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Note

The measurement of affinity between ligand and antibody using ligand-induced antibody fluorescence change

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Abstract

Computational analysis shows that affinity measurements using ligand-induced protein fluorescence change [*Carbohydr. Res.* **33** (1974) 377–382] give reliable data for K_as for values in the order of from *ca.* 10^2 M^{-1} to *ca.* 10^7 M^{-1}. © 1997 Published by Elsevier Science Ltd.

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In our original report [1] on the use of hapten-induced protein fluorescence changes to measure the binding constant (K_a) between proteins and UV-transparent haptens, seven reasons were given why the procedure is preferable over equilibrium dialysis, the foremost alternative method. We recently published [2] measurements of binding constants at different temperatures for four systems of homologous antigen-(monoclonal) antibodies and used van 't Hoff plots to derive enthalpies of binding. Of necessity, a few of the binding constants were measured at low temperatures (278 K and 286 K), and this significantly increased affinity. From this we have come to realize that for systems having affinity constants significantly above the range 10^6 M^{-1} to 10^7 M^{-1}, hapten-induced change of the antibody's tryptophanyl (or tyrosyl) fluorescence can lead to scattered data points and less certain results. Thus we found the practical limit of the method to lie with K_a's that are less than approximately 10^7 M^{-1}. Therefore, of the seven reasons previously given in favor of the method, the fifth one ("It is versatile: there is no restriction on the K_a value measured") must now be qualified. Carbohydrate antigens rarely—if ever—have binding constants at room temperature for their antibodies that significantly exceed 10^6 M^{-1}. Except for the very few measurements at low temperatures referred to above, that is certainly the case in all of our previous work. The reason for the method's reduced accuracy at high K_a values is illustrated here: For the equilibrium between an antibody site (s) and a ligand (lg) capable of binding to it we have: $s + lg \rightleftarrows s - lg$, and the equilibrium constant $K_a = C\,s - lg / C\,s_f \times C\,lg_f$, where C represents the concentration, and the index f stands for 'free' (i.e., unbound). When ν is the fraction of antibody sites occupied by ligand (ranging from 0 to 1), it follows that $K_a = \nu \cdot C\,s/(1 - \nu) \cdot C\,s \times C\,lg_f$ where $C\,s$ is the total antibody site concentration. Thus we have $K_a = \nu/(1 - \nu) \times C\,lg_f$ **(1)**, or $K_a(1 - \nu) = \nu / C\,lg_f$ and a plot of $\nu / C\,lg_f$ (y-axis) versus ν (x-axis) directly yields the

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K_a as the intercept on the y-axis. The fraction of antibody bound (ν) is easily measured at each addition of ligand: it is the change in the protein fluorescence at that point (ΔF), divided by the maximally attained change of antibody fluorescence at infinite ligand concentration (ΔF_{max}), when all antibody sites are of necessity occupied by ligand i.e., $\nu = \Delta F/\Delta F_{max}$. Now consider an antibody/ligand system having the relatively high $K_a = 10^7\ M^{-1}$. When half the sites carry ligand, i.e., $\nu = 0.5$, expression **1** becomes: $K_a = 1/C\lg_f$, or $C\lg_f = 1/K_a = 10^{-7}$ M. Antibody solutions employed in titrations using ligand-induced fluorescence change frequently have an A_{280} of 0.07, i.e., they are 6.67×10^{-7} M in antibody sites. At $\nu = 0.5$, the concentration of bound ligand is $0.5 \times C$ s, or 3.34×10^{-7} M. Thus the total added C lg at half-saturation of antibody sites would be 4.34×10^{-7} M, of which 77% is bound. The correction (subtraction of two large numbers to obtain the relevant $C\lg_f$) is large, and that data point could be inaccurate. Thus, in the plot to obtain the K_a, where the x-axis for ν goes from 0 to 1, it puts the more reliable points past $\nu = 0.5$ and towards $\nu = 1$, making the extrapolation for the K_a less accurate. We found that data points for K_a values near to, or in excess of $10^7\ M^{-1}$ can be poor. [1] Thus, when the K_a to be measured is near the same order of magnitude as the inverse of the antibody site-concentration this limitation can arise. Hence reducing the antibody site-concentration would appear as an obvious solution. However, our practical observation is that protein solutions of significantly lower concentrations than indicated above, can show unacceptably high scatter of their fluorescence intensity, probably due to a relatively high instrument background. It may well be that future improvements in instrumentation could obviate this problem.

A second point to be made is the following: it must be kept in mind that the term $C\lg_f$ stands for the *free* ligand concentration, not the added ligand concentration. As pointed out [1], the free ligand concentration is the ligand concentration added, minus the ligand concentration bound. The concentration of bound ligand is, of course, equal to the concentration of antibody sites that carry bound ligand, i.e., $C\lg_f = C\lg_{added} - \nu \cdot C$ s. The antibody solution in the cuvette is diluted by the addition of ligand stock solution. Consequently its fluorescence is corrected for this, as was stated in the original report. What was not pointed out is that in the cuvette the antibody's dilution by the addition of ligand solution also affects the concentration of sites, C s. Fortunately, it turns out that ignoring the latter dilution has no discernable effect [2] on the computation of the K_a. It is only mentioned here as a matter of record. Thus, the use of ligand-induced protein fluorescence change to determine affinity constants remains an outstandingly accurate, fast, economical (little protein and ligand is used) and simple method for proteins possessing K_a's for the hapten in question of from $\sim 0.5 \times 10^2$ to near $10^7\ M^{-1}$.

References

[1] M.E. Jolley and C.P.J. Glaudemans, *Carbohydr. Res.*, 33 (1974) 377–382.
[2] E.M. Nashed and C.P.J. Glaudemans, *J. Biol. Chem.*, 271 (1996) 8209–8214.

[1] When we omitted the few less reliable K_a values and considered all other K_a's anew from the data used for our recent report [2], we found that the enthalpy values derived from these new computations were generally comparable (from ca. −40 kJ of the lower oligosaccharides to −75–90 kJ for the higher ones), and thus the binding was again shown to be enthalpy driven. In addition, the unitary free energy did not vary much with temperature, as found before, again indicating that the unitary entropy change associated with the binding was relatively small, since $\delta(\Delta G_u)/\delta T = -\Delta S_u$. However the distinct correlation of $-\Delta S^\circ$ in the binding to the two differing sets of antibodies with the chain length of the oligosaccharides studied was no longer observable, and $-\Delta S^\circ$ generally varied upwards with chain length of the haptens studied, from around −60 J to around −170 J, and in one case to around −210 J. The effect of salt concentrations on the K_a values for the various haptens, indicative of either charged, hydrophilic or hydrophobic binding patterns, remained the same.

[2] When we compute values for K_a's at both low and higher affinity, with and without this correction, we found that this omission has virtually no affect on the final value obtained for the K_a (i.e., for titrations done at protein concentrations of from $5–7 \times 10^{-7}$ M, an error was found of 0.0018% for a low K_a of $2.14 \times 10^4\ M^{-1}$, and 0.1% for a K_a of $2.15 \times 10^6\ M^{-1}$). The reason for the K_a's insensitivity to this correction is that in the beginning of the titration the dilution of the antibody concentration by ligand addition is very small since the volume of the addition is very small, while at the end–when it is larger–the subtraction hardly matters, since the concentration of free ligand is already so very much larger than the antibody concentration.