

Accepted Manuscript

Ultra-Weak Reversible Protein-Protein Interactions

Arthur J Rowe

PII: S1046-2023(11)00042-9
DOI: [10.1016/j.ymeth.2011.02.006](https://doi.org/10.1016/j.ymeth.2011.02.006)
Reference: YMETH 2723

To appear in: *Methods*

Accepted Date: 7 February 2011



Please cite this article as: A.J. Rowe, Ultra-Weak Reversible Protein-Protein Interactions, *Methods* (2011), doi: [10.1016/j.ymeth.2011.02.006](https://doi.org/10.1016/j.ymeth.2011.02.006)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Full title: Ultra-Weak Reversible Protein-Protein Interactions

Author and Affiliation:

Arthur J Rowe
University of Nottingham
NCMH
School of Biosciences
Sutton Bonington
Leicestershire
LE12 5RD
UK

Contact details:

Tel: +44 115 9516156

Email: arthur.rowe@connectfree.co.uk / arthur.rowe@nottingham.ac.uk

Abstract:

Ultra-weak interactions ($K_d > 100 \mu\text{M}$) between proteins have in the last decade become an increasing focus of attention in cell biology, especially in relation to cell-cell interactions and signalling processes. Methods for their quantitative definition are reviewed. NMR spectroscopy plays a major role in this area, as it not only can define interactions as weak or weaker than 3 mM, but in favourable cases structural information concerning the complex can be yielded.

Free solution technologies mostly fail when addressed to such systems. The AUC has the highest practical capability, but evaluation of the data to yield K_d values is complicated by the presence of thermodynamic/hydrodynamic effects of a comparable order of magnitude. These effects can however be computationally removed by means of suitable algorithms, and K_d values of up to 50 mM can be characterised. The relative merits of velocity and equilibrium approaches are discussed, and both are shown to have particular advantages.

Keywords:

Analytical ultracentrifugation; interactions; NMR; protein-protein; ultra-weak

Ultra-Weak Reversible Protein-Protein Interactions

1 Introduction

1.1 The AUC as a probe for protein-protein interaction (PPI)

The Analytical Ultracentrifuge (AUC) has an established place in the development of protein science. Through the pioneering work of Thé Svedberg and colleagues, celebrated recently in a Symposium and published volume [1] it came to be understood that proteins are discrete, defined chemical species, and not merely polymers of narrow size distribution [2]. Without this insight an essential foundation stone for the construction of sequence and crystallographic methods would have been lacking.

Used in transport (sedimentation velocity, SV) mode, the homogeneity of a protein preparation could be analysed, the purification of a given protein might be monitored throughout its course, and the level of homogeneity of the final product estimated.

Used in equilibrium (SE) mode, an estimate could be made of the molecular weight of the protein species present in a preparation. With the realisation that the time to attain sedimentation equilibrium was inversely related to the square of the length of the solution column used [3] came the ability to complete experiments in this mode in a usable time scale (< 24 hours in most cases). The advent of modern instrumentation, and then of linked software, enabling AUC analyses to be performed on a routine basis, has resulted in a host of studies being performed using this technology, on protein systems of course, but also on other biopolymers and on inorganic systems. All these developments have been comprehensively reviewed, and the reader is referred to this literature [4-10].

It is not clear as to who first noticed that the AUC might be of use in characterising protein-protein (and other) interactions. It must have been clear quite early on however that when two different proteins (for example) were mixed together, then the sedimentation properties of that mixture might not always be simply the averaged sum of the properties of the individual proteins. An early example would be the identification of a complex, named 'actomyosin', when the extended polymer F-actin was mixed with the soluble protein myosin [11]. Almost all early work on interactions was performed in SV mode, and was generally semi-quantitative at best. The advent of the computer age brought with it not only online data capture [4], but immensely

more powerful offline data analysis software, making practicable especially the (then quite novel) use of non-linear fitting algorithms. The most significant of these latter developments was the writing of the NONLIN SE software, subsequently incorporated into the commercial software set of the Beckman XL-A instrument [4]. Since then a range of software for both SV and SE has been written, notably SEDFIT/SEDPHAT [12,13], ULTRASCAN [14,15], SVEDBERG [16], time derivative methods including SEDANAL and DC/DT+ [17,18,19] and LAMM [20]. The utility of this methodology has been well proven by the wide range of biological systems which have been studied over the years, and recent reviews of the results obtained have been written [21,22]. Interaction constants, K_a and from thence K_d values over the range $K_d = 40$ nM to 2 μ M have been defined by a range of physical methods [23]. The range for the AUC has generally been recognised to from around 100 nM to 100 μ M, although the newly available fluorescence optics do in suitable cases extend the lower end of the range considerably [24].

