

HUMAN AGLYCOSYL-IgG EXHIBITS INCREASED HYDROPHOBICITY

Binding/Fluorescence Studies with 8-Anilino-naphthalene-1-sulfonic Acid (ANS)

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SUMMARY: Human monoclonal, aglycosyl-IgG produced *in vitro* in the presence of tunicamycin, was compared with its native and acid pH-altered counterparts for their respective abilities to bind the fluorescent hydrophobicity probe, 8-anilino-naphthalene sulfonate. A novel technique based on continuous-flow dynamic dialysis (Sparrow et al., 1982, *Anal. Biochem.* 123:255-264) allowed binding studies under non-equilibrium conditions. While the native IgG conformation exhibits two, weak ANS binding sites (ca. 10^3 l/mol), aglycosyl-IgG has one weak and one moderate affinity (least squares average $K_a = 2 \times 10^4$ l/mol) site, and the acid conformer binds yet another two ANS molecules with moderate affinity (4×10^4 l/mol). Increases in affinity and in the number of sites correlate roughly with increased relative percent fluorescence by conventional fluorimetry. The fluorescence lifetime of ANS bound to altered IgGs is about 10% longer ($T_2 = 15$ nsec by time-resolved fluorimetry) than that for native IgG. All populations also exhibit a rapid decay component ($T_1 = 3$ nsec) analogous to that seen for ANS in 50% aqueous dioxane. Results are discussed in relation to structural role(s) for IgG-linked heterosaccharides.

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While the existence of hydrophobic sites on the surface of IgG molecules was reported in 1970 (1), only recently have investigators begun to probe these sites in relation to antibody structure and function. Chromatographic studies show that hydrophobic region(s) exist on the surface of IgG somewhere in the Fc (2,3,4). The fact that ANS² binds with enhanced fluorescence to IgG and Fc when exposed to acid pH (5) suggests that the acid conformation has either an increased probe-binding capacity, or there is an increase in quantum yield/probe. Acid treatment followed by neutralization irreversibly alters

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²Abbreviations used: ANS, 8-anilino-naphthalene-1-sulfonic acid; C1, C1q the first component, subcomponent of the classical C pathway; C γ 2, second constant-region domain of IgG.

complement C1-activating potential (6) and C1q binding sites (antecedents) in the Fc region (7) either directly or indirectly by altering $C_{\gamma}2:C_{\gamma}2$ juxtapositioning. As the major oligosaccharide for IgG of all animal species is found in this domain (reviewed in 8), and since ANS is an inhibitor of C1q binding in $C_{\gamma}2$ (9); the probe may be useful for studies of experimentally-induced changes in this region of IgG.

With the molecular basis for enhanced ANS fluorescence (increased binding sites versus increased probe quantum yield) essentially unresolved and because we wished to develop a simple, sensitive method for distinguishing probe binding after experimental deletion of IgG-linked oligosaccharides, the present study was undertaken. Of particular interest was any potential change in the hydrophobic nature of monoclonal IgG produced in vitro in the presence of tunicamycin, an antibiotic which inhibits glycosylation but not, to a great extent, secretion of IgGs (10,11).

In the present study, the binding of ANS to human IgG2, its acid and its aglycosyl conformers was determined by a simple but novel method for deriving ligand:protein binding data. The method, based on a continuous-flow, dynamic dialysis technique (12), was adapted to use a nitrogen-pressurized dialysis cell. By compiling large numbers of data points into a least squares fit equation for a polynomial over short sections of the data, it was possible to derive meaningful and accurate binding isotherms without having to establish either steady state or equilibrium conditions (12). Since it was also important to correlate changes in probe binding with changes in fluorescence intensity observed when ANS interacted with the various IgG populations, ANS fluorescence lifetimes were determined by time-resolved spectrophotofluorimetry.

