

Circular Dichroism reveals evidence of coupling between immunoglobulin constant and variable region secondary structure[☆]

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ABSTRACT

Antibodies (Ab) are bifunctional molecules with two domains, a constant region (C) that confers effector properties and a variable (V) region responsible of antigen (Ag) binding. Historically the C and V regions were considered to be functionally independent, with Ag specificity being solely determined by the V region. However, recent studies suggest that the C region can affect Ab fine specificity. This has led to the proposal that the C_H domain influences the structure of the V region, thus affecting Ab affinity and fine specificity. An inference from this proposal is that V region identical monoclonal Abs (mAbs) differing in C region (eg isotype) would manifest different secondary structures arising from isotype-induced variation in the V–C regions after Ag binding. We hypothesized that such effects could translate into differences in Circular Dichroism (CD) upon Ag–Ab complexes formation. Consequently we studied the interaction of a set of V region identical IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ mAbs with glucuronoxylomannan (GXM). The native CD spectra of the pairs IgG₁/IgG_{2a} and IgG₃/IgG_{2b} were strikingly similar, implying similar secondary structure content. GXM binding by IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ produced different CD changes, with the pairs IgG₁/IgG_{2a} and IgG₃/IgG_{2b} again manifesting qualitatively similar trends in secondary structure changes. The magnitude of the changes differed among the isotypes with IgG_{2a} > IgG₃ > IgG_{2b} > IgG₁. These differences in CD changes were interpreted to reflect differences in V–C secondary structures.

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Immunoglobulin molecules are products of the adaptive immune response that function as antimicrobial proteins. They are heterodimers in which each monomer contains both a heavy (H) and a light (L) chain. The H and L chains each has a V domain (V_H and V_L, respectively) that forms the Ag binding site. The rest of the chains are composed of C domains (C_H and C_L). Classically, the structure of the V region is thought to confer Ag specificity directly, while the class of C_H domain determines the effector functions of the Ab such as interaction with Fc receptors, half-life and complement actions (Ravetch and Kinetic, 1991). Both domains were thought to act separately, with distinct individual roles. Ab isotypes are defined by the C_H domain. During the generation of IgG from IgM, through a process known as class switch recombination, the avidity and effector functions of the Ab were thought to change while maintaining the fidelity of Ag specificity (Tonegawa, 1983). Recently, this concept was challenged by reports of V region identical Abs that manifest differences in idiotypic

(Id) recognition, fine specificity and the magnitude of Ag binding (Greenspan et al., 1989; Cooper et al., 1993; Morahan et al., 1983; McCloskey et al., 1996; Torres et al., 2005, 2007a; Dam et al., 2008). The occurrence of isotype-related specificity changes would represent a new mechanism for the generation of diversity in Ab responses.

To understand the differences in Ag specificity conferred by the Ab C_H domain requires detailed structural information. Circular Dichroism (CD) Spectroscopy measures protein secondary structure by detecting differences in the absorption of left and right handed circularly polarized light using far ultraviolet absorption spectra. The spectra of proteins in this region is defined by the amide group transition of n → π* and π → π*. This transition is further influenced by the structural geometry of the polypeptide backbone, which results in different spectra depending on the content of secondary protein structures (Whitmore and Wallace, 2007).

In this analysis, four different mAb isotypes, IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ of the 3E5 family that share identical V_H and V_L sequences (Torres et al., 2005) were analyzed by CD spectra. Secondary structural characteristics of the mAbs showed differences both with and without Ag binding. To our knowledge these are the first data showing that Abs differing in isotype undergo different structure changes upon binding to a common Ag. The results provide strong evidence for a tight structural linkage between the C and V domains such that the C region can affect V region structure, and hence speci-

Abbreviations: C, constant; V, variable; Ab, antibody; mAb, monoclonal Ab; CD, Circular Dichroism; Ag, antigen; GXM, glucuronoxylomannan; H, heavy (chain); L, light (chain).

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ficity (Torres et al., 2007b), and the V region can affect C region structure upon Ag binding.

