0026-895X/98/050926-07$3.00/0

MOLECULAR PHARMACOLOGY, **53:**926–932 (1998).

Interferon-γ-Inhibitory Oligodeoxynucleotides Alter the Conformation of Interferon-γ

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Received November 25, 1997; Accepted February 5, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

The aptamer mechanism of action involves the direct interaction of oligonucleotide with protein and is responsible for the biological effects of many pharmacologically active oligodeoxynucleotides. In the work reported here, we have determined the effects of aptamers on the secondary, tertiary, and quaternary structures of the proteins with which they interact using interferon-γ and the interferon-γ-inhibitory aptamer oligonucleotide, 5′-GGG GTT GGT TGT GTT GGG TGT TGT GT, as a model system. CD, fluorescence spectroscopy studies, and antibody binding studies in this system demonstrate that the interferon-γ-inhibitory aptamer oligonucleotide causes significant changes in secondary and tertiary structures of interferon-γ. These structural changes do not result in, or resemble, protein denaturation or aggregation, and the results suggest that aptamer oligodeoxynucleotides can significantly alter the structure of the proteins they interact with.

Single-stranded oligonucleotides that exert pharmacological effects by directly interacting with proteins have been termed "aptamers" (Bock *et al.*, 1992). Considerable recent evidence suggests that the vast majority of pharmacologically active oligonucleotides exert their effects via the aptamer mechanism and not through the antisense or antigene mechanisms, which involve interactions with nucleic acids (Stein, 1995).

We have previously demonstrated that certain oligonucleotides inhibit the biological effects of the proinflammatory cytokine, IFN-γ (Fedoseyeva *et al.*, 1994; Ramanathan *et al.*, 1994; Tam *et al.*, 1994). These IFN-γ inhibitors may have therapeutic uses because the biological effects of this cytokine are undesirable in inflammatory and autoimmune diseases such as multiple sclerosis, insulin-dependent diabetes and septic shock. The IFN-γ-inhibitory oligonucleotides act by an aptamer mechanism because they prevent binding of IFN-γ to the IFN-γ-binding subunit of the IFN-γ receptor (Ramanathan *et al.*, 1994; Lee *et al.*, 1996).

With the exception of the thrombin aptamer, the mechanisms of aptamer action, particularly the effects of aptamers on the proteins with which they interact, are poorly understood. The thrombin aptamer inhibits clotting by binding to the anion binding exosite of thrombin, and NMR (Macaya *et al.*, 1993; Schultze *et al.*, 1994) and X-ray crystallographic (Padmanabhan *et al.*, 1993) evidence suggests that the aptamer forms an intramolecular G-quartet. Much of the research on aptamers has focused on the structural and sequence characteristics of aptamer oligonucleotides (Bock *et al.*, 1992; Ojwang *et al.*, 1994; Rando *et al.*, 1995), and relatively little data on the effects of aptamers on the target protein is available.

In this article, using IFN-γ-inhibitory oligonucleotides as a model system, we highlight the effects of aptamers on the structures of the proteins with which they interact. We address four specific questions. Do IFN-γ-inhibitory oligonucleotides alter (i) the secondary structure, (ii) the tertiary structure, (iii) the quaternary structure, and (iv) a functional epitope of IFN-γ?

Our results demonstrate that oligonucleotides cause large structural changes in the secondary structure of IFN-γ and that the magnitude of the structural changes is surprising given the relatively weak (micromolar to submicromolar) dissociation constant for the interaction between oligonucleotides and IFN-γ. To our knowledge, aptamer-induced structural changes have not been previously reported and these findings therefore represent a novel mechanism for aptamer action. Because aptamer-induced protein structural changes have not usually been considered a potential mechanism for aptamer oligonucleotide activity, its prevalence may be more widespread than currently suspected.

Experimental Procedures

Oligonucleotides and interferon-γ. The phosphodiester oligonucleotide 5′-GGG GTT GGT TGT GTT GGG TGT TGT GT (Oligo I) and its reverse complement, 5′-ACA CAA CAC CCA ACA CAA CCA

This work was supported by Grant 1R29-GM54087–01 from the National Institute of General Medical Sciences. Funding from the National Multiple Sclerosis Society is also gratefully acknowledged. M.R. is a member of the University-to-University Cooperative Research Project between Kanazawa University, Japan, and the State University of New York at Buffalo.

