The Unreliability of Estimates of Group Dissociation Constants

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If a molecule contains two groups that bind a common ligand, then to determine their group dissociation constants it is necessary (1) to assume that some property of the molecule is affected by the degree of ligation of only one of the groups, and (2) to determine how this property varies with the concentration of free ligand. When assignment of group dissociation constants made on this basis suggests that the degree of ligation of each group greatly affects the dissociation constant of the other, then the original assumption that the chosen property is completely unaffected by ligation of the second group is rendered doubtful. Dissociation constants of interacting groups can therefore not be reliably determined.

Standard treatments of multiple ligations, e.g. protonations, make use of the quantities defined (Adams, 1916) in Scheme 1. The molecular (titration, macroscopic, stoichiometric) dissociation constants K_1 and K_2 are first established by titration. It is then necessary to measure the ratio between the tautomeric forms II and III in order to establish the group (microscopic, site) constants K_A etc. When these are known, the ratio K_A/K_A' , i.e. the interaction between the groups, can be found. It is the purpose of this paper to point out: (1) that such an assignment depends on an assumption about the properties of the tautomeric forms II and III; (2) that this assumption is difficult to test and has seldom been tested or even critically discussed; (3) that it is unlikely to be valid if the groups interact strongly. Group constants of interacting groups can therefore not easily be reliably determined. It therefore seems preferable to express the properties of systems in terms of molecular rather than of group constants.

Argument

To determine group constants, such as K_A in Scheme 1, it is necessary first to determine the molecular constants K_1 and K_2 . As pL , i.e. $-\log[L]$, is raised, the properties of the substance change in a manner that is, in general, doubly sigmoid (Dixon & Tipton, 1973), with points of inflexion at pK_1 and pK_2 , if these pK values differ greatly. Even if they do not, the relation between any property and $[L]$ can be expressed in terms of three parameters, and these can be chosen so that two of them (1) have the dimensions of dissociation constants, (2) are independent of the property chosen, and (3) permit calculation of K_1 and K_2 (see also Dixon, 1974, 1975). It is therefore molecular constants that are determined in any such titration. The third parameter derivable from a titration is the

fraction a of the total change in property obtainable by removing two ligand molecules (i.e. passing from form I to form IV) that is obtained on removing the first (i.e. passing from form I to an equilibrium mixture of forms II and III). (If the property measured is the degree of ligand release, which is possessed by forms II and III equally, then the fraction a is 0.5; knowledge of this fraction a *a priori* makes titration of ligand binding particularly valuable for determining K_1 and K_2 .)

To determine the group constants it is then necessary to determine the ratio $[II]/[III]$ (which is independent of $[L]$). This is done by choosing a property whose change can be assigned solely to the deligation of one of the groups A and B. If the group is A, then it is assumed that forms II and IV of the molecule possess the property to exactly the same extent, and likewise forms I and III. For such a property the fraction a must represent $[II]/([II] + [III])$.

On this assumption the individual group constants can be calculated and hence the ratio K_A/K_A' . What does not seem to have received much attention is that a finding that this ratio differs greatly from unity throws doubt on the initial assumption. If the ligation of group B has such a great effect on the dissociation of ligand from group A, then it seems unwise to assume that it will have absolutely no effect on the properties of group A (more precisely, that it will have no appreciable fraction of the effect that ligation of group A itself would have).

The classic study of cysteine by Benesch & Benesch (1955) may be taken as an example. This can be represented by Scheme 1 where $-AL = -SH$ and $-BL = -NH_3^+$. They found that the molecular pK values of cysteine were 8.36 and 10.53. They assigned a value of 2.1 to the ratio $[II]/[III]$ on the assumption that forms II and IV had identical extinction coefficients at 230–240 nm, i.e. that the $-S^-$ form alone

Scheme 1. Double ligation

This scheme is essentially that of Adams (1916). A-B represents a molecule with two binding sites, A and B, for a ligand L.

Group constants:

$$K_A = \frac{[L][A-BL]}{[LA-BL]} \quad K_B = \frac{[L][LA-B]}{[LA-BL]}$$

$$K_A' = \frac{[L][A-B]}{[LA-B]} \quad K_B' = \frac{[L][A-B]}{[A-BL]}$$

Molecular constants:

$$K_1 = \frac{[L]([A-BL] + [LA-B])}{[LA-BL]} \quad K_2 = \frac{[L][A-B]}{[A-BL] + [LA-B]}$$

Relations:

$$\text{Hence } K_1 = K_A + K_B, \text{ and } 1/K_2 = 1/K_A' + 1/K_B'$$

contributed to this extinction and that the value of the extinction was unaffected by the state of ionization of the amino group. Benesch & Benesch (1955) specifically drew the attention of readers to the assumption. The value of the ratio they found led to pK values for the thiol group of 8.53 (pK_A) when the amino group was protonated and 10.03 (pK_A') when it was not. With such a large effect on the dissociation constant, it seems questionable to assume that the u.v. extinction of the -S⁻ chromophore (or indeed any other property of the -S⁻ group) is completely unaffected by protonation of the amino group. The doubt is reinforced by the finding of Benesch & Benesch (1955) that the absorption maximum is slightly shifted, from about 237 nm to about 231 nm, when one proton is added to form IV.