Materials and Methods

Human IgG2(K) myeloma protein was produced in vitro by the cell line GM4672 (American Type Culture Collection) grown in RPMI 1640 medium (13) supplemented with 20 uCi/ml D-[^{14}C]-N-acetylglucosamine (5 mCi/mmol, ICN), 10 uCi/ml [3H]-L-amino acid mixture TMM-236 (50 Ci/mmol, Research Products, International Corporation), 2 mM L-glutamine, 12 mM glucose, 1.0 mM pyruvate, 10 mM HEPES and 10% agamma fetal bovine serum. For tunicamycin incubations, the antibiotic (Calbiochem) was added at 1.0 ug/ml to cells in serum-free

medium HB101 (Hanna Biological) supplemented with 2 mM L-glutamine. IgGs produced after 4 days of culture were purified by combined ion exchange/molecular sieve chromatography (DE-52, Whatman/Sephacryl S300, Pharmacia). Desired protein was detected by scintillation and ultraviolet spectroscopy and by means of a human K/ λ chain-specific, enzyme-linked immunosorbant assay (Taves and Winkelhake, manuscript in preparation). The acid form of protein 4672 was obtained by treating samples (3-5 mg/ml) in 0.01 M sodium acetate, 0.15M NaCl, pH 5.5 with 0.1M HCl to achieve the desired pH. The pH was then adjusted back to 7.2 by dialysis against 0.1M tris or 0.1M sodium phosphate.

Reagent grade ANS (Sigma) was recrystallized twice (14) providing a reagent with a molar extinction coefficient of $5.03 \times 10^3 \text{ cm}^{-1}$ at 350 nm. The binding of ANS was quantitated in an Amicon 3.0 ml, rapidly-stirred pressure/dialysis cell (PM30 membrane) containing 5-150 μM IgG by applying a linear gradient of 50 μM -1.0 mM (250 ml total) of ANS in PBS from two 1 liter pressure cylinders. Flow rate was kept constant at 40-42 ml/hr under 40 psi of N_2 pressure and 0.5 ml fractions were collected automatically. A standard curve relating effluent ligand to free ligand concentration was established in control experiments (no protein in dialysis cell). Subsequent test experiments with proteins then rely on this set flow rate/volume collection regimen to differentiate free and bound ligand. Samples were assessed in an Aminco-Bowman SPF500 spectrophotofluorimeter (Ex 370 nm/ Em = 480 nm) after adding 1.5 ml dioxane at room temperature with appropriate dilutions using 67% dioxane at ANS concentrations exceeding 100 μM . Data were fitted to a ligand permeating/binding isotherm program (12) modified for the Apple II+ computer. The Scatchard expression was used in a least-squares refinement program (STEPIT)(15) to evaluate binding parameters which are reported as K_a (association constant) and n (number of sites) constrained to integer values.

Fluorescence excited state lifetimes of ANS were measured with a PRA time-correlated, single photocounting fluorescence lifetime instrument (Ex 376 nm, Em 460 nm). All measurements were made at $25^\circ \pm 0.2^\circ$. Data was analyzed for exponential decays (from 1 to 3 decays) by a PRA deconvolution (lamp profile) and non-linear regression program on a PDP-11/23 computer. Concentrations of ANS and IgG were 50 μM respectively. Lifetimes of ANS in buffer solutions and in dioxane or 50% dioxane-water were measured at ANS concentrations of 300 μM and 20 μM respectively. Deoxygenation of several samples indicated that O_2 did not effect the excited state lifetimes under the conditions used in this work.

Results and Discussion

The effects of tunicamycin on synthesis/secretion of IgG2 by GM4672 cells is shown by Figure 1. Tunicamycin rapidly produced greater than 95% inhibition of D-[^{14}C]-N-acetylglucosamine incorporation into secreted protein while having much less effect on [^3H]-amino acid incorporation. Total inhibition of secretion of IgG by the antibiotic ranged from 10 to 40% (day 1 and day 4 respectively) of the 25 $\mu\text{g}/\text{ml}/24$ hr routinely synthesized by this cell line (data not shown). Thus, the GM4672 cell line exhibits similar sensitivity to tunicamycin as do murine plasmacytomas (10,11).