ABBREVIATIONS: IFN-γ, interferon-γ; ANS, 1,8-aminonaphthalene sulfonate.

ACC CC (Oligo II) were synthesized using standard phosphoramidite protocols by Oli-to-Go (Dulles, VA) and Genosys (Woodlands, TX). Oligodeoxynucleotide concentrations were determined from spectrophotometric absorbance measurements at 260 nm by using a conversion factor of 33 μg of oligonucleotide per unit of absorbance.

Interferon-γ, free of macromolecular additives such as human serum albumin, was a gift from Genentech (South San Francisco, CA). The IFN-γ was dialyzed against several volumes of phosphate-buffered saline in small volume Slide-a-lyzer dialysis cassettes (Pierce, Rockford, IL) according to the manufacturer's instructions. After dialysis, protein concentrations were measured using the Bio-Rad protein assay reagent (BioRad, Richmond, CA). Bovine serum albumin (Fisher Scientific, Springfield, NJ) solutions were used as calibration standards.

Thermally aggregated IFN-γ for fluorescence experiments was obtained by heating IFN-γ to 70° for 45 min. The aggregation was independently confirmed by monitoring the absorbance at 360 nm, which increases because of increased scattering by aggregates.

CD spectroscopy. The CD studies were carried out in 1 mm path length quartz cuvets using a Jasco J500 spectropolarimeter (Jasco, Easton, MD) calibrated with d 10-camphorsulfonic acid (Sigma Chemical, St. Louis, MO). The instrument time constant and sensitivity were set at 4 sec and 1 millidegree, respectively, and samples were scanned over the wavelength range of 260–195 nm for secondary structure analysis.

For titration, two solutions were prepared in phosphate-buffered saline, pH 7.4: solution A contained 50 μg/ml IFN-γ and solution B contained 25 μM oligonucleotide plus 50 μg/ml IFN-γ. The spectra of solution A were recorded and solution B was added in aliquots to solution A. After each addition, the solutions were mixed by inversion and the spectra were recorded.

The ellipticity data over the wavelength range of 195–240 nm were analyzed for secondary structure with the use of the convex constraint analysis program (Perczel *et al.*, 1991, 1992) with a reference set consisting of 30 proteins.

The percent reduction in ellipticity was plotted against total oligonucleotide concentration, [Oligo], and the data were fitted to the quadratic binding equation:

$$\%\ \text{Ellipticity Reduction} = \text{Maximum \% Ellipticity Reduction} \times \frac{S - \sqrt{S^2 - 4[\text{Oligo}][\text{IFN}]}}{2[\text{IFN}]}$$

In the equation, [IFN] is the total IFN-γ concentration, K_d is the dissociation constant, and $S = [\text{Oligo}] + [\text{IFN}] + K_d$.

The quadratic binding equation was used instead of the Michaelis-Menten type simple binding hyperbola because the free oligonucleotide concentration cannot be made equal to the total oligonucleotide concentration. The least squares curve fitting routine in Kaleidagraph 3.08 (Synergy Software, Reading PA) was used to determine the Maximum % Ellipticity reduction and the dissociation constant terms in the equation.

Fluorescence spectroscopy. All fluorescence studies were done at room temperature on an SLM Aminco 8000 fluorometer (Spectronics Instruments, Rochester, NY) with 4-mm excitation and emission slits.

Tryptophan fluorescence emission spectra over the 300–400 nm wavelength range were recorded with the excitation wavelength set at 280 nm. A 295-nm long pass filter was used during the measurements to minimize the effect of Raman bands on the emission maxima. Spectra were recorded in I-shaped, 2 mm/10 mm dual path length cuvets so that corrections for the inner filter effect could be made. Oligonucleotide titrations were carried out as described for the CD studies. For the urea denaturation experiments, a phosphate-buffered saline solution containing 16 M urea plus 50 μg/ml IFN-γ was substituted for solution B.

The percent reduction in fluorescence was plotted against total oligonucleotide concentration, and the data were fitted to the quadratic binding equation using the method used for the CD studies.

The fluorescence of ANS (Sigma Chemical, St. Louis, MO) is sensitive to quaternary structure and was used to determine the effects of oligonucleotides on the homodimeric quaternary structure of IFN-γ. The final IFN-γ and the ANS concentrations were 10 μg/ml and 10 μM, respectively. Emission spectra for ANS between 400 and 600 nm were obtained with excitation set at 380 nm.