1. Materials and methods

1.1. Glucuronoxylomannan (GXM) preparation

GXM was isolated from *C. neoformans* strain 24067 (serotype D). *C. neoformans* was grown with shaking at 150 rpm at 30 °C in Sabouraud dextrose broth. GXM was then isolated and purified with minor modifications (Cherniak et al., 1998). Supernatant was filtered through 0.45 μm filters to remove any cryptococcal cells remaining after low speed centrifugal separation. The polysaccharide was then precipitated from the supernatant with 10% (w/v) sodium acetate and 2.5 volumes of ethanol. Determination of the amount of carbohydrate in the water-solubilized precipitant was done using the phenol-sulfuric acid method (Dubois et al., 1956). The solution was adjusted to 0.2 M NaCl, and GXM was selectively precipitated using 3 g of hexyldecyltrimethylammonium bromide (Sigma) per gram of carbohydrate present. GXM was then dissolved in 2 M NaCl, dialyzed extensively against 1 M NaCl to remove hexyldecyltrimethylammonium bromide, followed by a 2 day dialysis against distilled water. GXM was finally collected by lyophilization (McFadden et al., 2006).

1.2. mAb preparation

The IgG₁, IgG_{2a}, and IgG_{2b} switch variants of 3E5 IgG₃ have been described earlier (Casadevall et al., 1992; Spira and Scharff, 1992). The murine mAbs were purified by protein A or G affinity chromatography (Pierce) from hybridoma cell culture supernatants and dialyzed against Phosphate Buffered Saline (PBS), pH 7.4. mAb concentration was determined by OD₂₈₀ measurement. mAb concentrations varied from 0.5–1 milligrams/milliliter (mg/ml), resulting in ~2 nanomoles (nmol) of mAb per sample.

1.3. ClustalW2 alignment

3E5 switch variant Fab primary sequences that include only the V_H and C_{H1} domains of the heavy chain were used in this comparison (Torres et al., 2007b).

1.4. GXM-mAb titration

GXM was added to each sample of 2 nmol of mAb after the initial measurement at 14, 28, 55, 110, 220 and 367 picomoles (pmol). The molar concentration of GXM was calculated assuming the molecular mass of 1200 kDa derived from light scattering measurements (Frasces et al., 2008). The mAb-GXM solution was then allowed to equilibrate at room temperature for 10 min before a spectral measurement was taken. All mAb analyzes labeled 'with GXM addition' were done using the spectra obtained from the final GXM titration of 368 pmol, corresponding to a molar ratio of 5.4 for Ab–Ag. Furthermore, as a negative control, Glycogen IX, with a molecular mass of 2520 kDa was titrated with the mAbs to a final molar ratio of 7.1 Ab–Ag.

1.5. Circular Dichroism spectroscopy

Circular Dichroism was performed in a Jasco-Spectrophotometer using a 350 μl rectangular quartz cuvette (Jasco) with a 0.1 cm path length. All scans were done in triplicate, at 25 °C using a 1 nm bandwidth, 0.1 nm step size and a 10 s averaging time. Data was measured from a range of 240–190 nm. Background corrections were done by subtracting GXM in PBS spectra which alone had no signal (data not shown). Machine