1.2 PPIs in Cell Biology

It has been recognised for some time that the number of PPIs in the living cell is very large indeed. The totality of these interactions defines the “Interactome”, which can be characterised – to an approximation – by methods such as the Yeast 2-Hybrid (Y2H) method [25] or by mass spectrometry-based proteomics [26]. However, the overwhelming majority of the interactions currently defined are of $K_d < 100$ μ M, and very few indeed have a $K_d > 1$ mM [27]. Very many ‘strong’ ($K_d < 1$ μ M) PPIs have been defined [28], and it is understandable that this situation would be appropriate in the context of the many PPIs involved in cellular structure. Yet it equally clear that many signalling and cell adhesion phenomena must require weak ($K_d < 100$ μ M) or ultra-weak ($K_d > 100$ μ M) interactions for their effective implementation.

1.3 Ultra-weak interactions: their definition, occurrence and importance

Clearly the definition of a level at which a PPI becomes ‘ultra-weak’ is arbitrary. Indeed it is far from clear as to the units at which the K_d itself should be measured. Almost universal custom calls for *molar* units to be employed, yet in general, as we will see below (2.2) interaction terms are often quoted in *mass* (per unit volume) terms, especially as it is evident that (in particular) the excluded volume *per gram* across a range of roughly similar proteins is approximately constant, whereas the excluded volume *per mole* is certainly not. Since consistency cannot be achieved, we

will simply follow custom and use molar units for K_d values, and mass units for the thermodynamic (virial) terms.

2. Methods for the characterisation of specific interaction

2.1 The variety of approaches

A plethora of techniques have been invoked for the study of homologous and heterologous interactions in protein, including techniques designed to define the nature of domain-domain interactions within a protein. For present purpose we will consider only those techniques which can (a) define K_d values at (or at least very near to) our chosen limit for ultra-weak interaction ($K_d > 100 \mu\text{M}$), and (b) be carried out on equipment widely found in biophysical facilities. On these criteria we do not consider the many optical methods such as e.g. FRET (Fluorescence Resonance Energy Transfer) [29], or BRET (Bioluminescence Resonance Energy Transfer) [31], powerful though such methods can be in specific application.

The four techniques most likely to be attempted in an attempt to characterise a weak interaction are (in no particular order) probably (i) Surface Plasmon Resonance (SPR); (ii) Isothermal Calorimetry (ITC); (iii) Nuclear Magnetic Resonance (NMR) Spectroscopy and (iv) Hydrodynamic Techniques -Analytical Ultracentrifugation. We will briefly consider each of these in turn as candidate techniques:

(i) SPR [30] is certainly capable under optimal use of detecting interaction at the $K_d = \sim 100 \mu\text{M}$ level, see for example the interaction between the cell-surface proteins CD2 and CD48 [44], for which consistent results between SPR and AUC were obtained. However neither published literature nor indeed manufacturer's specifications give any reason to suppose that interactions as weak as $\sim 1 \text{ mM}$ or weaker can be defined by this technique

(ii) ITC [32,33] is a powerful technique for the study of heterologous interactions, whether protein-protein or protein-ligand. A problem for our present purpose is that a relatively high concentration of protein is required: $30 \times K_d$ is considered to be optimal, whilst $10 \times K_d$ is the minimal concentration feasible [33]. For a 30 kDa protein in the sample cell, binding to a second protein with $K_d = 3 \text{ mM}$, a minimum concentration of 30 mM, equal to 900 mg/ml is required. Even before we consider the issue of the (higher) concentration of the second protein needed in the injection syringe, it is obvious that this technique cannot be applicable to ultra-weak interactions.

(iii) Nuclear Magnetic Resonance (NMR) Spectroscopy has in recent years started to realise the potential, which has always been there in theory [34], for detecting and interpreting inter- (rather than just intra-) protein resonance signals. A very full review of modern approaches has been given by Vaynberg & Qin [35]. In common with other optical/electromagnetic methods, the signal generated is a function solely of the physical interaction (through proximity) of the macromolecules concerned, and of no other property or properties of the system as a whole (see below). And – the sensitivity of the technique extends into the range of ultra-weak interactions (2.3).

The only obvious drawback is that a study of an interaction via NMR is only likely to happen if a detailed NMR investigation of a system is already being undertaken. This arises from the complexity and resource implications of the use of NMR technology.

(iv) Hydrodynamic Techniques – the AUC in particular – offer established and well-defined approaches to tackling interacting systems. The equipment required is widely available worldwide, it is accessible to those with good generic (as opposed to highly specialised) skills. As we will see, the sensitivity of the technique extends further into the ‘ultra-weak’ zone than any other. There are however problems of interpretation, which it has only recently become at all simple to solve (see Sections 2.2, 2.4 & 3)

2.2 A problem – two types of interaction in solution

Macromolecules in solution undergo multiple types of interaction. Interaction with solvent is clearly of importance, and this includes important topics such as solvation (hydration) and the binding of inorganic and other smaller ions and substances, such as sugars. For our present purpose, however, we will confine our attention to macroparticle to macroparticle interactions. These can be of two types. In the first, there is transient binding on a timescale which is significant in relation to the methodology being applied. The term ‘transient’ is used in recognition of the fact that by convention any binding which is indefinite in its timescale is referred to as “complex formation”. We will use the term “specific interaction” to denote an interaction of this type. We note that there need be no fine-structural implications in the use of the term: at it’s simplest it can mean just that A binds to B, rather than to C or other potentially interacting partners. There may or may not be one or more specific sites of binding which can be identified.