Antibody binding assay. Antibody 202 (Seelig *et al.*, 1994) and antibody 3125 (Alfa and Jay, 1988) were generous gifts from Dr. Gail F. Seelig (Schering Plow Research Institute, Kenilworth, NJ) and Dr. Francis T. Jay (University of Manitoba, Winnipeg, Canada). IgG control antibody, recombinant-human IFN-γ, and ^{125}I-labeled IFN-γ were purchased from Biodesign International (Kennebunk, ME), PeproTech (Rocky Hill, NJ), and Amersham (Arlington Heights, IL), respectively.

Control IgG antibody and anti-interferon-γ antibodies for different epitopes were dissolved in 0.2 M carbonate buffer and immobilized on enzyme-linked immunosorbent assay plates overnight at 4°. The plates were treated overnight with 200 μl of D-phosphate-buffered saline containing 1% bovine serum albumin at 4° to block nonspecific binding. ^{125}I-Labeled IFN-γ containing 0.1, 1, or 10 μM of either experimental or control oligonucleotide (Oligo I or II) was added followed by incubation for 1 hr at 4°. An aliquot of supernatant was removed for measuring the unbound ^{125}I-IFN-γ and the plates were washed with 0.05% Tween in phosphate-buffered saline. The radioactivity in the supernatant and that bound to the wells was quantified in a Minaxi γ Auto-gamma 5000 series γ-counter (Packard, Downers Grove, IL).

The ratio of the bound to free radioactivity was calculated and expressed as percentage of the ratio obtained in the absence of oligonucleotide. The results were fitted to a Hill equation:

$$\text{Bound/Free Ratio (as \% of control)} = 100 - \frac{100 \times [\text{Oligo}]^{n_H}}{\text{IC}_{50}{}^{n_H} + [\text{Oligo}]^{n_H}}$$

Again, the least squares curve fitting routine in Kaleidagraph 3.08 was used to determine the IC_{50} and n_H, the Hill coefficient.

Results

Evidence that oligonucleotides alter the secondary structure of IFN-γ. To determine the effect of oligonucleotide on the secondary structure of IFN-γ, far ultraviolet-CD spectra of the protein in the presence and absence of oligonucleotides were acquired. The CD spectrum of IFN-γ (Fig. 1) exhibited a negative band around 220 nm, a shoulder at 208 nm, and a positive band at 190 nm, all of which are characteristic of an α-helix rich protein (Fasman, 1993). Convex constraint analysis (Perczel *et al.*, 1991, 1992) showed 58% α-helical content and some random coil and aromatic contributions. These are consistent with the reported crystal structure data on IFN-γ (Ealick *et al.*, 1991) and with previously reported far ultraviolet-CD data (Hogrefe *et al.*, 1989).

The data in Fig. 1 are IFN-γ CD spectra in the absence and presence of varying concentrations of oligonucleotide and are corrected for ellipticity of the added oligonucleotide. Fig. 1 shows that the IFN-γ CD spectrum changes significantly from the native structure upon addition of Oligo I. The ellipticity value at 220 nm decreased with the addition of oligonucleotide, indicating secondary structure changes in the protein, and quantitative estimation using convex constraint analysis indicated a reduction in α-helix content to 40% and an increase in random coil content. CD spectra of oligonucleotide alone were also acquired at each concentration, but because the spectra were corrected for ellipticity of oligonu-

cleotide alone, the data are not shown. At the highest concentration of Oligo I, 10 μM, the ellipticity of Oligo I alone at 220 nm was less than 15% of the ellipticity of IFN-γ alone. The ellipticity of IFN-γ at 220 nm decreased rapidly with increasing Oligo I concentration but became independent of the oligonucleotide concentration at the higher Oligo I concentrations (Fig. 1, *inset*). A similar decrease in the ellipticity values was observed with Oligo II (data not shown).

Evidence that oligonucleotides alter the tertiary structure of IFN-γ. Tryptophan fluorescence emission spectra were monitored to determine the effects of oligonucleotide treatment on the tertiary structure of IFN-γ. Because IFN-γ has only one tryptophan, W31, per monomeric subunit, fluorescence spectroscopy yields information about the site at which oligonucleotide interacts. The tryptophan is located at the end of the B helix and takes part in the formation of a cleft that accommodates the carboxyl-terminal F helix of the other monomer (Ealick *et al.*, 1991). Changes in tryptophan fluorescence therefore provide information about the effects of oligonucleotide on dimeric interface of IFN-γ.