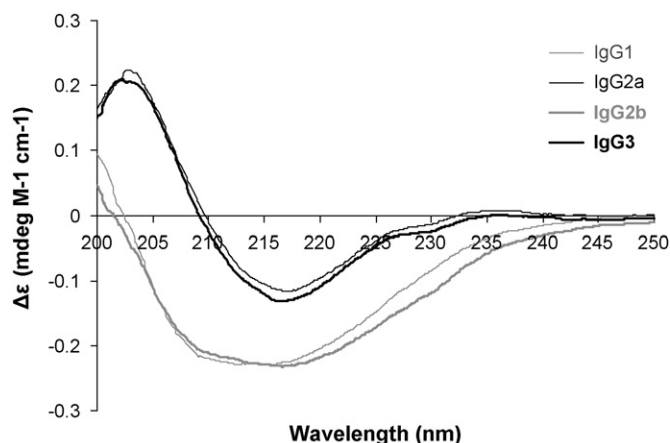


Fig. 1. CD Spectra for the four IgG isotypes without GXM antigen. mAb molar ellipticity is expressed in delta epsilon units as an average of three separate analyzes. Alpha helix absorbance shows as negative bands at 208 and 222 nm, β sheet shows as a negative band at 218 nm and random coils show as positive bands at 212 nm, where Δε = delta epsilon (previously described), mdeg = millidegrees and M = molarity.

units θ_{obs} (millidegrees) were converted to delta epsilon ($\Delta\epsilon$) for secondary structure prediction by the relationship.

$$De = \frac{\theta_{\text{obs}} \cdot MRW}{10 \cdot c \cdot l \cdot 3298}$$

where MRW is the mean residual weight of the mAbs (MW/number of amino acids in sequence), c is the mAb concentration in mg/ml and l is the path length in cm. Results were reported from CONTIN/LL, and analyzed using the CDPro suite. The CONTIN/LL program was established and modified by (Sreerama and Woody, 2000) by using a basis set of 42 soluble proteins whose crystal structures are known. The reference set of proteins includes those with high α helical content, high β sheet content and mixed α/β content. The program uses CD spectra of a set of proteins for which secondary and tertiary structures are known to compare to the CD spectra of the protein being studied. Based on this comparison, a readout is provided of estimated percentage α helix, β sheet, turn and 'disordered' content in the protein under study. Furthermore, the CONTIN/LL program has been compared to the other CDPro analytical programs and has been found to give superior predictions for spectral measurements between 190 and 240 nm due to its larger reference range (Sreerama and Woody, 2000).

1.6. Statistical analyzes

Data shown in Figs. 1 and 2 was analyzed by GraphPad Prism. Ellipticity data for all four isotypes was analyzed by 1-way ANOVA for each secondary structure category using GraphPad Prism (GraphPad Software, La Jolla, CA). This was followed by a Tukey Multiple Comparison Test from which p -values and significance was obtained by the comparison of the raw data of each mAb isotype with that of each of the other isotypes independently.

2. Results and discussion

We analyzed the secondary structures of four 3E5 mAb isotypes before and after the addition of GXM by CD spectroscopy. Specifically we measured changes in molar ellipticity as a function of GXM concentration and calculated the relative changes in various protein secondary structure components. The origin of these experiments was based on an attempt to test the prior existing paradigm in Ag–Ab interactions, which posited that all of the paratope, or Ag-combining surface on the Ab molecule, was determined exclusively by V region secondary structure. If this were the case we

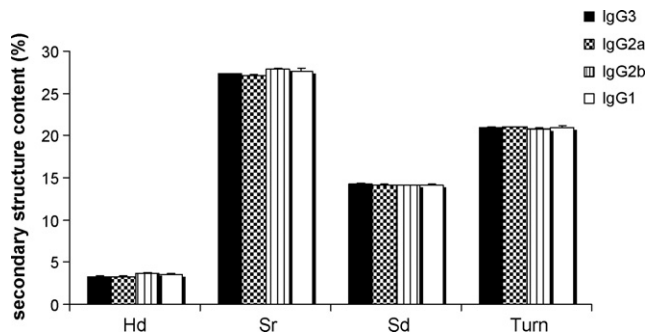


Fig. 2. Baseline percent secondary structure content for each of the four isotypes averaged from five separate data collections (each done in triplicate) analyzed by the CDPro program COTIN/LL. H(d) represents α helix (disordered) secondary structure, S(r) represents β sheet (regular) secondary structure, S(d) represents β sheet (disordered) secondary structure. Error bars represent \pm standard deviation. 1-way ANOVA analysis with a Tukey Multiple Comparison Test revealed statistical significance ($p < 0.05$) in the comparison between all mAb pairs except IgG₁/IgG_{2b} for Hd values, IgG_{2a}/IgG_{2b} and IgG₃/IgG_{2b} mAb pairs for Sr values, the IgG₃/IgG_{2b} mAb pair for Sd values and no statistically significant difference between mAb pairs for turn values.

