1996–2000 *Nucleic Acids Research, 1998, Vol. 26, No. 8*

A high throughput method to investigate oligodeoxyribonucleotide hybridization kinetics and thermodynamics

Abhijit Mazumder, Mehrdad Majlessi and Michael M. Becker*

Gen-Probe Inc., 10210 Genetic Center Drive, San Diego, CA 92121-4362, USA

Received November 6, 1997; Revised and Accepted February 20, 1998

ABSTRACT

We describe a high throughput microtiter-based assay to measure binding of oligodeoxyribonucleotides to nucleic acid targets. The assay utilizes oligodeoxyribonucleotide probes labeled with a highly chemiluminescent acridinium ester (AE). Reaction of AE with sodium sulfite renders it non-chemiluminescent. When an AE-labeled probe hybridizes to a target nucleic acid AE is protected from reaction with sodium sulfite and thus remains chemiluminescent. In contrast, unhybridized probe readily reacts with sodium sulfite and is rendered non-chemiluminescent. Hybridization of an AE-labeled probe to a target nucleic acid can therefore be detected without physical separation of unhybridized probe by treatment of the hybridization reaction with sodium sulfite and measurement of the remaining chemiluminescence. Using this method we measured hybridization rate constants and thermodynamic affinities of oligodeoxyribonucleotide probes binding to simple synthetic targets as well as large complex biological targets. The kinetic and thermodynamic parameters were measured with a high degree of accuracy and were in excellent agreement with values measured by other established techniques.

INTRODUCTION

Rapid and accurate measurement of hybridization of oligodeoxyribonucleotides to complementary nucleic acid targets is fundamental to the design of nucleic acid probes for mechanistic, diagnostic and therapeutic purposes. Measurements of hybridization can be either heterogeneous or homogeneous in nature. In heterogeneous assays, such as gel electrophoresis (1) or chromatography (2), physical separation steps are usually required when distinguishing unhybridized nucleic acid from hybridized nucleic acid. A major limitation of these techniques is that they are incapable of directly monitoring hybridization in solution. In homogeneous assays unhybridized and hybridized nucleic acids exhibit different physical properties that allow them to be distinguished from one another without physical separation steps. However, homogeneous approaches, such as absorbance spectroscopy (3,4), calorimetry (5–7) and nuclear magnetic resonance (8,9), are insensitive, require large amounts of purified nucleic acids and are often limited to thermodynamic measurements.

More recently fluorescent measurements have been used to monitor hybridization reactions in a homogeneous format. Although relatively small amounts of nucleic acids labeled with a fluorescent label can be probed by fluorescence, these measurements are often restricted to either short (10,11) or long (12) or specially labeled synthetic targets (13,14) in order to observe significant changes in fluorescence or diffusion times and they often require expensive and complicated equipment.

Here we describe a homogeneous assay to quantitatively analyze, in solution, both hybridization kinetics and thermodynamics that does not suffer from the above restrictions and which is amenable to a high throughput format. The approach utilizes nucleic acid probes labeled with a highly chemiluminescent acridinium ester (AE) (15,16) and an adduct protection assay to distinguish unhybridized and hybridized AE-labeled probes from one another (Fig. 1). When an AE-labeled probe hybridizes to a target nucleic acid, the carbon atom at the 9 position of AE is protected from attack by various adduct-forming nucleophiles, including sodium sulfite (17). Because the 9 position of AE must react with basic hydrogen peroxide to emit chemiluminescence, prior reaction with sodium sulfite prevents chemiluminescence. Thus unhybridized and hybridized AE-labeled probes in a hybridization reaction can be distinguished from one another by brief treatment with sodium sulfite and measurement of the chemiluminescence that is emitted from the hybridized probe. Here we demonstrate that this approach can accurately measure both thermodynamic and kinetic parameters of oligodeoxyribonucleotide hybridization reactions.

MATERIALS AND METHODS

Oligodeoxyribonucleotide synthesis, purification and AE labeling

Oligodeoxyribonucleotides were synthesized using standard phosphoramidite chemistry (18). For AE-labeled probes an amine-terminated arm was incorporated at a predetermined position in each oligodeoxyribonucleotide during synthesis using an abasic arm chemistry (19).

