_{\text{off-comp}} = P_1 \bullet \tau_{\text{off-1}} + P_2 \bullet \tau_{\text{off-2}}$, and k_{on}. Inserting the values for P_1, P_2, $\tau_{\text{off-1}}$, $\tau_{\text{off-2}}$ and τ_{on} into

$$K_{\text{d-comp}} = k_{\text{off-comp}}/k_{\text{on}} = 1/k_{\text{on}} \bullet (P_1 \bullet \tau_{\text{off-1}} + P_2 \bullet \tau_{\text{off-2}})$$

yields a value of $K_{\text{d-comp}} = 9.0 \times 10^{-8}$ M. Clearly, the composite stability constant is dominated by the stability constant for the second types of events, $K_{\text{d-2}} = 9.2 \times 10^{-8}$ M. The strong influence of the second type of events can also be concluded from a rearranged equation for $K_{\text{d-comp}}$

$$K_{\text{d-comp}} = k_{\text{off-1}} \bullet k_{\text{off-2}}/(k_{\text{on-1}} \bullet k_{\text{off-2}} + k_{\text{on-2}} \bullet k_{\text{off-1}})$$

In the case of a low $k_{\text{off-2}}$ value, the term $k_{\text{on-2}} \bullet k_{\text{off-1}}$ dominates over $k_{\text{on-1}} \bullet k_{\text{off-2}}$, and the composite stability constant approximates the stability constant for the second type of events

$$K_{\text{d-comp}} \approx k_{\text{off-2}}/k_{\text{on-2}} = K_{\text{d-2}}$$

2) Derivation of Thermodynamic Parameters and Activation Energies for DNA Duplex Formation in the Lumen of a Nanopore

Thermodynamic values for duplex formation by tethered strand oligo-A and free oligo-B in the internal cavity of a single αHL pore were obtained by using the temperature dependence of the equilibrium formation constant (van't Hoff method). Similarly, the activation enthalpies and the activation entropies for the formation of duplex oligo-A•oligo-B in the nanopore were derived from the temperature dependence of the association and dissociation rate constants (Arrhenius plot).

The thermodynamics of duplex formation can be described by:

$$\Delta G° = \Delta H° - T \cdot \Delta S° = -RT \cdot \ln K_f$$

where $\Delta G°$ is the standard Gibbs free energy change, $\Delta H°$ the standard enthalpy change, $\Delta S°$ the standard entropy change, and K_f the equilibrium formation constant. This equation can be rearranged to

$$1/T = \Delta S°/\Delta H° - R/\Delta H° \cdot \ln K_f$$

Thus, $\Delta S°$ and $\Delta H°$ can be obtained from a plot of $\ln K_f$ vs. $1/T$. K_f values for temperatures between 10 and 40°C were derived by combining the association and dissociation rate constant k_{on} and k_{off} obtained from single channel current recordings using $K_f = k_{on}/k_{off}$.
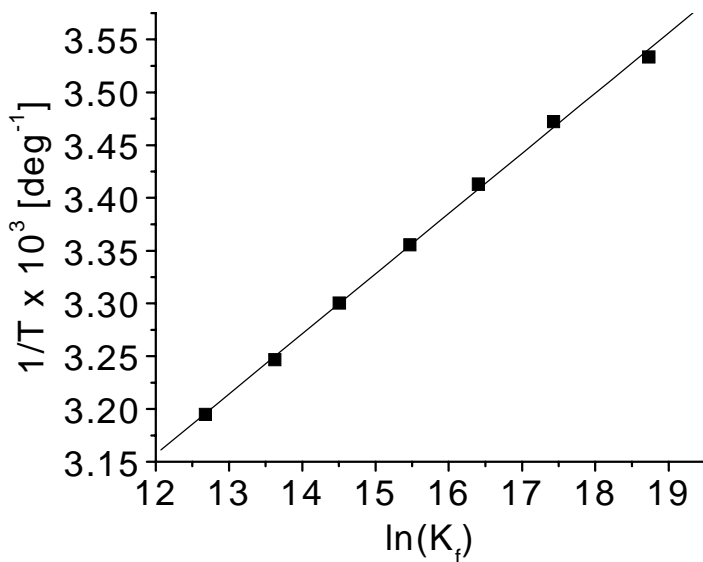

Fig. 4. From the plot of $\ln K_f$ vs. $1/T$, the following values were obtained:
$\Delta H° = -34$ kcal mol^{-1}
$\Delta S° = -84$ cal mol^{-1} deg^{-1}
$\Delta G° = -9.4$ kcal mol^{-1} at 20°C
$K_f = 1.4 \times 10^7$ M^{-1} at 20°C
$K_d = 7.1 \times 10^{-8}$ M at 20°C