Samples were excited at 280 nm and the emission spectra were obtained between 300–400 nm. Fluorescence spectra for the IFN-γ and for IFN-γ in the presence of various concentrations of Oligo I are shown in Fig. 2. The fluorescence spectra of the oligonucleotide alone were also acquired at each concentration but are not shown because the analysis included correcting for the fluorescence of oligonucleotide. Over the 320–370 nm wavelength range, the fluorescence contribution of Oligo I alone at its highest concentration, 10 μM, was less than 15% of the fluorescence of IFN-γ alone.

The IFN-γ spectrum shows a peak maximum at 343 nm, suggesting that the tryptophan is partially shielded from the surrounding aqueous environment, which is consistent with the crystal structure data. The fluorescence of the protein is reduced by the addition of Oligo I in a dose dependent manner. A similar reduction in fluorescence was observed with Oligo II (data not shown). Fig. 2, *inset*, is a plot of percent of fluorescence quenched versus Oligo I concentration. The quenching of IFN-γ fluorescence in the presence of oligonucleotide shows saturation at higher oligonucleotide concentrations.

The quadratic binding curves for the CD data (Fig. 1, *inset*) and the fluorescence quenching data (Fig. 2, *inset*) were very similar and the fitted parameters were not statistically different. The data were therefore pooled and the dissociation constant was estimated from fitting to be 0.15 ± 0.10 μM. These results show that Oligo I interacts with W31 and suggest that the interactions either cause or are associated with changes in the tertiary structure of IFN-γ.

Evidence that the conformational changes do not involve denaturation of IFN-γ. Changes in secondary structure and quenching of fluorescence intensity can also occur when a protein denatures in the presence of agents such as urea. To determine whether the conformational changes induced by Oligo I were distinct from denaturation, we compared the fluorescence emission spectra of IFN-γ obtained in the presence of various concentrations of either urea or Oligo I (Fig. 3).

In the absence of urea, IFN-γ shows a peak maximum of 343 nm. However, with increasing urea concentration, the fluorescence intensity is quenched (Fig. 3, *inset*) and the peak maxima are shifted to longer wavelengths (Fig. 3). Thus, the denaturation of IFN-γ is associated with both fluorescence quenching and shift in the peak maximum. In contrast, peak position does not shift in the Oligo I treated IFN-γ samples, suggesting that the molecular events associated with oligonucleotide addition are different from protein denaturation and that the solvent exposure of the tryptophan residue, W31, is not altered during the interaction with Oligo I.

Evidence that Oligo I does not detectably alter the quaternary structure of IFN-γ. The conformational changes observed using CD and fluorescence could potentially also be associated with the loss of quaternary structure of the IFN-γ and this could result in the formation of larger aggregates or dissociation to individual subunits. To investigate the aggregation or dissociation of the dimeric protein, we probed the quaternary structure by ANS fluorescence.