*To whom correspondence should be addressed. Tel: +1 619 410 8832; Fax: +1 619 410 8870; Email: mickb@gen-probe.com

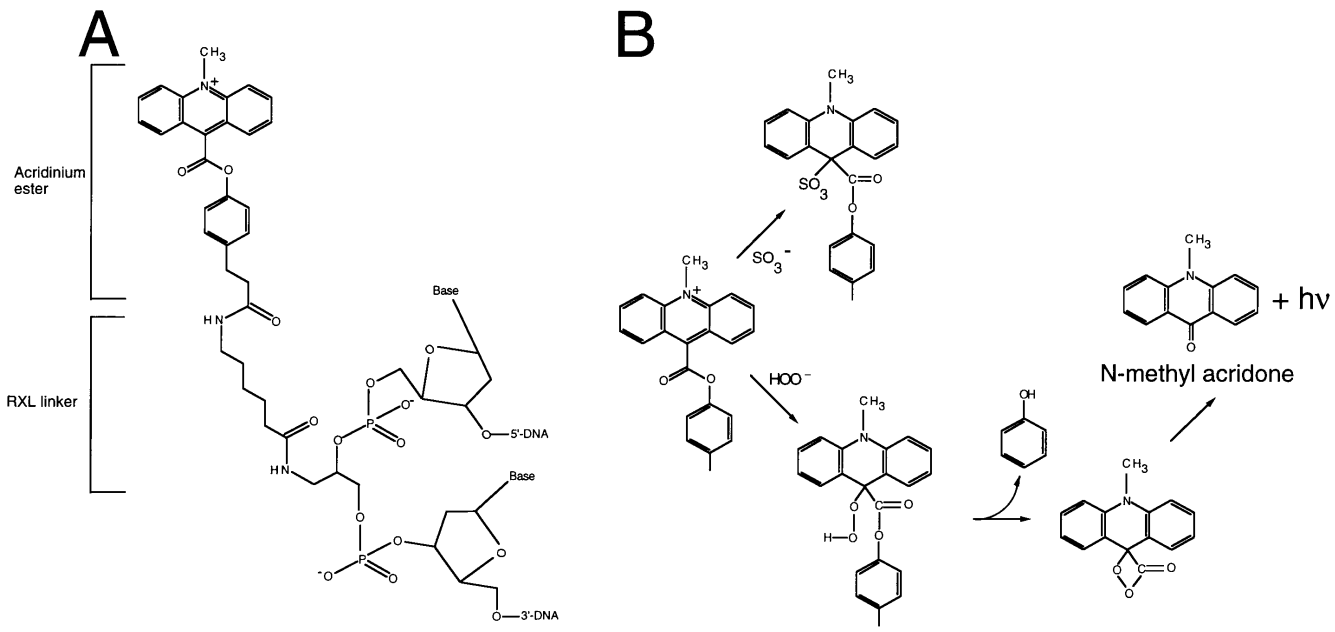

Figure 1. The acridinium ester (AE) and its chemistry. (**A**) The structure of AE and the linker arm covalently attached to the phosphodiester backbone of an oligodeoxyribonucleotide are indicated. (**B**) Reaction of AE with basic hydrogen peroxide or sodium sulfite. (Lower pathway) Basic hydrogen peroxide (OOH^-) reacts with AE to form a 1,2 dioxetanone that decomposes to liberate *N*-methylacridone and a photon (chemiluminescence). (Upper pathway) Sodium sulfite reacts with AE to prevent subsequent reaction with peroxide ion (no chemiluminescence).