However, as will consider in more detail below (Section 3.2), there are also non-specific interactive forces between macroparticles to be considered. These are often referred to as ‘thermodynamic’ forces. They arise from the fact that a macroparticle

always occupies volume in solution, and very often possesses charge, both by way of net charge and site-localised charge(s). These non-specific interactive forces are always present to some degree, and are additional – although not necessarily in the simple arithmetical sense – to the specific interactive forces. The understanding of ‘colligative’ methods such as light-scattering, osmotic pressure, sedimentation equilibrium requires a consideration, and if need be the disentangling, of all interactions, specific and non-specific. By way of contrast, the ‘optical’ methods including NMR acquire signal exclusively from specific interaction, and are thus in principle easier to interpret.

2.3 NMR methods isolate specific interaction and can define structure

A very comprehensive review of the possibilities in this area has been given by Vaynberg and Qin [35]. To illustrate the power and potential of this technology, and also to illustrate further its potential for elucidating mechanisms in cell biology we refer to further work by Vaynberg, Qin and co-workers [27], the clarity and significance of which are best served by quotation: “. . . For example, the interactions between lymphocyte cell surface molecules are known to be multivalent and avidity driven. The affinity between each individual contacting pair is very low, between 2×10^{-4} and 10^{-6} M, and such low affinity is favorable for reversible cell-cell adhesion process” [36]. Many enzyme-substrate interactions are also weak, which is advantageous for broad substrate specificity and rapid turnover during catalysis [37]. Different combinations of transcription factor NF κ B subunits result in different weak affinities, which may differentially regulate gene expression [38]”. These authors [27], whilst thus illustrating the biological importance of ultra-weak interactions, draw attention to the very minimal amount of structural data available for systems of this type: and they present an analysis by NMR Spectroscopy of an ultra-weak interaction between the adaptors Nck-2 and PINCH-1, of importance in integrin-mediated focal adhesions [39]. Consequent to its discovery by Y2H analysis [40], Vaynberg, Qin and co-workers [27] define both the affinity ($K_d = \sim 3\text{mM}$) and structural nature of this complex (found to be Nck-2 SH3 domain to PINCH-1 LIM4 domain). This well illustrates the power and range of methods based upon NMR Spectroscopy.

2.4 AUC Methods: advantages and limitations

The AUC is, at heart, a weighing machine. The binding of protein A to protein B (or indeed of protein A to itself) results in the appearance of a new species in the solution, a species whose weight exceeds that of the monomeric species A, B . . . The fact that all species present in an analytical ultracentrifuge rotor are subjective to accelerative forces operating centrifugally (or in the case of particles of density lower than that of solvent centripetally) means that a change occurs in the sedimentation potential arising from the presence of the solute particles, as a result of the presence of new species. Here there is an important distinction to be made between specific and non-specific (thermodynamic) interaction. The sedimentation potential associated with a given species is linear in the concentration of that species, i.e. the potential per unit mass is constant, showing no ‘concentration dependence’. By contrast, as we will explore below (Section 3.2), non-specific interactions give rise to a chemical potential (or gradient of chemical potential) of a given species whose magnitude changes significantly with concentration.

It is often said that specific interactive forces between particles are attractive, whereas thermodynamic forces are repulsive. This is a simplification. As we will see later there is one term (the ‘third virial term’) in the series making up the algebraic series defining the thermodynamic terms which is normally attractive, but under ‘normal’ conditions it is of minor significance only.

The use of the AUC to define specific interaction is attractive. It is a ‘free solution’ technology, involving no surface interaction nor (usually) calling for any chemical modification of proteins or other macromolecules under study. The quantities of protein required are not large, and (at least when sedimentation equilibrium is the method of choice) samples used can be retrieved. For specific interactions which are of magnitude $K_d < 10 \mu\text{M}$, the thermodynamic interactions (which are of the approximate order of $K_d \sim 1\text{mM}$) can be safely ignored or roughly computed. But if one is to attempt to define interactions which are ultra-weak, where the strength of the specific interaction may be even weaker (e.g. $K_d \sim 10 \text{ mM}$) than the thermodynamic terms, then it is imperative to eliminate the effect of the latter, if the specific interactive properties of the system are to be described objectively.

Of course the AUC may be used to study the transport properties of a system (Section 4) and this gives an alternative way in which K_d values may be defined. Here, in a system in constant flux, it is the hydrodynamic properties of the system which now

must be considered. We discuss later (Section 5) the relative advantages and drawback of the two AUC methods (SE & SV).