The fluorescence of ANS is sensitive to the hydrophobicity

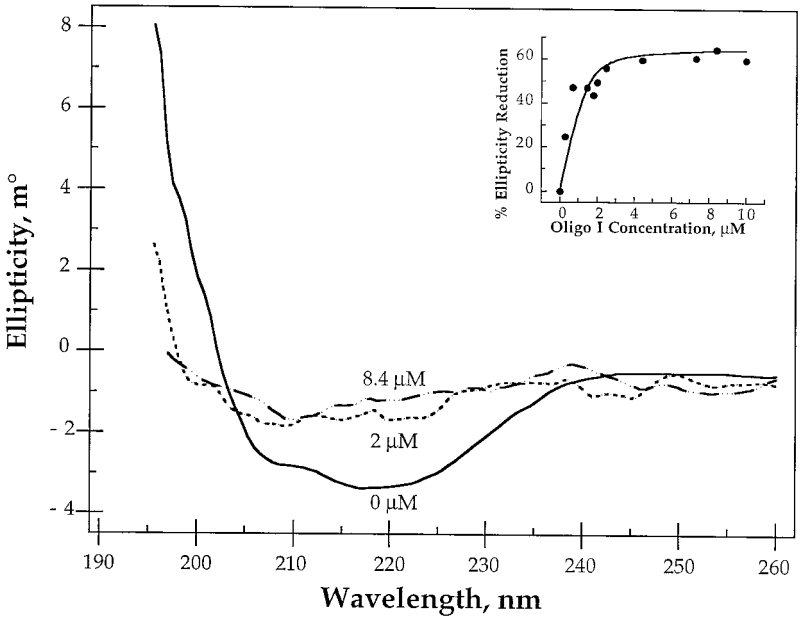

Fig. 1. CD spectra of IFN-γ in the presence of Oligo I. The IFN-γ concentration was 50 μg/ml and the oligonucleotide concentrations are indicated. The y-axis is the ellipticity in millidegrees. The IFN-γ spectra shown have been corrected for the ellipticity of the added oligonucleotide. *Inset*, dependence of the IFN-γ ellipticity at 220 nm on oligonucleotide concentration. *Solid line,* least squares fit of a quadratic binding equation to the data.

of proteins and its use for quaternary structural determination and subunit affinity has been well documented (Aloj *et al.*, 1973; Merz, 1988). Thus, ANS fluorescence in the presence of IFN-γ can be expected to increase if aggregation occurs, and decrease if dissociation to subunits occurs.

However, the ANS fluorescence cannot be used to draw conclusions regarding quaternary structure unless these structural changes are accompanied by hydrophobicity changes. The accuracy of this underlying premise was confirmed by measuring the fluorescence of ANS in presence of thermally aggregated IFN-γ. The aggregation was independently confirmed by measuring the absorbance at 360 nm, which was almost 10-fold higher than the absorbance of native IFN-γ. This positive control sample also had a 4-fold higher ANS fluorescence intensity (Fig. 4) than native IFN-γ, showing that ANS fluorescence is sensitive to IFN-γ quaternary structure.

The fluorescence intensity of ANS in the presence of native IFN-γ is higher than the fluorescence of ANS in buffer, indicating possible binding of the probe with the dimeric IFN-γ. As shown in Fig. 4, the ANS fluorescence intensities for IFN-γ-oligonucleotide mixtures were independent of the Oligo I concentration, indicating that the extent of IFN-γ aggregation or dissociation upon interaction with Oligo I is either small or not accompanied by detectable changes in surface hydrophobicity.

Evidence that Oligo I alters the recognition of epitopes on IFN -γ. Antibody 202 recognizes an epitope that is spread across amino acids (1–29, 75–96, 104–111, 118–125, 131–139) (Seelig *et al.*, 1994) and antibody 3125 recognizes epitope E2′ (Alfa and Jay, 1988). The sequence corresponding to E2′ is not known, but it mediates antiviral activity (Alfa and Jay, 1988).

As shown in Fig. 5 A, binding of IFN-γ to antibody 202 was

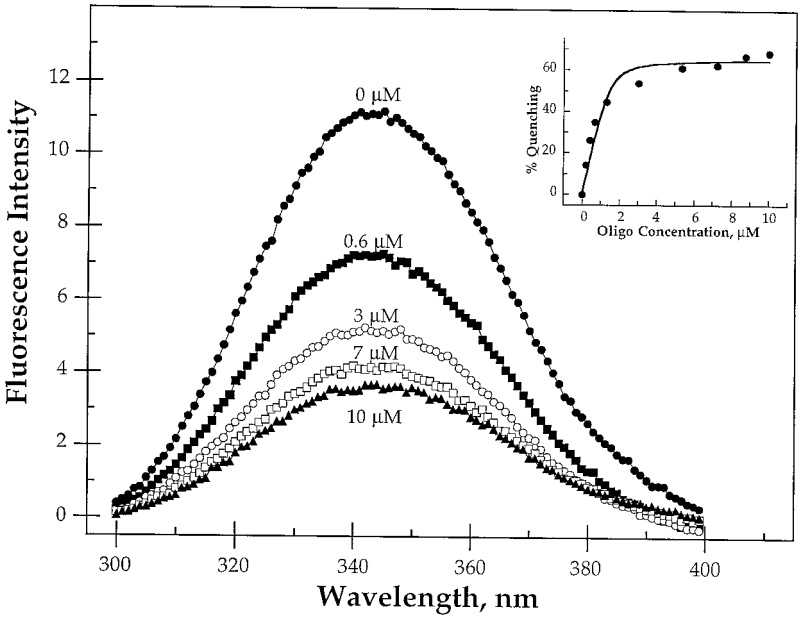

Fig. 2. Tryptophan fluorescence spectra of IFN-γ in the presence of Oligo I. The IFN-γ concentration was 50 μg/ml and the oligonucleotide concentrations are indicated. The samples were excited at 280 nm and the emission spectra between 300–400 nm were recorded. The IFN-γ spectra shown have been corrected for the fluorescence background of the added oligonucleotide. *Inset*, dependence of the fluorescence intensity at 340 nm on oligonucleotide concentration. *Solid line,* least squares fit of a quadratic binding equation to the data.