Oligodeoxyribonucleotides were purified and labeled with the *N*-hydroxysuccinimide (NHS) ester of AE (20) by one of two methods. In the first method the 5′-dimethoxytrityl (DMT) protecting group was cleaved from the oligodeoxyribonucleotides. The resultant oligodeoxyribonucleotides were then purified by standard PAGE and labeled with AE (21). Labeling was performed by precipitating and resuspending 10 nmol oligodeoxyribonucleotide containing the amine-terminated linker in 10 µl 0.25 M HEPES, pH 8. In a separate tube 7.5 µl 0.25 M HEPES, 15 µl DMSO and 5 µl 25 mM NHS-AE were mixed together. Aliquots (25 µl) of this solution were added to the oligodeoxyribonucleotide solution. The reaction was allowed to proceed at 30°C for 90 min and precipitated with ethanol. The labeled oligodeoxyribonucleotide was then purified by reversed phase HPLC (Vydac C4 column; Western Analytical Products, Hisperia, CA) using a binary solvent system consisting of 0.1 M triethylammonium acetate (TEAA), pH 7.0, and acetonitrile (ACN). The ACN was increased from 5 to 25% (v/v) over 40 min at a flow rate of 1.0 ml/min and the desired pooled fractions precipitated with ethanol.

In the second method AE-labeled probes were prepared without gel or HPLC purification steps. The DMT group was left on the oligodeoxyribonucleotides and they were purified by absorption to a SEP-PAK cartridge (Waters Corp, Milford, MA) as follows. The cartridge was washed with 10 ml 100% ACN and 5 ml 2 M TEAA. After loading the sample the cartridge was washed successively with 10 ml 10% (v/v) ACN/0.1 M TEAA, 10 ml 12% (v/v) ACN/0.1 M TEAA and 10 ml water. The sample was detritylated by passing 10 ml 2% trifluoroacetic acid through the cartridge for 3 min. The cartridge was then neutralized by washing with 5 ml 5% ACN/0.1 M TEAA and 10 ml water. The sample was eluted with 3 ml 20% ACN and then evaporated, precipitated with ethanol and labeled with AE as described above. AE labeling reactions were adjusted to a total volume of 500 µl with water, extracted once with an equal volume of water-saturated *n*-butanol and precipitated with ethanol. AE-labeled probes prepared by either method gave identical hybridization rates as measured by our high throughput method (results not shown).

Hybridization reactions

Hybridization reactions were performed in white 96-well flat-bottom microtiter plates (Labsystems, Needham Heights, MA). The sequences of the RNA target, 2′-*O*-methyl probe and deoxy probe were 5′-AUGUUGGGUUAAGUCCCGCAACGAGC-3′, 5′-GCUCGUUGCGGGACUU(AE)AACCCAACAU-3′ and 5′-GCGGGACTT(AE)AACCCAACAT-3′ respectively, where (AE) denotes the site at which AE was inserted. Hybridization was conducted by adding various concentrations of RNA target to different wells that contained 0.25 fmol AE-labeled probe to give a total volume of 50 µl hybridization buffer containing 125 mM LiOH, 95 mM succinic acid, 8.5% lithium lauryl sulfate (LLS), 1.5 mM EDTA, 1.5 mM EGTA, pH 5.1. Hybridization to rRNA (for the experiments in Table 1) was performed in the presence of 1.5 pmol helper oligodeoxyribonucleotides (5′-ACCGCGTGCAACAAAGGATAAGGGTTGC-3′ and 5′-ACAACACGAGCTGACGACAGCCAGTACGCACCTGTCTCACGG-3′). Following addition of 80 µl silicone oil the plate was placed in a microtiter plate incubator/shaker (Labsystems) and hybridization was performed without shaking at 60°C for 1 (C_0t analysis) or 15 h (equilibrium binding studies). To stop hybridization the plates were then placed on ice for 20 min. Less than 10% additional hybridization was found to occur during this time (data not shown). After briefly warming the samples to room temperature chemiluminescence was measured on a chemiluminescent plate reader (EG & G Microlumat) by automated injection of 50 µl freshly prepared 20 mM sodium sulfite, 60 mM sodium tetraborate, pH 8.8, followed 30 s later by automated injection of

50 µl 1.5 N NaOH, 0.15% H_2O_2. Chemiluminescence was read for 2 s.

Radiolabeling and desalting of oligodeoxyribonucleotides

Eleven picomoles of oligodeoxyribonucleotide were labeled to a final specific activity of 1.5×10^6 c.p.m./pmol with 105 µCi [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) and 10 U T4 polynucleotide kinase (Pharmacia, Piscataway, NJ) at 37°C for 1 h. Oligodeoxyribonucleotides were then purified on a NENSORB column (NEN Life Sciences, Boston, MA) by eluting with 1% lithium dodecyl sulfate, 50 mM LiCl.