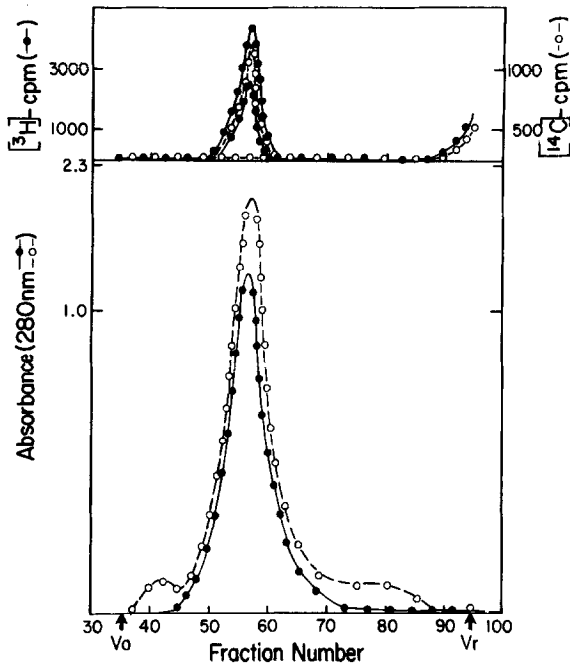


Figure 1:

Molecular sieve (Sephacryl S300) chromatograph of protein GM4672 produced in the presence (solid lines) and absence (dashed lines) of tunicamycin. Biosynthetically-labeled ($[^3\text{H}]$ -L amino acids \bullet and $[^{14}\text{C}]$ -N-acetylglucosamine \circ) human IgG2 was monitored by counting 100 μl samples in Aquasol 2 (New England Nuclear). The column void (V_0) and retention (V_r) volumes are shown by arrows indicating the elution peak for blue dextran and phenol red respectively. Protein was applied after elution from a DEAE-cellulose column in 0.025M phosphate pH 7.2 (4 mmhos). The molecular sieve column (2.5 x 90 cm) was pumped (1.0 ml/min) and fractions (2.0 ml) were collected automatically. Fractions containing protein which reacted in the anti-human chain ELISA (#42-65) were pooled for these studies.

When the resulting aglycosyl-IgG2 protein was compared with control GM4672 protein and its acid pH 2.0 or pH 3.5 conformers by their abilities to exhibit enhanced fluorescence of ANS, results (Table I) showed increases of about 50 and 150% (aglycosyl and acid conformers) relative to controls. The latter results are analogous to those previously reported for the acid conformer (16).

While there have been several interpretations of such ANS spectral changes (17,18), increased fluorescence has generally been associated with the existence of hydrophobic site(s) on the protein (5,16). In order to gain a finer appreciation for this interpretation, binding and fluorescence probe

TABLE I
**Comparative Fluorescence of ANS
 Bound to Various Solutions of Monoclonal Human IgG**

Sample	Relative Percent Fluorescence (+1 S.E.M.)
Normal (control)	25*
Aglycosyl IgG2 (pH 7.2)	41
pH 4.8	29
pH 3.5	59
pH 2.0	83
pH 3.5 (reneutralized to pH 7.2)	47

* Set arbitrarily by photomultiplier for IgG at 10 μ M and 100 μ M ANS (Ex. = 370 nm; Em = 480 nm) in an Aminco Bowman SPF500 spectrophotofluorimeter at 22 $^{\circ}$.

lifetime studies were performed. As shown by Figure 2, the continuous-flow dynamic dialysis technique (12) provides binding isotherms in the form of