reasoned that any changes in secondary structure following Ag binding would originate from induced fit changes in the V region domain. If the final structure of the GXM–Ab complex was solely determined by the V region, similar if not identical CD changes for all isotypes would be expected given that each of the 3E5 isotypes has identical V regions. On the other hand, if the secondary structure of the V region was affected by the associated C_H domain, whose primary sequence differs among the isotypes, then differences in secondary structure must originate in the C regions and/or V–C joining regions. Hence, similar CD changes would be consistent with and favor the older view of Ag–Ab interactions, while different CD changes would be consistent with and favor the view that the associated C region imposes structural constraints on the V region (Torres and Casadevall, 2007).

First, we analyzed the CD spectra of the four isotypes. Each isotype has identical V regions but the C domains have different amino acid variations that produce proteins with different effector functions (Spira and Scharff, 1992). To our knowledge this is the first comparison of secondary structure among immunoglobulins with identical V regions and differing C region domains. CD spectra of the four isotypes revealed that these isotypes fell into two groups, one consisting of IgG₁ and IgG_{2b} and the other of IgG_{2a} and IgG₃ (Fig. 1). The calculated protein secondary structure content for the four isotypes is very similar and given in Fig. 2. De units were used to correct for any differences in mAb concentration and baseline buffer spectra was subtracted from all data. In general, the spectra suggest that IgG₁ and IgG_{2b} have a greater α helical content than IgG_{2a} and IgG₃, which, in turn, have greater β sheet content. Along with the ‘unread’ data, which denotes structure that does not fall into the defined categories of α helix, β sheet and turn, the percent composition for each mAb sums to 100% (data not shown), and the bandwidth used was below 2 nm, indicating a high level of confidence in analysis (Hennessey and Johnson, 1982). Previous secondary structure analyses of murine IgG molecules have produced results that when compared across studies are inconsistent with one another as well as with the data presented in this paper (Vermeer and Norde, 2000; Vermeer et al., 1998; Jiskoot et al., 1991). Hence, the four isotypes studied here were more similar to one another with regards to secondary structure content than to other Abs of the same isotype reported in the literature. This was unexpected given that the C region makes up the preponderance of the mass of intact IgG. However, the IgGs used in other studies each had different V regions that may have conferred varying secondary structural characteristics to the entire immunoglobulin.

Table 1
% homology of 3E5 IgG FAb amino acid sequences.

Isotype	Percent homology ^a			
	IgG ₁	IgG _{2a}	IgG _{2b}	IgG ₃
IgG ₁	100	92	91	85
IgG _{2a}	92	100	93	88
IgG _{2b}	91	93	100	87
IgG ₃	85	88	87	100

^aClustalW2 homology alignment calculation using the amino acid sequences of the Fab portions of the IgGs (this includes all of the L chain and the V_H and C_{H1} domains of the H chain) (Larkin et al., 2007).

To further investigate the origin of the two separate groups of mAbs, the ClustalW2 alignment program was used to calculate the amino acid sequence homology between the four isotypes (Table 1). When the results of homology are compared to secondary structure findings by CD we note discordance, such that IgG_{2a} and IgG_{2b} are most similar by sequence yet the pairs IgG₁/IgG_{2b} and IgG_{2a}/IgG₃ are most similar by secondary structure. These results bear upon our understanding of the evolution of the IgG isotype family in mice, which is thought to have arisen by a process of gene duplication of an ancestral gene with subsequent drift through mutation. In mice, IgG₁ has been proposed to be the oldest and it may have diverged from IgG₂ before speciation (Adetugbo, 1978). However, though IgG₁ is most similar to IgG_{2a} by sequence, it manifests considerable difference from IgG_{2a} with regards to secondary structure content. On the other hand, IgG_{2a} is closest in secondary structure to IgG₃, despite the latter having the least sequence homology from the other IgG isotypes. These results suggest that the evolution of secondary structure and amino acid sequence have diverged from each other such that IgG_{2a} and IgG_{2b} are most closely related in primary sequence while each of these isotypes is closest in secondary structure content to IgG₃ and IgG₁ respectively. Despite these differences, each of the individual isotypes manifests the common immunoglobulin structural features of β sheet (>40%) > unread/other/random (30–40%) > turn (10–20%) > α helix (little to none) (Vermeer and Norde, 2000; Vermeer et al., 1998; Jiskoot et al., 1991).