Hydroxyapatite kinetic and thermodynamic measurements

Radiolabeled probe. Hybridization reactions were performed as described above using 0.25 fmol radiolabeled probe and increasing amounts of *Escherichia coli* rRNA. Reactions were allowed to proceed at 60°C for 1 (kinetic measurements) or 15 h (thermodynamic measurements). Reactions were then quenched by the addition of 5 ml hydroxyapatite (HAP) suspension (0.12 M Na_2PO_4, pH 6.8, 0.02% w/v sodium azide, 0.02% w/v sodium lauryl sulfate, 2% w/v HAP). The nucleic acids were bound to HAP at 45°C for 5 min and then centrifuged at 2000 r.p.m. for 2 min. The resultant pellet was washed once with 5 ml wash solution (0.12 M Na_2PO_4, pH 6.8, 0.02% sodium azide, 0.02% sodium lauryl sulfate) and the pellet and supernatants were subjected to Cerenkov counting. The extent of hybridization was then determined from the fraction of counts bound to HAP.

AE-labeled probe. Hybridization reactions were performed as described above using 0.25 fmol AE-labeled probe and increasing amounts of *E.coli* rRNA. Reactions were allowed to proceed at 60°C for 1 (kinetic measurements) or 15 h (thermodynamic measurements). Reactions were then quenched by addition to 4 ml HAP suspension and processed as described above. The HAP pellet was mixed with 200 µl hybridization buffer containing 125 mM LiOH, 95 mM succinic acid, 8.5% LLS, 1.5 mM EDTA, 1.5 mM EGTA, pH 5.1, vortexed, spun at 2000 r.p.m. for 2 min and the eluted hybrid collected. Fourteen microliters of 3.2 N HNO_3 were added and the chemiluminescence of each reaction measured as described above.

RESULTS AND DISCUSSION

To measure hybridization kinetics and thermodynamics we first optimized the adduct protection assay to suppress chemiluminescence from unhybridized AE-labeled probes. An AE-labeled probe was hybridized to a complementary target and then equimolar amounts of the resultant hybridized or unhybridized AE-labeled probe were reacted with 10 mM sodium sulfite for different amounts of time. Following reaction with sodium sulfite basic hydrogen peroxide was injected into the solutions to initiate chemiluminescence. As shown in Figure 2, hybridized probe molecules were protected from adduct formation and emitted chemiluminescence, while unhybridized probe molecules formed an adduct and failed to emit chemiluminescence. From these data it was determined that automated injection of 10 mM sodium sulfite 30 s prior to a second injection of basic hydrogen peroxide gave adequate discrimination between hybrid and probe. Maximal discrimination between hybrid and probe could be further optimized by varying the position of the AE linker site (17) or lowering the amount of detergent in the hybridization reaction (results not shown).

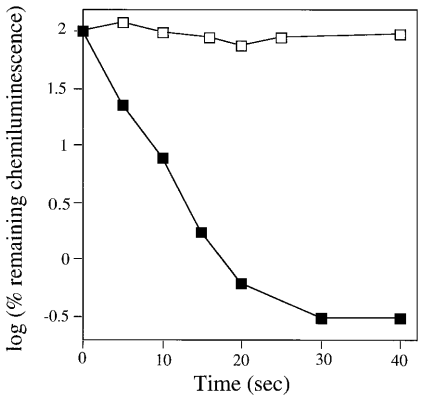

Figure 2. Kinetic analysis of the reaction of sodium sulfite with unhybridized AE-labeled probe or AE-labeled probe hybridized to complementary target. The loss of chemiluminescence from AE resulting from addition of sodium sulfite was measured and plotted as the log of percent remaining chemiluminescence versus time (s) between injections of sulfite and peroxide. Open and filled squares represent hybridized and unhybridized AE-labeled probe respectively.