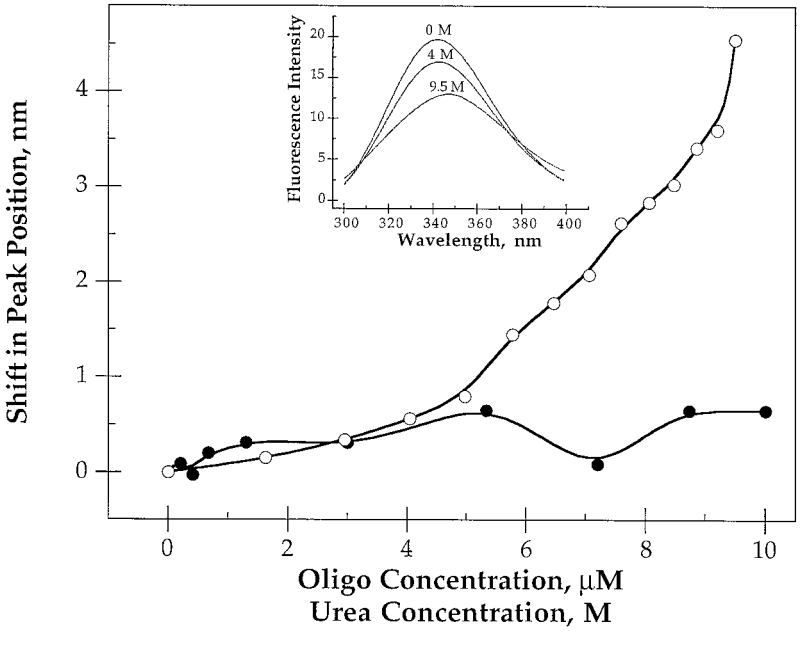

Fig. 3. The shift in position of the IFN-γ fluorescence emission peak in the presence of the indicated concentrations of urea (○) or Oligo I (●). The IFN-γ concentration was 50 μg/ml. Note that the position of the emission maximum shifts as the protein denatures in the presence of urea. *Inset*, fluorescence spectra of IFN-γ in the presence of the indicated concentrations of urea.

inhibited in a dose-dependent manner by Oligo I and II. Oligo I had a greater inhibitory effect compared with Oligo II and the IC_{50} value for Oligo I (1.3 ± 0.24 μM) was 10-fold lower than that for Oligo II (13 ± 8.3 μM). However, both Oligo I and II show similar inhibition of IFN-γ binding to antibody 3125 (Fig. 5 B) and the IC_{50} values were not statistically different. This suggests that Oligo I and II modify epitope E2′ to a similar extent.

These results further confirm the structural changes obtained by the spectroscopic methods and show that the structural changes are either associated with or can cause altered recognition of IFN-γ by biological macromolecules.

Discussion

In this work, we have investigated the effects of oligodeoxynucleotides on the structure of IFN-γ and our results demonstrate that the interaction with oligonucleotides causes a loss of α-helical content and alterations in the tertiary structure of IFN-γ.

In our previous studies, we have shown that Oligo I is more active than Oligo II in inhibiting the biological effects of IFN-γ and in inhibiting the binding of IFN-γ to cells (Ramanathan *et al.*, 1994) and to the IFN-γ-binding subunit of the receptor (Lee *et al.*, 1996). However, in the spectroscopic assays used in this work, both oligonucleotides altered the structure of IFN-γ, suggesting that the structural changes are necessary but not sufficient for activity. In the results displayed in Fig. 5, however, Oligos I and II show different effects on binding of IFN-γ to antibody 202 but similar effects on IFN-γ binding to antibody 3125. These findings are consistent with the notion that contributions from both sequence and backbone elements are necessary for IFN-γ inhibitory activity of oligonucleotides and supports the hypothesis that the two oligonucleotides have certain similarities and certain dissimilarities in their actions that may contribute to the differences in biological activity. The mechanisms by which bases of the oligonucleotide inhibit the interaction between IFN-γ and its receptor are currently unknown but several possibilities can be envisioned. For example, the bases may cause steric hindrance to interaction or alternatively, the IFN-γ may not fully recover its native structure after perturbation caused by aptamer.