3. Sedimentation Equilibrium Approach

‘Classical’ methods for data analysis to yield K_a values

The basis of the SE method is very simple. If we consider the equation of state which describes any system in which both accelerative forces leading to movement (usually centrifugally) of particles in solution are opposed by opposing diffusive forces, this gives a relationship for a single solute species (generally known as the Lamm equation):

$$(\partial c / \partial t)_r = -(1/r) \{ \partial / \partial r [s \omega^2 r^2 c - D r (\partial c / \partial r)_r] \}_t \quad [1]$$

where c is the solute concentration at given radial position r , ω the angular velocity of the rotor, s is sedimentation coefficient, D the translation diffusion coefficient [41]. Noting that in the equilibrium state the two inner bracketed terms must be equal at all radial positions in the cell, we can then derive an equation [41] which describes the equilibrium distribution of solute as a function of radius

$$c_r = c_a \exp[0.5 \sigma (r_a^2 - r^2)] \quad [2]$$

in which (c_a, r_a) denote the concentration (c_a) at a chosen reference position (r_a) and

$$\sigma = M(1 - \bar{v}\rho) \omega^2 / RT \quad [3]$$

where M is the solute molecular weight, R is the gas constant, \bar{v} is the partial specific volume of the protein, ρ the solvent density and T is the temperature (K). For a simple ‘molecular weight determination’ of a single macromolecular solute, conducted at a dilution such that all interactive (solute-solute) effects can be ignored, non-linear fitting of equation [2] to a $[c, r]$ data set suffices to yield an estimate for M . This is the original use of the SE method. With the advent of recombinant proteins, however, it is unusual for an investigator to be in ignorance of the molecular weight of a well-defined monomeric protein. Contemporary interest centres therefore on the information which can potentially be retrieved from studies carried out on solutions in which the various interactive terms, driven by solute concentration, can not be ignored, and indeed are themselves an object of study. We consider how SE (present Section) and SV (Section 4) can be utilised in practice, with particular reference to the ‘ultra-weak’ range of specific interaction ($K_d > 100 \mu\text{M}$) for which the importance in

cell biology has been stressed, and for the analysis of which a range of NMR methods have been successfully defined [27, 35].

Clearly a difficulty in the numerical analysis of an SE distribution appears when we have to consider c-driven interaction effects. Looking at equation [2], we see that the inclusion of any term in c in the RHS must cause the equation to become transcendental, i.e. the LHS (the c_r value) must appear in some way as a term in the RHS. Simple fitting by non-linear methods of a transcendental equation is not possible. We now explore briefly the ‘classical’ approaches which have been adopted over the years to cope with this problem, at least under conditions where the certain approximations can be tolerated [22], before considering how exact fitting methods capable of defining ultra-weak specific interactions ($K_d > 100 \mu\text{M}$) even in the presence of strong non-specific interactions, can be defined (3.2, 3.3).

3.1.1 Analysis via c-regression of ‘apparent’ molecular weight values

Possibly the oldest approach to the study of interaction has been via an ‘isotherm’ approach, in which the c-dispersion of a set of values of the ‘apparent’, usually weight-averaged molecular weight is analysed to yield estimates for a virial term (no specific interaction assumed) or for a K_a value (virial terms either assumed to be negligible or calculated on the basis of prior knowledge [42,43]). By ‘apparent molecular weight’ is meant the value resulting from ignoring the contribution of all interaction, specific or other, and using algorithms for analysis which are based on the properties of an ‘ideal’ macromolecular solute. Although hardly elegant, this approach can be perfectly effective, and capable of yielding estimates for K_d which are in the ultra-weak range. An example is the definition of the CD2 – CD48 interaction, where both an ISOTHERM approach and direct fitting using a computed value for the second virial term were employed to yield an estimate of $100 \pm 30 \mu\text{M}$ for K_d , an estimate which accorded well with SPR estimates on the same samples [44].

An approximate equation which can be used for fitting purposes [42], based upon the assumption of ‘first order in c’ is

$$(1/\sigma_{app}) = (1/\sigma)\{1 + 2(BM - K_a/M)c\} \quad [4]$$

in which B is the second virial coefficient (BM is the ‘second virial term’). This equation makes it clear that if we consider only the limiting slope of the c-dependence resulting from the totality of the interaction terms, then the specific interaction effect

(K_a) is numerically additive to the non-specific (or ‘thermodynamic’ terms, attributable to both excluded volume and charge effects). The two effects can thus never be resolved in this way: one or other will need to be either assumed or calculated to an approximation.

The ISOTHERM approach can be compared with the same method applied to the c-regression of the sedimentation coefficient (4.2). However, the errors involved the estimation of an apparent molecular weight will almost invariably larger than those involved in the estimation of a sedimentation coefficient. Overall, the only clear advantage which the approach possesses, and which it shares with SV, is that self-interaction of very narrow dispersions can be analysed, i.e. the method need not be confined to proteins.