To measure hybridization kinetics of an oligodeoxyribonucleotide to a complementary target we incubated a 2′-*O*-methyl probe labeled with AE with increasing concentrations of a complementary synthetic RNA target for 1 h at 60°C. Percentage of hybridization for each solution was calculated by dividing the chemiluminescence of that solution by the chemiluminescence observed at high target concentrations where hybridization was complete. A plot of the log of percent hybridization versus log of $C_o t$, where C_o is the initial concentration of target (nt/l) and t is the time of hybridization (s), is shown in Figure 3A. When hybridization is 50% complete the corresponding $C_o t$ value is inversely proportional to the rate constant of hybridization ($k_2 = \ln 2/C_o t_{½}$). These data were then fitted to the equation % hyb = $100 \times [1 - \exp(-k_2 \times C_o \times t)]$ to calculate the hybridization rate constant k_2. The rate constants measured for six identical hybridizations (mean 0.73×10^5 $M^{-1}s^{-1}$) differed from one another by only ±5% (1 SD), demonstrating the high reproducibility of the method.

Table 1. Calibration of kinetic and thermodynamic parameters obtained from the high throughput and HAP methods

Constant	[^{32}P]Oligodeoxyribonucleotide on HAP	AE-oligonucleotide on HAP	AE-oligodeoxyribonucleotide in high throughput
Rate constant ($M^{-1}s^{-1}$)[a]	$7.32 \times 10^4 \pm 10\%$	$5.19 \times 10^4 \pm 14\%$	$4.86 \times 10^4 \pm 7\%$
Equilibrium dissociation constant (M)[a]	$3 \times 10^{-11} \pm 2\%$	$3.1 \times 10^{-11} \pm 29\%$	$4.9 \times 10^{-11} \pm 5\%$

[a]Error limits represent ± 1 SD and are the result of two to four experiments.

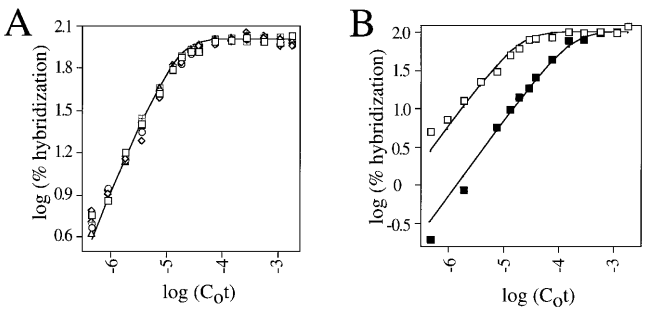

Figure 3. Hybridization of a 2′-*O*-methyl probe labeled with AE to a complementary synthetic RNA target (**A**) or a complementary synthetic RNA target (open squares) and *E.coli* rRNA (filled squares) (**B**). The results of six independent experiments are shown in (A). In each experiment 250 amol probe were incubated with increasing concentrations of RNA target for 1 h at 60°C. The log of percent hybridization (where 100% hybridization is the chemiluminescence measured in the plateau region at high C_ot) was plotted against the the log of C_ot, where C_o is the initial concentration of RNA target (nt/l) and t is the hybridization time (s). In (A) and (B) the solid lines are the best fit to the experimental data according to the equation % hyb = $100 \times [1 - \exp(-k_2 \times C_o \times t)]$, where k_2 is the hybridization rate constant. Values of k_2 are 6.99×10^4, 6.38×10^4 and 7.39×10^3 $M^{-1}s^{-1}$ for hybridization to the synthetic target in (A), to the synthetic target in (B) and to rRNA respectively.

The measurements in Figure 3A examined hybridization of a 2′-*O*-methyl oligodeoxyribonucleotide probe to a small synthetic complementary RNA target. To demonstrate that the chemiluminescent method could also be used to measure hybridization of oligodeoxyribonucleotide probes to complex RNA molecules we repeated these experiments using the same synthetic RNA target used in Figure 3A or an rRNA containing the same target sequence as the synthetic RNA. Both RNA targets exhibited similarly shaped C_ot curves (Fig. 3B). As expected, rRNA, which contains the target sequence in a partially double-stranded conformation, hybridized significantly more slowly (8.5-fold) to the oligodeoxyribonucleotide probe than the synthetic target.