At first sight, the sensitivity of the secondary structure of IFN-γ to oligonucleotides is somewhat surprising, considering that the biological roles of IFN-γ do not involve DNA or RNA binding. In contrast, the inhibitory effects of phosphorothioate oligonucleotides on the activities of the DNA polymerases, RNases H (Gao *et al.*, 1992), and retroviral reverse transcriptases (Ojwang *et al.*, 1994) are, in retrospect, not surprising because the physiological roles of these enzymes require interactions with nucleic acids (Gao *et al.*, 1992). Basic fibroblast growth factor (Jellinek *et al.*, 1993) and vascular endothelial growth factor (Jellinek *et al.*, 1994) are among the non-DNA binding proteins that are inhibited by RNA aptamers. The inhibition of these growth factors can be rationalized, in part, by their heparin binding ability (Stein, 1995). We speculate that the interaction of oligonucleotides with IFN-γ is also perhaps an unexpected consequence of a biologically relevant interaction between IFN-γ and some negatively charged component of the extracellular matrix (Rider, 1993; Tanaka *et al.*, 1993). Heparin sulfate and heparin bind IFN-γ with nanomolar dissociation constants (Lortat-Jakob and Grimaud, 1991, 1992; Lortat-Jacob *et al.*, 1991), and depending on experimental conditions, these interactions can either enhance (Sylvester *et al.*, 1990; Lortat-Jakob and Grimaud, 1991) or inhibit (Daubener *et al.*, 1995; Douglas *et al.*, 1997) the activity of IFN-γ. In *in vivo* experiments, these extracellular matrix interactions of IFN-γ appear to enhance the stability and activity of IFN-γ (Sylvester *et al.*, 1990; Lortat-Jacob *et al.*, 1996).

Are protein structural changes a common feature in aptamer-protein interactions or are these changes unique to the IFN-γ system? Few protein structural data are available for most aptamers, but in the thrombin-thrombin aptamer system, the aptamer adopts a G-quartet structure. Significant changes to the secondary structure of thrombin were not reported, however, in the X-ray crystal structure (Padmanab-

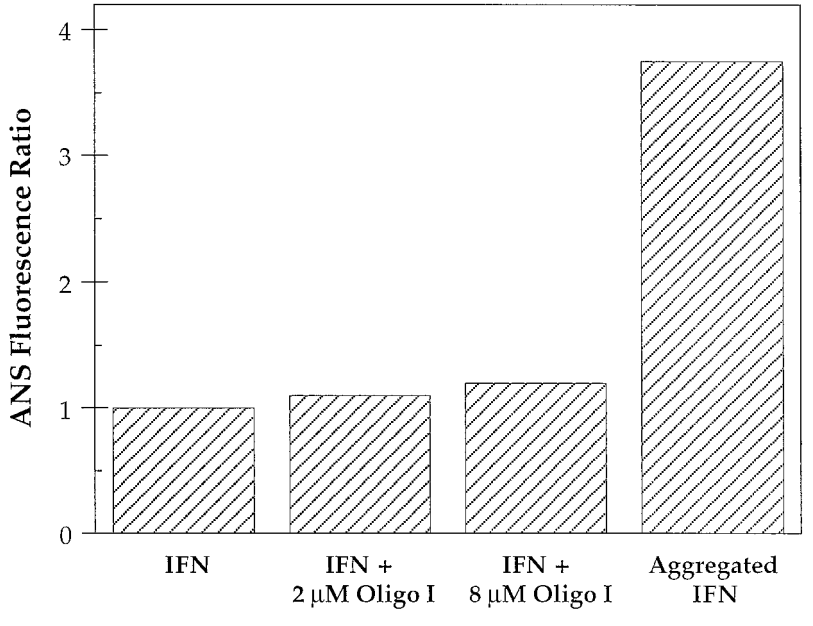

Fig. 4. Normalized ANS fluorescence at 482 nm in the presence of IFN-γ, heat-aggregated IFN-γ, or IFN-γ plus the indicated concentrations of Oligo I. The y-axis is the ratio of the ANS fluorescence intensity of the sample to the ANS fluorescence intensity in the presence of IFN-γ. The IFN-γ and the ANS concentrations were 10 μg/ml and 10 μM, respectively. The samples were excited at 380 nm and the emission spectra between 400–600 nm were recorded.