3.1.2 Direct data fitting to both single and global data sets

The most widely employed method for the analysis of SE distributions is the NONLIN algorithm and program [42], for which a detailed examination of the error analysis necessary to ensure confidence in results obtained has been performed. We emphasise the necessity for the latter, since by general experience and agreement the error surface of the fit is complex. A distinctive feature of the NONLIN approach is the use of the concentration of the monomeric species at a reference radial position as a floated parameter. For a single, ideal, non-interacting solute, this of course results in an equation identical to eqn [2] above. But if specific self-interaction to give a dimeric species is assumed, then the result for a monomer-dimer system is given by:

$$c_r = c_{monomer,a} \exp[0.5\sigma(r_a^2 - r^2)] + K_a (c_{monomer,a})^2 \exp[2 * 0.5\sigma(r_a^2 - r^2)] \quad [5]$$

whilst the effect of ‘non-ideality’, designated here as Q and attributable to a single second (subtractive) virial term, results in the equation

$$c_r = c_{monomer,a} \exp[0.5\sigma(r_a^2 - r^2) - Q] + K_a (c_{monomer,a})^2 \exp[2 * 0.5\sigma(r_a^2 - r^2) - Q] \quad [6]$$

where

$$Q = BM(c_r - c_a) \quad [7]$$

A very full and accessible account of how this approach can readily be extended to higher order of interaction (e.g. monomer-trimer) is given in a Beckman booklet [45].

Global fitting of multiple experiments over a range of rotor speed and solute concentration has routinely been applied, and has –with care - obvious application where a system with a range of n-mers are present.

The ability of the NONLIN approach to analyse systems containing multiple interactions is impressive, and has found widespread application. It is clear however, by simple inspection of eqns [6] & [7], that if the BM term is floated then the equation is transcendental, and non-linear fitting cannot succeed. The computation of BM cannot hope to yield accurate values. It has long been appreciated that other than very close to the isoelectric point the computation of the excluded volume term – in itself involving assumptions concerning solvation – is insufficient. The charge term must in most cases be taken into account, and since a parallel investigation designed to elucidate the charge on the (various) species is unlikely to be conducted, another approximation is required.

None of these approximations, either in the equations used or in the computation of non-ideality terms, need be fatal if these terms are small in comparison to K_a . But it is simple to calculate that for typical proteins the non-ideality term is of the order of 1 mM in K_a : i.e. for a system in which $K_d = 10$ mM, the non-ideality term is an order of magnitude greater than K_a , and this means that the approximations which have to be made in its use result effectively in zero precision in K_a . Thus ultra-weak interactions can never be defined in this way.

At this juncture we may briefly mention that direct data fitting on a Lamm equation basis is also possible [12-20]. However, the situation with regard to the analysis of ultra-weak interaction may actually be even worse than the older approaches outlined above. This stems from the fact that the Lamm equation (eqn [2] above) incorporates both the sedimentation and the diffusion coefficients as parameters. Thus at finite concentration we are forced to define the concentration dependence of both of these. For sedimentation, the dependence is ‘hydrodynamic’ and a treatment is available [46]. Although this strictly involves the assumption that the primary charge effect can be ignored – a fair assumption under many conditions – one just could treat k_s (the c-dependence coefficient) as a purely empirical parameter, to be floated. However one would also need to float a c-dependence coefficient for diffusion, and here the theory is less well developed. And it seems doubtful on general numerico-analytical considerations that these two parameters could be floated in addition to all the other floated terms.

3.2 Direct data fitting with both K_a values and virial terms floated

The problem is simply stated, for present purpose we consider a monomer-dimer interacting system. Where there is non-ideality at a level which needs to be

considered (see above) then for each of the i ($=2$) two species present (monomer and dimer) we can write

$$c_r = c_a \exp[0.5\{\sigma_i/(1 + (\partial \ln(\gamma_i)/\partial c_r)c_r)\}(r_a^2 - r^2)] \quad [8]$$

where γ is the appropriate activity coefficient. We must then effect a summation over the species present, with the fraction (α) of the weight associated with each individual species computed from the K_a value via the usual quadratic equation derived via the Law of Mass Action. The value for σ_i in eqn [8] then becomes the weight average over monomer and dimer.. Finally, we must parameterise the inner bracketted term in eqn [8]. This can be done on any basis which is capable of being written out explicitly.

However, it matters little how one writes the final equation out: it will always be transcendental, and thus not readily amenable to non-linear fitting of data sets.

3.2.1 The INVEQ algorithm for direct data fitting

Clearly eqn [8] must be parameterised, i.e. expressed in terms of discrete meaningful parameters, before any algorithm for attempted fitting can be defined. That however does not in itself suggest any practical route to data fitting. That practical route has come from a simple observation of the nature of the basic equation to be tackled, and the benefits which come from a simple re-arrangement of the terms. That practical route is the INVEQ algorithm.