To validate that the C_ot values measured by the chemiluminescent method were accurate we repeated hybridization to rRNA and analyzed the results by the established HAP method (22) using a probe that was either AE-labeled or radiolabeled. Hybridization rates of the AE-labeled probe using either the high throughput or HAP method were very similar, validating the high throughput method (Table 1). In contrast, the radiolabeled oligodeoxyribonucleotide was found to hybridize 1.4-fold faster than the AE-labeled oligodeoxyribonucleotide, demonstrating that the linker and AE cause a small decrease in hybridization rate.

An advantage of measuring hybridization rates by the chemiluminescent approach is that the method is relatively insensitive to environmental conditions. Thus hybridization rates can be measured over broad ranges of temperature and buffer conditions. One such application that we have investigated concerns hybridization of oligodeoxyribonucleotides in high concentrations of a denaturant such as urea. If secondary structure exists in either a probe or target sequence hybridization rates will be accelerated by the presence of denaturants. In Figure 4A the same probe and target combination examined in Figure 3A was hybridized in five different concentrations of urea (0.1–3.6 M). The observed rate constants varied by only 20% over this range of urea. In contrast, the hybridization rate of the same probe to rRNA increased 2.2-fold when the urea concentration was increased from 0 to 3.5 M (Fig. 4B).

Because AE is highly chemiluminescent, very small concentrations of a hybridized AE-labeled probe can be detected. For example, as little as 20×10^{-18} mol (amol) hybridized AE-labeled probe can be detected by the approach described here (17). Because of its high sensitivity, the chemiluminescent approach can be adapted to measure the thermodynamic affinities of oligodeoxyribonucleotides for targets, affinities that are often quite high and difficult to measure. To measure the affinity with which a 19 base deoxy probe bound a synthetic RNA target we first used the kinetic method shown in Figure 3 to determine how long it took a large molar excess of probe to hybridize to a very small amount (250 amol) of target. Under our hybridization