We then studied the CD spectra of the mAbs with the addition of Ag. An advantage of this experimental system is that GXM is composed of sugars and this polymer has little or no CD signal relative to that of immunoglobulins. Consequently, any change in the CD spectra following mAb binding to GXM comes from immunoglobulin structural changes only. Upon binding GXM, all four isotypes manifested secondary structure changes as evidenced by changes in CD spectra. When Glycogen IX was added to antibody solutions as an irrelevant antigen negative control there was no change in mAb secondary structure, implying that the observed changes were specific to GXM, the cognate antigen of this mAb set (data not shown). The magnitude of the changes was relatively small and ranged from 0–1.1% within the four structure categories (see Fig. 3), suggesting that induced fit structural changes were relatively small and/or did not have a large effect on the overall secondary structure content of the isolated IgG vs the GXM–IgG complex. The isotype pairs that had the greatest similarities in their native spectra manifested similar changes upon binding GXM (Fig. 3). Hence the isotype pairs IgG₁/IgG_{2b} and IgG_{2a}/IgG₃ had the same pattern of changes in protein structure, although the magnitudes of the individual protein structural features varied.

With the addition of small amounts of GXM, each isotype followed a different path to the final Ag–Ab secondary structure (Fig. 4). The final equilibrium of secondary structure change was reached at different GXM concentrations for each of the four isotypes. β sheet change trends were similar for all mAbs, and are reciprocated by α helix changes (Fig. 4). Therefore, not only is

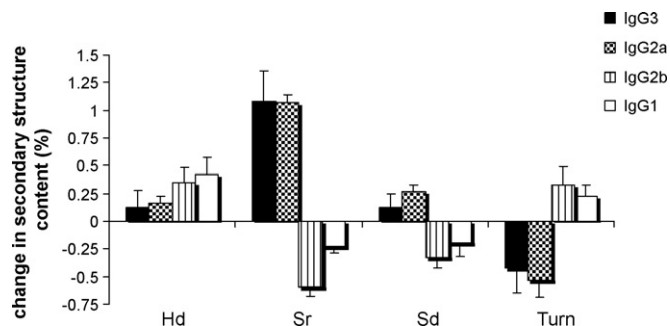


Fig. 3. Changes in predicted secondary structure upon 3E5 IgG mAb binding to GXM. Helix (r) is not included as its value was 0 for all samples. H(d) represents α helix (disordered) secondary structure, S(r) represents β sheet (regular) secondary structure, S(d) represents β sheet (disordered) secondary structure. Error bars represent standard deviation. 1-way ANOVA analysis with a Tukey Multiple Comparison Test revealed statistical significance ($p < 0.05$) in the comparison between IgG₃ and IgG₁ for Hd values, all pairs except IgG₁/IgG_{2b} for Sr values, all pairs except IgG₁/IgG_{2b} and IgG_{2a}/IgG₃ for Sd values and all mAb pairs except IgG₁/IgG_{2b} and IgG_{2a}/IgG₃ for turn values.