3.2.1.1 Definition of the INVEQ algorithm

The simplest possible approach to the parameterisation issue is to assume that the term in c , ($=\partial \ln(\gamma_i)/\partial c_r$) within the inner bracket in eqn [8] can be treated as single-valued and equal to 2BM for both species. This is often called the ‘Adams-Fujita approximation’ [47]. For cases where the excluded volume term is sensibly the same for monomer and dimer, and charge is not dominant, then it is likely to be reasonably accurate. It is important to stress however that one could write this term in any desired form, with any number of parameters. For instance, more than one 2nd virial term (BM) can be included, or indeed 3rd or even higher order terms can readily be incorporated. Of course it is important to be aware that however soundly based in theory such additional terms may be, the precision of real data may not be sufficient for them to be resolved in a fitting process.

The approach to the core problem of the transcendentality of eqn [8] and all directly derived formats, which has proved successful, comes from noticing that although eqn [8] is transcendental, its inverse ($r = f(c)$ rather than $c = f(r)$) is not:

$$r = \{(\ln(c_r/c_i) + 0.5*(\sigma_w/(1+2BMc_r))*r_i^2) / (0.5*(\sigma_w/(1+2BMc_r)))\}^{0.5} \quad [9]$$

This simple inversion of the basic equation (the INVEQ algorithm), without any approximations of the type noted above (typically ‘working to first order in c ’) has achieved separation of variables, and provides a simple basis for non-linear fitting in the standard way. It is of course very clearly not a new equation, merely an inversion without other modification of a standard equation which in its general form is universally accepted. And, as noted above, the non-ideality term can be written out in any way which can be justified on thermodynamic grounds: in no way is the ‘Adams-Fujita’ approach mandatory. These two points (a) avoiding approximations; and (b) maintaining generality of approach in writing out of virial terms; are stressed, inasmuch as they seem on occasion not to have been fully understood [50].

3.2.1.2 Implementation of fitting procedures

Eqn [9] should in principle be simple to fit using a non-linear least squares fitting engine. But the fact that the error surface, in this as in other approaches to SE analysis is found to be complex [49] means that care must be taken to minimise the danger of ‘false minima’ being identified as optimal solutions. Following accepted good practice, we provide error estimates for the data parameters, normally ± 0.0002 cm in r and 0.007 in fringe increment. The value of σ is fixed: this is essential if the baseline offset is to be floated, as it must be when interference optics are used. We then employ a 3-stage fitting procedure: (a) an interactive manual fit is performed to obtain initial estimates; (b) improvement of these estimates is then achieved by re-fitting (without statistical analysis but with input of data error estimates) using a ROBUST algorithm; (3) final re-fitting using the Marquardt-Levenberg algorithm is carried out with 500 iterations and a confidence interval of 68.4%. Finally histograms of the parameter estimates are plotted, from the table of parameter estimates generated by the last fit. In using this multi-stage fitting procedure we follow long-established good practice in this and in other fields. The particular ROBUST algorithm implemented within pro Fit™ is closely related to a Monte Carlo algorithm, inasmuch as random small increments in the fitting parameters are tested for ‘improvement being yielded’ on the current parameter set via a chi-squared criterion: however unlike an actual

Monte Carlo algorithm, truly random new guesses are not permitted. It is thus a form of search algorithm with a condition set for termination of the search, dictated by a cessation of the decrementing of chi-squared values. The ROBUST algorithm is appreciably slower than Marquardt-Levenberg, but much faster than Monte Carlo.

All these operations are carried out within the curve-fitting package pro Fit™ (Quantum Soft, Zürich) on an Apple Macintosh computer. Individual functions within the INVEQ package are designated by the prefix inveq. . . , e.g. inveq5a (for estimating BM, K_a); inveq5c (for estimating two 2nd virial terms, K_a); inveq5aBC (for estimating BM, CM, K_a); inveq6 (for 1:1 heterologous interaction).

Finally, it is always good practice to carry out a SV analysis prior to SE work. In the present case, this is needed to give an indication as to the presence of small amounts of irreversible dimer, which would be confused with reversible dimer in an analysis conducted at a single protein concentration. If small amounts are present, then performing several analyses over a solute concentration range and extrapolating to $1/c = 0$ (i.e. to infinite concentration) removes the effect of the presence of irreversible dimer on the estimate for K_a . An example of this is the estimation of the ultra-weak self-association of lysozyme [49], where the extrapolated value K_d has been found to be 17.5 mM, in a system where ~3% irreversible dimer had been found to be present. In principle, extrapolation to zero concentration will remove the effect of incompetent monomer on K_d values, although this has yet to be demonstrated. The presence and quantitation of incompetent monomer can be demonstrated more easily – as compared to that of incompetent dimer – by sedimentation velocity analysis, as it forms a ‘trailing boundary, behind and separated from higher s-value components.