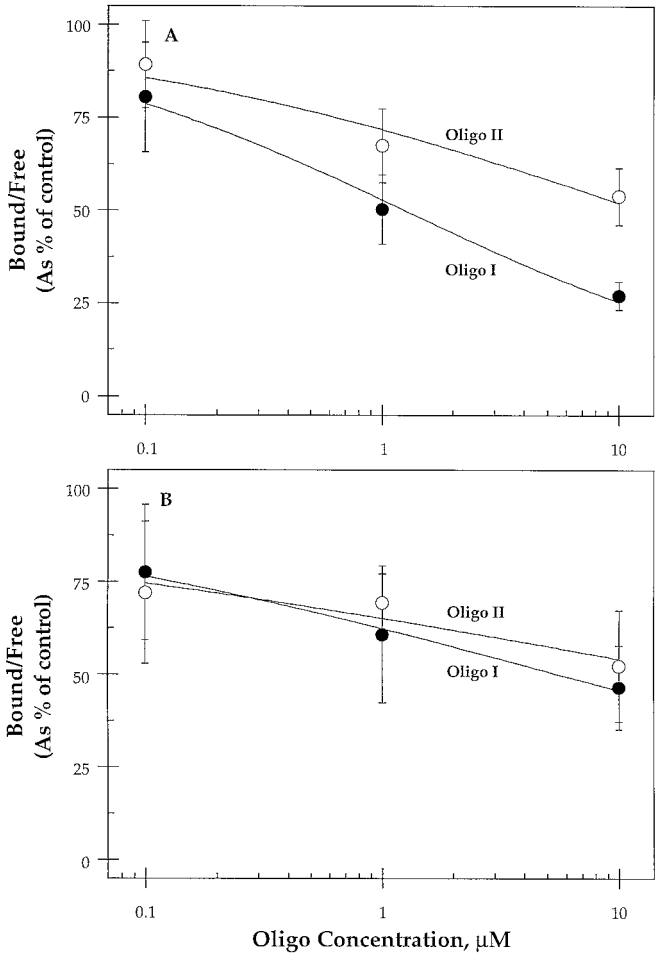

Fig. 5. A, Effect of Oligo I (●) and II (○) on IFN-γ binding to antibody 202. B, Effect of Oligo I and II on IFN-γ binding to antibody 3125. The x-axis in both graphs is oligonucleotide concentration in micromolar. The data are the summary of four experiments. *Error bars*, standard deviation. *Solid lines*, best fit to the Hill equation.

han *et al.*, 1993) and using molecular modeling (Macaya *et al.*, 1993).

In this work, we have used spectroscopic methods that allow the structural changes associated with the interaction to be monitored and do not require separation of the bound and unbound species. Other techniques such as mobility shift assays and gel filtration are also frequently used to demonstrate binding, but in our hands, these assays have not proven useful possibly because the binding is relatively weak.

The spectroscopic changes reported here may be useful in the design of high throughput screening strategies for identifying more potent, specific, therapeutically useful inhibitors for IFN-γ from combinatorial chemistry libraries. Effective inhibitors of IFN-γ are likely to be clinically useful in diseases such as multiple sclerosis, graft rejection, and diabetes where the proinflammatory effects of IFN-γ are disease-promoting.

In conclusion, our results may potentially provide a novel mechanistic basis for the activity of aptamers because they show that oligonucleotides can cause structural changes in proteins.

Acknowledgments

We thank Dr. Gail F. Seelig (Schering Plough, Kenilworth, NJ) for providing antibody 202; Dr. Francis T. Jay (University of Manitoba, Winnipeg, Canada) for supplying antibody 3125; and Dr. Robert Straubinger (State University of New York, Buffalo, NY) for use of the spectropolarimeter. We thank Dr. Gerald D. Fasman (Brandeis University, Waltham, MA) for providing us with the CDANAL software used for the analysis of circular dichroism spectra and we gratefully acknowledge Genentech Inc. for generously providing us with the interferon-γ used in these studies.

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