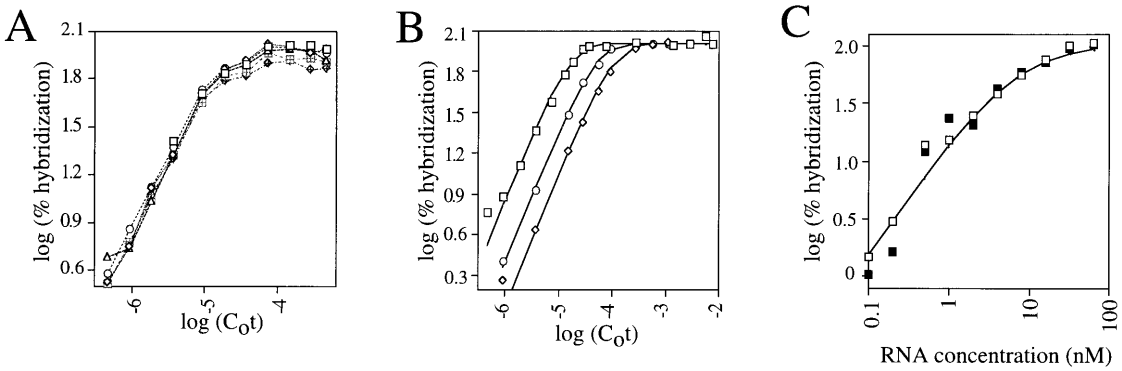

Figure 4. Kinetic and thermodynamic measurements of binding of AE-labeled probes to complementary RNA targets. (**A**) Hybridization of a 2′-*O*-methyl AE-labeled probe to a synthetic target in five different concentrations of urea [0.1 (open diamonds), 0.3 (open circles), 0.9 (open triangles), 2.7 (crossed squares) and 3.6 M (crossed diamonds)]. (**B**) Hybridization of a 2′-*O*-methyl AE-labeled probe to a synthetic target in the absence of urea (open squares) or to *E.coli* rRNA in the absence (open diamonds) or presence (open circles) of 3.5 M urea. The solid lines are the best fit to the experimental data according to the equation % hyb = $100 \times [1 - \exp(-k_2 \times C_o \times t)]$, where k_2 is the hybridization rate constant. Values of k_2 are 7.2×10^4, 1.15×10^4 and 2.46×10^4 per $M^{-1}s^{-1}$ for hybridization to the synthetic target, to rRNA in the absence of urea and to rRNA in the presence of urea respectively. (**C**) Thermodynamic measurements of binding of a deoxy AE-labeled probe to a synthetic RNA target. The results of two independent experiments are shown. The solid line is the best fit to the experimental data according to the equation % hyb = $100 \times [KT/(1 + KT)]$, where K is the equilibrium association constant and T is the target concentration (in strands). K_{eq} was determined to be 0.15×10^9 M^{-1}. In (A)–(C) 250 amol probe was incubated with increasing concentrations of RNA target for 1 h at 60°C (A and B) or 15 h at 55°C (C).

conditions equilibrium binding was reached in 7 h (results not shown). We then added 10 different concentrations of the RNA target to two different rows of a microtiter well, with the lowest amount being 250 amol. After addition of 250 amol AE-labeled probe to each well hybridization was allowed to proceed for 15 h at 55°C and chemiluminescence of each well measured by injection of sodium sufite and basic hydrogen peroxide as before. Percent hybridization was calculated as described above. The resulting equilibrium binding isotherms (Fig. 4C) were then used to calculate the dissociation constant (K_d) for the reaction ($6.55 \pm 0.35 \times 10^{-9}$ M) and the corresponding change in free energy ($\Delta G^\circ = RT\ln K_d = -12.6 \pm 0.1$ kcal/mol).

To validate that the change in free energy measured by the chemiluminescent approach was accurate we compared our measurements to free energy measurements of the same equilibrium measured by melting analysis. The same probe lacking an amine linker and AE label was hybridized to the same RNA target and the concentration dependence of T_m (23) of the resulting probe–RNA hybrid, as measured by absorbance spectroscopy, was then used to calculate the free energy of the hybrid and K_{eq} of association. The change in free energy calculated at 55°C, –12.8 kcal/mol (results not shown), was in excellent agreement with the chemiluminescent method. Thus the chemiluminescent method can accurately measure thermodynamic affinities and the values so measured are apparently not perturbed by the presence of the AE label.

To verify the latter conclusion we analyzed equilibrium binding isotherms for hybridization of both AE-labeled and radiolabeled probes to *E.coli* rRNA by the high throughput and HAP methods (Table 1). The radiolabeled oligodeoxyribonucleotide was found to bind with a dissociation constant 1- to 1.6-fold lower than the AE-labeled oligodeoxyribonucleotide, demonstrating that the linker and AE have little or no effect on affinity of the oligodeoxyribonucleotide.

Two additional advantages are inherent in the direct measurement of thermodynamic affinities described here. First, in contrast to approaches which estimate affinities from T_m measurements based on absorbance spectroscopy our technique allows one to calculate binding constants for probes whose affinities are not amenable to analysis by T_m measurements. For example, the affinity of the 2′-*O*-methyl probe shown in Figures 2 and 3 for a complementary synthetic target was readily measured by the equilibrium binding method (results not shown). However, thermodynamic parameters for this hybridization could not be obtained by the T_m method (23) because the ΔH° and, therefore, the ΔG° of association were too high to exhibit a measureable concentration dependence. Second, extrapolation of affinities measured from melting analysis to temperatures other than the T_m value is less accurate than direct measurement of the affinity at a desired temperature (24). Because our technique can be performed at different temperatures, it should provide a more accurate assessment of the thermodynamic parameters of a hybridization reaction than thermal melting curves.

Two disadvantages of this method exist. First, each probe can only be used once due to irreversible destruction of acridinium ester by peroxide chemistry (Fig. 1B). However, because of the small amounts of probe required for each experiment, this limitation should not affect utility of the method. Second, high temperatures (90°C and above) and high pH buffers (pH 9 and above) cannot be used, because the AE ester will undergo hydrolysis during hybridization. However, these conditions can also be detrimental to RNA targets and are not likely to be used.