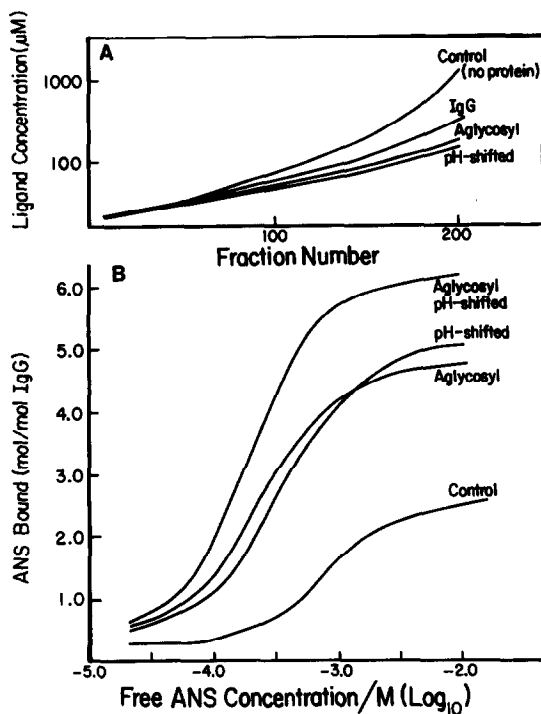


Figure 2: (A) Representative elution profiles for the dialysis of ANS in the presence of aglycosyl-IgG (150 μ M).
 (B) Typical Bjerrum plots of ANS:IgG interactions isotherms at 22 $^{\circ}$ (+1 S.E.M.).

TABLE II
Binding of ANS to Experimentally-Altered Human IgG

Sample	K _a (1/mol)	n*	a**
Control	5.2 x 10 ³	2	0.9
Aglycosyl IgG2 (pH 7.2)	2.3 x 10 ⁴	2 (Biphasic)	0.6
pH 3.5	4.1 x 10 ⁴	4 (Heterogenous curve)	0.5
pH 2.0 neutralized to 7.2	2.1 x 10 ⁴	2	0.6

* Absissa intercept value from Scatchard plot (ref. 25)

** Sipps (heterogeneity) distribution function derived from plots of log r/n-r versus log c (ref. 26). A value of 1.0 indicates homogeneity in binding site(s).

parabolas. With a least-squares fit analysis over short (20 point) sections of the data, a linear/log plot of these isotherms or Bjerrum plot (19) is derived and shown representatively in Figure 2B. By utilizing the Scatchard expression for this system (20), $v = \frac{\sum_{i=1}^2 n_i k_i C}{(1+k_i C)}$ the values for n_i, the number of binding sites and k_i, the protein:ligand K_a can be generated by a refined least squares fit program (15). Results of such an analysis of ANS binding by IgG2 are shown in Table II.

From these results it appears that the initial weak ANS interaction with native IgG is enhanced for the aglycosyl-IgG conformer without any apparent increase in the number of ANS binding sites. However, the acid pH conformer exhibits two new ANS binding sites, and to verify this conclusion, the fluorescence lifetimes of ANS bound to the various IgG populations were evaluated by time-resolved fluorimetry. Figure 3 shows a typically profiled fluorescence decay curve for ANS during interaction with IgG2 at pH 3.5. From this type of data, computer analyses resolves two discrete lifetime components of 15.0 and 2.43 nsec respectively. Similar results are obtained for IgG at pH 2.0 and for aglycosyl IgG at neutral pH as shown in Table III. Native IgG:ANS interactions show the probe to have lifetimes of about 13 and 3.5 nsec and buffer controls show that the enhanced T₂ for ANS when bound to the