native CD spectra different for each mAb, but the pathway of secondary structure change upon Ag titration is also different, again suggesting that C region structural differences are being propagated to the V region. Averaging the amount of GXM needed to reach secondary structure equilibrium, gives magnitudes in the order of IgG_{2b} > IgG_{2a} = IgG₃ > IgG₁ with the average value being 24 mol mAb per 1 mol GXM (values used not shown). Since each of these Abs are bivalent, the differences in molar saturation ratio probably reflect differences in the Ag–Ab complex tertiary structure given variation in the hinge angle of the various isotypes, which could translate into differences in the number of contacts made between the two molecules. Currently, only two complete murine IgG structures have been solved crystallographically, murine IgG_{2a} 231 and murine IgG₁ 61.1.3. Comparison between the hinge regions of these antibodies reveals that though they both have extended conformations, the hinge of IgG₁ 61.1.3 is shorter and significantly more compact than that of IgG_{2a} 231 and their torsion angles are variable (Saphire et al., 2002). Differences in hinge geometry could affect the number of epitopes bound by an Ab molecule through steric constraints. Furthermore, differences in saturation ratio could also reflect the differences in fine specificity that have been documented

for this antibody set (Torres et al., 2005, 2007a; Dam et al., 2008; Spira and Scharff, 1992; Torres and Casadevall, 2007).

The finding of the molar saturation ratio of $\sim 24:1$ for Ab: GXM complexes has important clinical implications. Cryptococcosis is a fungal infection that is characterized by the presence of large amounts of GXM in serum and in tissue. Cryptococcal infections are seldom accompanied by a significant Ab response, a phenomenon that has been attributed to polysaccharide-induced Ab unresponsiveness (Kozel et al., 1977). However, our finding that a single GXM molecule can bind up two dozen immunoglobulin molecules implies that the shed polysaccharide in tissue constitutes an enormous Ab binding sink that could contribute to specific Ab depletion. The high saturation ratio also suggests an explanation for the observation that large amounts of Ab were required in patients with cryptococcosis before immunoglobulin therapy affected serum Ag levels (Larsen et al., 2005). This ratio could also be useful for estimating therapeutic Ab doses from serum GXM levels in future clinical trials of passive Ab therapy.

At Ag saturation, considerable changes were apparent in the CD spectra (Fig. 4). The increase in the depression at 218 nm upon GXM binding suggests either an exposure of beta sheet structure or a shift in secondary structure toward beta sheet formation (Fig. 5). The magnitude of percent secondary structure change upon GXM binding was IgG_{2a} > IgG₃ > IgG_{2b} > IgG₁, with values being $2.03 > 1.75 > 1.60 > 1.08$. The pairs IgG_{2a}/IgG₃ and IgG₁/IgG_{2b} again cluster together with regards to manifesting the largest and smallest secondary structural changes, respectively, with the stipulation that IgG_{2a} and IgG₁ are at the two extremes of this spectrum. The relatively small percent of secondary structural change upon Ag binding is consistent with induced fit changes affecting the V and possibly associated C_H regions. These results are consistent with the finding that of the 3E5 set, IgG₁ and IgG_{2b} behaved most similar with respect to thermodynamic parameters when forming the Ab–Ag complexes when the process was studied by ELISA and Immunofluorescence studies as well as Isothermal Titration Calorimetry studies (Torres et al., 2005; Dam et al., 2008). Furthermore, we note that in all these measurements there is discordance in the fact that IgG_{2a} and IgG_{2b} are most similar in primary sequence homology yet they are more similar to IgG₃ and IgG₁ respectively with regard to secondary structure changes.

These results support the hypothesis that amino acid differences in the 3E5 IgG mAb C_H region behave allosterically, perhaps