3.2.1.3 Simulation and the definition of error in returned parameter estimates

Figure 1 illustrates the type of final parameter distribution which can be obtained, using simulated data. It is notable that especially when the conditions employed are ‘borderline’ (see below) then the distribution of parameter estimates tends to be very non-Gaussian. Indeed it may correspond to no clear distribution at all. Where there is a single, more-or-less Gaussian distribution of parameter estimates, then we invariably find that fitting a single Gaussian single function to the estimate returns an estimate for the standard deviation of the distribution which is close to that provided by the Levenberg-Marquardt method.

The results of simulated experiments have been given in some details recently [50]. In summary of these and other findings, for proteins of a size range which might overlap with that used in NMR studies (~10-30 kDa) it is found that :

- (i) fitting for BM, K_a requires 3+ mg/ml loading concentration
- (ii) fitting for BM, CM, K_a requires 10+ mg/ml loading concentration
- (iii) the limit on fitting for BM, K_a requires K_a to be no weaker than 50-100 mM {of course BM has a lowest possible limit, given by the volume term}
- (iv) attempting to fit for more than a single second virial term gives no stable answer for ultra-weak interaction
- (v) fitting for heterologous reversible interaction ($A + B$) is little different from the homologous dimerisation case, provided that the two monomers are of a similar order of size.

The most remarkable of these findings is that interactions which are weaker by at least an order of magnitude than those detected by NMR techniques [27,35] are possible. Indeed these K_a values approach a limit set by systematic error, ~100 mM, caused by inevitable lack of certainty in the σ value to be used in fitting, arising mainly from a limitation on knowledge of the precise value to be used for the partial specific volume of the protein(s) in the solvent used. This systematic error would be expected to be greater in (for example) a high salt buffer, because of protein-solvent interactions.

Nonetheless, experimental data is found to be in reasonable accord with expectation based upon simulation (3.3).

3.3 Practical application of INVEQ fitting procedures

Since they were first defined [53] INVEQ procedures have been used in a range of applications [51-53], with K_d values ranging from 67 – 106 mM for a fragment of choline binding protein, CbpA-R2 [51] to 101 μ M for Yellow Fluorescent Protein [55]. They have additionally been validated in practice work using a standard protein RNase A.

3.3.1 Results from a test system: RNase A

This protein has been employed as a test system for the application of INVEQ analysis [50]. It is well studied, well defined, and an earlier thorough analysis of its self-interaction properties at relatively high concentration has been published by the Minton Laboratory [56]. In this latter work, a system featuring a relatively long-

column equilibrium in a preparative ultracentrifuge a over a period of time (1 week) was employed.

The particular sample used [50] had essentially undetectable levels ($<0.3\%$) of irreversible dimer present. Sets of single SE experiments were therefore acceptable for the task of demonstrating whether or no the level of precision and accuracy suggested by simulation could actually be achieved with a real protein system. It was found that estimates for K_d and for 2BM over a solute concentration range of 3 mg/ml to 40 mg/ml varied only slightly (Table 1). Although there appears to be some tendency for progressive increase in K_d with solute concentration, it needs to be borne in mind that (a) the two higher concentrations are stated to have been analysed at lower rotor speeds, to retain the fringe pattern and are thus of lower precision, and (b) the issue of non-linearity between fringe displacement and concentration increment (as is given in the full Svennson equation [59]) can become significant in the presence of high solute gradients, unless very critical conditions of focus of the camera lens are met. Of course an obvious interpretation of the rise in estimated K_d with concentration would be that a very small amount of irreversible dimer, not detectable by SV analysis, was present. Other experiments [49] have suggested that a clear dependence is only established when 2-3% of such irreversible dimer species is present. However, these experiments were conducted over a lower solute concentration range. And it might be postulated that when solute concentrations greater than ~ 10 mg/ml are employed, then the sensitivity to the presence of very small levels of irreversible dimer is enhanced: but the fact is that the elevation of solute concentration actually removes (in the limit) the effect of irreversible dimer (see [49] Figure 2 insert). Overall, the conclusion reached from simulation, that 3-10 mg/ml is adequate for the simultaneous definition of the specific interaction term K_d and the thermodynamic interaction term 2BM, is validated when applied to a real system. There are also predictions which can be made concerning the behaviour of a soluble protein when solvent conditions are changed. The effect of lowering ionic strength has also been monitored [50], and in this case an extension to the simple model has been incorporated. This extension involved the incorporation of a third virial term (3CM) which simulation had suggested as being feasible for analyses involving protein concentrations of at least ~ 10 mg/ml [50]. Basic theory of particle-particle interaction would suggest that as ionic strength is lowered towards zero the repulsive forces between particles should increase due to loss of 'shielding' of charge [58],

whilst the second virial term should equally increase for the same reason. As regards the third virial term, limited theory [59] suggests that 3CM should be very small and of negative algebraic sign: and possibly more significant at low salt levels. The actual estimates for RNase A under a range of ionic strength conditions do accord well with these predictions (Table 2). The K_d values seen at the higher ionic strengths are consistent with the results reported by Zorilla et al [56]. These authors equally report that a single second virial term is not quite adequate to explain their results: however, given the differences in the experimental approaches, it is hard to press a comparison further.