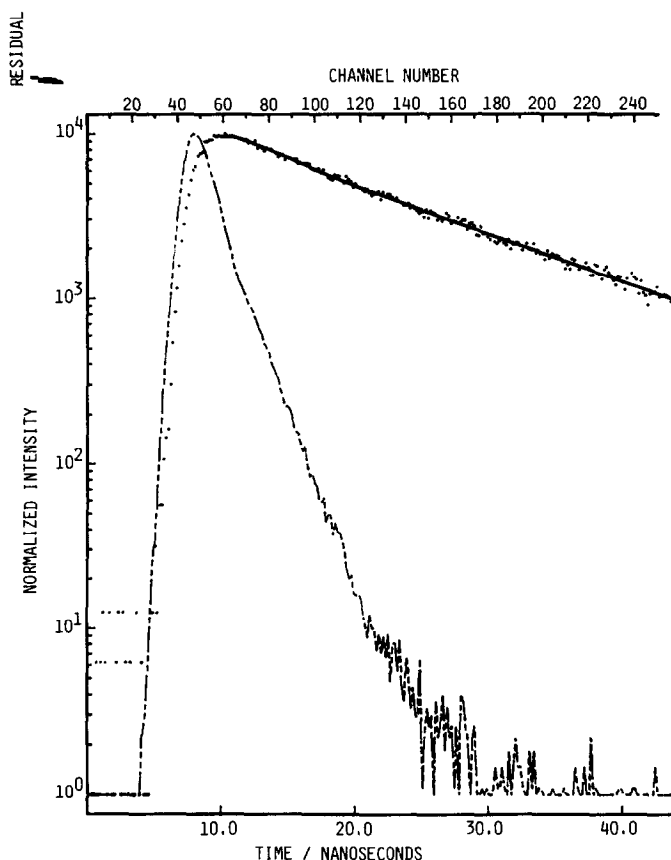


Figure 3: Fluorescence decay curve of 50 μ M ANS in a solution containing 25 μ M IgG in acetate buffer pH 3.5. The sharp pulse is a hydrogen lamp profile. The solid line is the best fit decay curve from the data (dots) with deconvolution of the lamp profile.

altered conformers is not a result of the low pH buffer effects. Interestingly, the environmental influences experienced by this probe when bound to altered IgG resemble those experienced by ANS in both dioxane and in 50% aqueous dioxane (Table III). These differences are statistically significant ($p \leq 0.001$) and, since quantum yield (Q) is directly proportional to the excited state lifetime of a fluorophore by: $Q = \frac{\tau}{\tau_0}$ (τ_0 is the excited state lifetime without nonradiative relaxation), then comparisons of Q for two samples A and B are valid since: $Q = \frac{\tau_A}{\tau_B}$.

Of interest in this study is the nature of the change in apparent hydrophobicity for aglycosyl-IgG compared with native IgG2. These changes

TABLE III
Fluorescence Lifetimes of ANS in Various Solutions of Human IgG

Sample	Lifetime* (nsec)	
	T ₂	T ₁
Normal IgG2 (Control)	13.0	3.75
Aglycosyl IgG2 (pH 7.2)	14.7	3.90
pH 4.8 IgG2	13.1	3.66
pH 3.5 IgG2	15.0	2.43
pH 3.5 reneutralized to 7.2 IgG2	12.9	2.84
ANS control (no IgG) pH 7.3		ca. 0.6
ANS control (no IgG) pH 3.5		0.6
ANS control (no IgG) in 50% dioxane/water		3.23
ANS control (no IgG) dioxane (neat)	11.6	--

* With IgG in solution, the fluorescence component with the longer decay time constant usually represents about 70% of the total fluorescence intensity.

evidently differ from those induced in the acid conformers where more probe molecules bind (Table II) and may reflect increased "exposure" of an hydrophobic region. It is likely that the changes occur in the hinge and C 2 regions since aglycosyl-IgG irreversibly loses some Clq anteceptor function (7,21) and since the Clq anteceptor is reversibly denatured in high ionic strength buffers (22) but irreversibly altered by low pH treatments (6,7). In the first two cases experimental modifications appear to be mild (7) whereas acid pH treatment provides a more highly denatured and more hydrophobic conformation. Such considerations are very important when interpreting studies on functional roles of immunoglobulin-linked heterosaccharides during antibody or immune complex metabolism (23,24) and in Fc receptor-mediated phenomena such as antibody-dependent cellular cytotoxicity (24).

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