Discussion

Examples

Similar considerations apply to the determination of group constants in other substances. Thus Metzler & Snell (1955) found an even larger difference between pK_A and pK_A', namely 2.89, for 3-hydroxypyridine, again by measurements of extinction. Similarly

Raman spectroscopy has been used (see Edsall, 1965) to confirm the work of Benesch & Benesch (1955) on cysteine, and nuclear magnetic resonance has been used to determine group pK values of cysteine and glutathione (Jung *et al.*, 1972; Rabenstein, 1973). Since the amino and thiol groups of glutathione are spatially separated, and the assumption that they do not affect the n.m.r. signal of each other's neighbouring carbon-bonded protons leads to a difference between pK_A and pK_A' of only 0.24 (Rabenstein, 1973), the assumption seems reliable for this substance.

Polgar (1975) has found that the monoprotonated thiol-imidazole system of glyceraldehyde 3-phosphate dehydrogenase has 40% of the absorbance at 250 nm of the deprotonated form. He believes that the monoprotonated form is overwhelmingly -S⁻ ··· HIm⁺, and that the fall in absorbance from the unprotonated form is entirely due to the effect of HIm⁺ on the extinction of -S⁻. It is equally reasonable to assume, however, that not all the monoprotonated form is -S⁻ ··· HIm⁺ and that the extinction falls for this reason. Hence the ratio between the -S⁻ ··· HIm⁺ and -SH ··· Im⁻ forms remains unknown, as Shipton *et al.* (1975) pointed out for the similar cysteine-histidine system of papain and related enzymes.

The direction of errors

The direction of errors due to the failure of the assumption is not easy to predict. If, as is usually true, ligation at one group diminishes the affinity of the other for ligand, the properties ascribable to group A in its unligated form might be weaker in form II than in form IV, because the propinquity of L on group B might modify group A by changing its properties towards those of AL. Nevertheless, it is also possible that group AL may develop in form III some of the properties of form IV, because L is less tightly bound. Errors produced by failure of the assumption may therefore tend to cancel each other out, although they are unlikely to cancel out exactly.

Analogy

The problem of assigning group constants is one example of the problem of distinguishing between tautomeric forms. It has been elegantly solved in an example where the tautomerism is slow (and where it is also linked with proton dissociation): Fersht & Requena (1971) and Fersht (1971) resolved the pK of about 9 in chymotrypsin into pK values below 8 in one (inactive) tautomeric form and nearly 10 in the other (active) form by rapid measurements.

Towards valid determination of group constants

The problem of finding which of forms II and III is a reactive intermediate (the problem of kinetic

ambiguity) can sometimes be solved by arguments from analogous situations, etc.; see Jencks (1969) and Dunford (1974). Such solutions would seldom be precise enough to determine the ratio of [II] to [III] exactly, but they may show that one form predominates enough to allow assignment of a molecular constant overwhelmingly to a particular group.

More exact determination requires first a careful choice of the property to be measured. The ratio of the effect of ligation of group A to that of ligation of group B may be greatest if the probe to which the property is due is as close as possible to the ligand L when it is bound to group A. Thus if n.m.r. is used to study the protonation of a $-\text{CH}_2-\text{NH}_2$ group, it may be better to observe the resonance of ^{15}N (cf. Hawkes *et al.*, 1975) than that of the carbon-bound protons. In this respect the u.v. absorbance of the $-\text{S}^-$ group used by Benesch & Benesch (1955) seems as good a property as can be found. However, the conclusions obtained from the use of several different properties should be compared. If they give the same answer, then the assumption that ligation of group B has no effect on the change of property when group AL is converted into group A is supported. Finally, the assumption seems more likely to be valid if the values it gives for K_A/K_A' are close to unity, as for glutathione cited above. Nevertheless, no direct test of the assumption is possible; this is because the ratio [II]/[III] cannot be varied except by means as gross as changing the solvent, which may alter the properties of the individual forms.

Usefulness of molecular constants

Klotz (1974) pointed out one superiority of molecular over group constants for describing a system; fewer of them are needed. Certainly this represents a sacrifice of potential knowledge of the system (see Dunford *et al.*, 1974), but in view of the additional facts that the dependence of particular properties of

the system on ligand concentration is more closely related to molecular than to group constants (see Dixon & Tipton, 1973), and that measurements of group constants are inherently unreliable (the present paper), molecular constants appear to be much more useful.

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