The activation enthalpies $\Delta H^{\ddagger\circ}_{on}$ and $\Delta H^{\ddagger\circ}_{off}$ and the activation entropies $\Delta S^{\ddagger\circ}_{on}$ and $\Delta S^{\ddagger\circ}_{off}$ for the association and dissociation of oligo-A and oligo-B in the nanopore were derived form the temperature dependence of k_{on} and k_{off} by using the Arrhenius equation.

$$k = A \bullet \exp(E_a/R \bullet T)$$

where k is the rate constant, A is the pre-exponential factor, and E_a is the activation energy. Under the assumption that the activation energy is very close to the activation enthalpy, the logarithmic form of the Arrhenius equation can be written to follow Eyring's Transition State Theory:

$$\ln k = \ln (k_B \bullet T/h) + \Delta S^{\ddagger\circ}/R - \Delta H^{\ddagger\circ}/R \bullet T$$

where k_B is the Boltzmann constant, and h is Planck's constant. Given the minimal temperature dependence of $\ln (k_B \bullet T/h)$, a plot of $\ln k$ versus $1/T$ (not shown) yields $\Delta H^{\ddagger\circ}$ and $\Delta S^{\ddagger\circ}$ from the slope and the intercept, respectively. From the temperature dependence of the association rate constant, k_{on}, and the dissociation constant, k_{off}, for the formation of duplex oligo-A•oligo-B in the nanopore, the following values were obtained:

$\Delta H^{\ddagger\circ}_{on} = -0.5$ kcal mol^{-1}
$\Delta S^{\ddagger\circ}_{on} = -29$ cal mol^{-1} deg^{-1}
$\Delta H^{\ddagger\circ}_{off} = 34$ kcal mol^{-1}
$\Delta S^{\ddagger\circ}_{off} = 57$ cal mol^{-1} deg^{-1}

3) Derivation of Thermodynamic Data for DNA Duplex Formation in Solution

Thermodynamic values for the formation of a duplex by oligo-A and oligo-B in solution were obtained using a modified van't Hoff method on the basis of the dependence of the melting temperature T_m on the concentration of DNA strands (1).

The thermodynamics for duplex formation in solution can be described by

$$\Delta G^{\circ\prime} = \Delta H^{\circ\prime} - T \bullet \Delta S^{\circ\prime} = -R \bullet T \bullet \ln K_f^{\prime}$$

where $\Delta G^{\circ\prime}$ is the standard Gibbs free energy change, $\Delta H^{\circ\prime}$ the standard enthalpy change, $\Delta S^{\circ\prime}$ the standard entropy change, and $K_f^{\prime}$ the equilibrium formation constant. Assuming an all-or-none model, the equilibrium formation constant can be written as

$$K_f = 2 \bullet f/[(1 - f)^2 \bullet c_t]$$

where f is the fraction of strands forming a duplex and c_t is the total concentration of all single strands under denaturing conditions. An equimolar concentration of the complementary strands is assumed. At T_m, $f = 1/2$ and, hence, $K_f^{\prime} = 4/c_t$.

Insertion of $K_f' = 4/c_t$ into the first equation and rearrangement yields:

$$+ 1/T_m = \Delta S^{\circ\prime}/\Delta H^{\circ\prime} - R/\Delta H^{\circ\prime} \cdot \ln(c_t/4)$$

Thus, $\Delta S^{\circ\prime}$ and $\Delta H^{\circ\prime}$ can be obtained from a plot of $\ln(c_t/4)$ vs. $1/T_m$. T_m values were derived from melting profiles for duplex oligo-A•oligo-B at various oligonucleotide concentrations in 2 M KCl, 12 mM MgCl$_2$, and 5 mM Tris•HCl, pH 7.4. In all experiments, the concentration of oligo-A was equal to the concentration of oligo-B. T_m was the temperature at which the first derivative of the change in the absorbance at 260 nm formed a maximum.
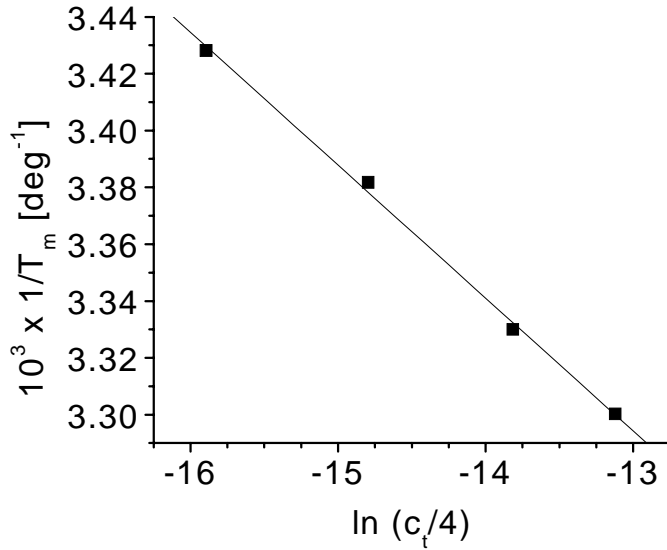