In conclusion, we have developed an accurate high throughput method to investigate oligodeoxyribonucleotide hybridization kinetics and thermodynamics. The method uses easily prepared probes, can be performed completely in solution, is highly reproducible, requires very small amounts (fmol quantities) of nucleic acid and can be performed with synthetic or biologically relevant targets with no restrictions on target length. Furthermore, because the method utilizes a highly chemiluminescent reporter molecule, very low concentrations of nucleic acid can be used in hybridization reactions, enabling the measurement of very fast kinetics or very large affinities.

ACKNOWLEDGEMENT

We thank Dr Norman C.Nelson for helpful discussions.

REFERENCES

1 Kumazawa,Y., Yokogawa,T., Tsurui,H., Miura,K. and Watanabe,K. (1992) *Nucleic Acids Res.*, **20**, 2223–2232.
2 Dewanjee,M. K., Ghafouripour,K., Kapadvanjwala,M. and Samy,A.T. (1994) *BioTechniques*, **16**, 844–850.
3 Bush,C.A. (1974) In Ts'o,P.O.P. (ed.), *Basic Principles in Nucleic Acid Chemistry.* Academic Press, New York, NY, Vol. 2, pp. 91–169.
4 Wang,S., Friedman,A.E. and Kool,E.T. (1995) *Biochemistry*, **34**, 9774–9784.
5 Zhong,M., Marky,L.A., Kallenbach,N.R. and Kupke,D.W. (1997) *Biochemistry*, **36**, 2485–2491.
6 Riccelli,P.V., Hilario,J., Gallo,F.J., Young,A.P. and Benight,A.S. (1996) *Biochemistry*, **35**, 15364–15372.
7 Law,S.M., Eritja,R., Goodman,M.F. and Breslauer,K.J. (1996) *Biochemistry*, **35**, 12329–12337.
8 Salazar,M., Thompson,B.D., Kerwin,S.M. and Hurley,L.H. (1996) *Biochemistry*, **35**, 16110–16115.
9 Gyi,J.I., Conn,G.L., Lane,A.N. and Brown,T. (1996) *Biochemistry*, **35**, 12538–12548.
10 Manoharan,M., Tivel,K.L., Zhao,M., Nafisi,K. and Netzel,T.L. (1995) *J. Phys. Chem.*, **99**, 17461–17472.
11 Li,Y., Bevilacqua,P.C., Mathews,D. and Turner,D.H. (1995) *Biochemistry*, **34**, 14394–14399.
12 Schwille,P., Oehlenschlager,F. and Walter,N.G. (1996) *Biochemistry*, **35**, 10182–10193.
13 Parkhurst,K.M. and Parkhurst,L.J. (1995) *Biochemistry*, **34**, 285–292.
14 Perkins,T.A., Wolf,D.E. and Goodchild,J. (1996) *Biochemistry*, **35**, 16370–16377.
15 Nelson,N.C., Cheikh,A.B., Matsuda,E. and Becker,M.M. (1996) *Biochemistry*, **35**, 8429–8438.
16 Nelson,N.C., Hammond,P.W., Matsuda,E., Goud,A.A. and Becker,M.M. (1996) *Nucleic Acids Res.*, **24**, 4998–5003.
17 Becker,M.M. and Nelson,N.C. (1995) US patent application 5731148.
18 Caruthers,M.H (1991) *Acc. Chem. Res.*, **24**, 278–284.
19 Arnold,L.J.,Jr, Bhatt,R.S. and Reynolds,M.A. (1988) WO patent 8803173.
20 Weeks,I., Beheshti,I., McCapra,F., Campbell,A.K. and Woodhead,J.S. (1983) *Clin. Chem.*, **29**, 1474–1479.
21 Arnold,L.,Jr And Nelson,N.C. (1993) US patent 5,185,439.
22 Britten,R.J., Graham,D.E. and Neufeld,B.R. (1974) *Methods Enzymol.*, **29**, 363.
23 Marky,L.A. and Breslauer,K.J. (1987) *Biopolymers*, **26**, 1601–1620.
24 Young,S. and Wagner,R.W. (1991) *Nucleic Acids Res.*, **19**, 2463–2470.