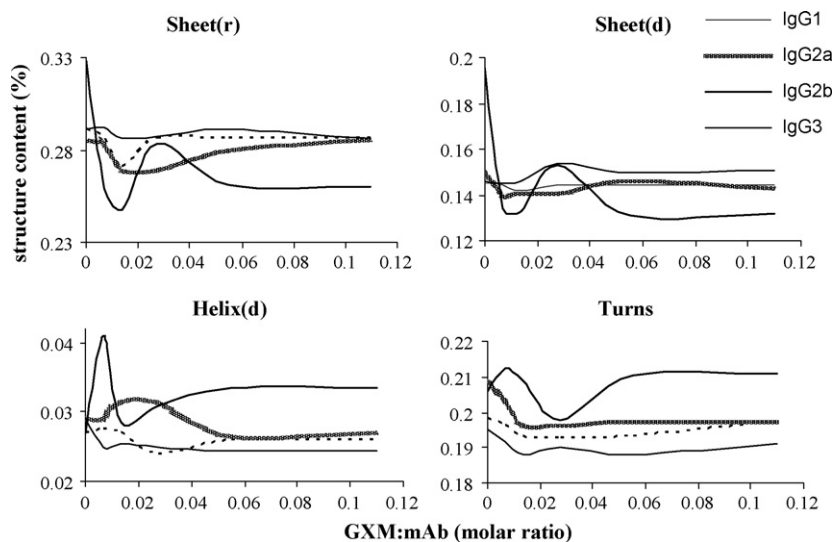


Fig. 4. Changes in secondary protein structure as a function of GXM:Ab molar ratio. Titration data. The y-axis represents the % secondary structure change upon GXM addition and the x-axis represents the GXM:mAb molar ratio. Helix (r) data remained constant throughout the CD experiments, so it has been excluded. Note: y-axis scaling differs.

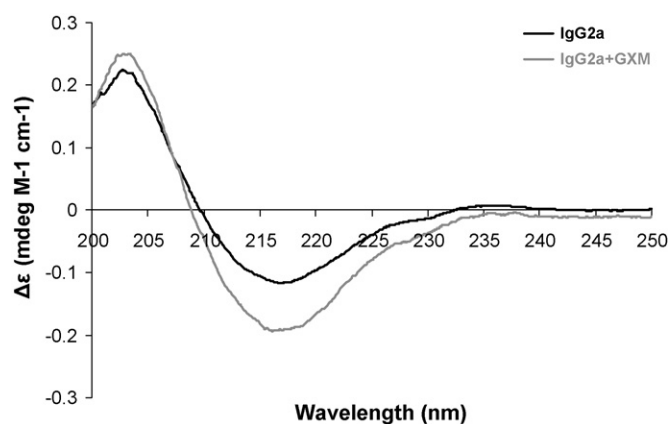


Fig. 5. CD spectra of IgG2a with and without GXM antigen shown to illustrate the type of changes observed. The spectrum with GXM was recorded at an Ab:GXM ratio of 5.4. Similar spectral changes were observed for the other isotypes (data not shown), where $\Delta\epsilon = \text{delta epsilon}$ (previously described), mdeg = millidegrees and M = molarity.

by constraining V region induced fit change and/or through tertiary protein conformation, resulting in subtle changes to the final V region conformation that manifest as differences in fine specificity. The differences in secondary structure changes observed between the isotypes upon Ag binding provide evidence for connectivity between the Ab C and V regions such that each can affect the other's secondary structure. Such connectivity has been predicted by modeling studies (Torres et al., 2007b). In the case of specificity this observation supports the notion that isotype differences can affect the fine specificity of V region identical Abs. In the case of the C region, this observation suggests that that V region changes associated with Ag binding can extend into adjacent C regions. Our findings suggest the need for further studies with more sensitive techniques such as X-ray crystallography to compare Ag–Ab structures and discern the mechanisms responsible for these differences.

In summary, our results provide the first comparison of secondary structure in solution for IgG isotypes with identical V regions in the presence and absence of their cognate Ag. Secondary structure content comparisons revealed a different correlation than would have been expected from primary sequence comparisons, suggesting divergence between primary and secondary structure in this mAb set. Structural changes upon Ag addition support the notion that the V and C regions have structural connections such that the conformations of the final Ag–Ab complexes differ depending on the isotype. This in turn is consistent with the emerging view that the configuration of the C region can impose structural constraints on the V region that translate into changes in fine specificity and idiotype, a finding that has major immunological implications (Torres and Casadevall, 2007).

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