Fig. 5. From the plot $\ln(c_t/4)$ vs. $1/T_m$, we obtained the following values:

$\Delta H^{\circ\prime}$ = −42 kcal mol^{1}
$\Delta S^{\circ\prime}$ = −110 cal mol^{-1} deg^{-1}
$\Delta G^{\circ\prime}$ = −9.8 kcal mol^{-1} at 20°C
K_f' = 2.8×10^{7} M^{-1} at 20°C
K_d' = 3.5×10^{-8} M at 20°C

The value for $\Delta G^{\circ\prime}$ (−9.8 kcal mol^{-1} at 20°C) is very similar to ΔG° for duplex formation of the same oligonucleotide pair in the nanopore (−9.4 kcal mol^{-1} at 20°C). Interestingly, the values for $\Delta H^{\circ\prime}$ (−42 kcal mol^{-1}) and $\Delta S^{\circ\prime}$ (−110 cal mol^{-1} deg^{-1}) for duplex formation in solution are lower than those obtained for the nanopore: ΔH° = −34 kcal mol^{-1}, ΔS° = −84 cal mol^{-1} deg^{-1}. It is known from other experimental systems that standard enthalpy and entropy values can "compensate" to yield similar free energy values when experimental conditions are varied (2, 3).

4) The Melting Temperature of an Individual DNA Duplex

Duplex formation in a single nanopore is an attractive model system to explore the macroscopic concept of melting temperature for individual DNA molecules. The melting temperature (T_m) of a solution of complementary DNA strands is defined as the midpoint of the transition from double-stranded to separated single-stranded DNA, as measured by the change of absorbance at 260 nm. Therefore, at T_m, 50% of the duplexes are separated. However, this meaning of T_m can not be applied to DNA duplex formation in the nanopore; the individual tethered DNA strand in the pore is either single-stranded or duplexed. To explore the meaning of the macroscopic melting temperature T_m for duplex oligo-A•oligo-B within the lumen of a single αHL pore, a simple kinetic scheme can be assumed.

$$\alpha\text{HL-A} + \text{B} \quad \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \quad \alpha\text{HL-A} \bullet \text{B}$$
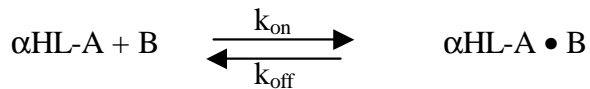
This two-state model is a simplification of Model II described in supporting information no. 1. The equilibrium dissociation constant for the kinetic scheme is given by

$K_d = k_{off}/k_{on} = P_{\alpha\text{HL-A}} \bullet [\text{B}]/P_{\alpha\text{HL-A}\bullet\text{B}}$

where $P_{\alpha\text{HL-A}\bullet\text{B}}$ and $P_{\alpha\text{HL-A}}$ are the probabilities for oligo-A to form or not to form a duplex with oligo-B, respectively. At the macroscopic melting temperature, 50% of the duplexes are separated. By analogy, at T_m for duplex formation in a single nanopore, it can be assumed that

$P_{\alpha\text{HL-A}\bullet\text{B}} = P_{\alpha\text{HL-A}}$

Therefore, at T_m:

$[\text{B}] = K_d$

T_m at the single molecule level is therefore the temperature, at which the concentration of oligo-B equals K_d. This is 20°C for a concentration of 71 nM.

References:

1. Martin, F. H., Uhlenbeck, O. C. & Doty, P. (1971) *J. Mol. Biol.* **57,** 201-215.

2. Grunwald, E. & Steel, C. (1995) *J. Am. Chem. Soc.* **117**, 5687-5693.

3. Rekharsky, M. V. & Inoue, Y. (1998) *Chem. Rev.* **98,** 1875-1918.