The finding, by both simulation and experimentation, that it is feasible on a routine basis to define not only K_a/K_d values but also second and higher order virial terms, is novel; and has clear implications for our knowledge of the behaviour of macromolecules in solution. In some ways systems where specific interactions are ultra-weak offer real advantages for study, inasmuch as the whole system is predominantly composed of monomer species. Typically only a few % (e.g. 1-5) of dimer will be found for practical working concentrations. This means that the use of a single second virial term ($= B_{1,1}M$) (and for that matter a third term) is plausible. Indeed it has been shown by simulation [50] that if an additional second virial term (essentially a $B_{1,2}$ term), related to the mass fraction of dimer species, is actually included, its presence cannot be detected by analysis of SE data of normal precision. This has established beyond doubt that the algorithm employed is a valid treatment, the empirical definition of a second virial term not resting on any approximation which is significant in terms of known precision levels.

3.3.2 Results from selected systems of biological importance

That ultra-weak interactions exist and are of importance to an understanding of cell biology is now generally accepted. The importance of NMR Spectroscopy in clarifying this issue, and in making specific measurements of K_d values as weak as ~ 3 mM has been reviewed above (2.3). The peculiar disadvantage of using SE in the AUC lies in the fact that ultra-weak K_a values lie in the same range of magnitude as do the thermodynamic interaction effects. It is thus necessary to determine two terms – the K_a value and the 2BM value - simultaneously. To do this calls for an unapproximated form of the relevant SE equation to be used, as the ‘to first order in c ’ approximations employed in widely used algorithms cause these two terms to be additive and hence – as a matter of basic arithmetic – to be inseparable (3.1). The

INVEQ algorithm derives from the simple observation that the desired fit can be achieved by direct fitting of an unapproximated SE equation via its inverse.

In this section we consider two selected examples of published characterisation of weak/ultra-weak interactions defined via the INVEQ algorithm.

- (i) in the characterisation of choline binding protein A, the major adhesin of *Streptococcus pneumoniae*, the self-interaction of various regions of this protein were explored [51], with a view to establishing the possible role of a dimeric form in adhesion. Over a range of ionic strength the R1 peptide shows an ultra-weak interaction ($K_d = 9.5 \text{ mM}$), whilst the value determined for the R2 peptide, although consistent over the ionic strength range ($K_d = 83 \text{ }\mu\text{M}$, range of 3 estimations = $65\text{-}106 \text{ }\mu\text{M}$), may be too weak to be identified as an interaction at all, given unavoidable uncertainty in the value of the (computed) reduced floatational weight of the solute.
- (ii) in a study of the self-association of forms of the human adrenaline synthesising enzyme, PNMT, and the role of dimerisation in its activity [52], it was found that rapid SEC could achieve an apparent separation of monomer from dimer. This implied that the equilibrium state was only slowly established. INVEQ analysis of the equilibrium state in both the 'monomer peak' and 'dimer peak' samples showed whilst in the former the K_d values were weak ($K_d = 30\text{-}70 \text{ }\mu\text{M}$), in the latter they were ultra-weak ($K_d = 300 - 550 \text{ }\mu\text{M}$). Clearly this is counter-intuitive, in terms of slow equilibration (why would starting with dimer lead to a weaker association?) and this interesting finding has yet to be explained. The presence/absence of a his-tag was shown to have no effect on K_d values.

3.4 General conclusions

As noted, the evaluation of ultra-weak K_d values from sedimentation equilibrium data requires the thermodynamic interaction terms (at the level of the second virial term in most instances) to be either known or estimated, as these terms can become of an order of magnitude similar to or even exceeding K_a values under these conditions. Since the precision with which experimental virial terms can be computed is limited, for ultra-weak interactions it is vital that they be empirically estimated. In current practice, only the INVEQ algorithm provides the basis for doing this. Of course this in no way invalidates long-established methods (such as NONLIN) which require the input of some prior value for a second virial coefficient. Where $2BM$ is very small

numerically as compared to K_a in compatible units, probably for interactions stronger than $\sim 10 \mu\text{M}$, then it may actually be disadvantageous to float the (much) smaller parameter. However, if it does seem preferable to use a calculated or assumed value for the second virial term, then it is marginally preferable to do that within the INVEQ function, as the value inserted is then employed in a non-approximated manner.

It thus seems entirely practicable to assay for the presence of ultra-weak interactions, under conditions of solute concentration which have been defined [51-55]. What is currently lacking is any validation of estimates of K_a by independent (i.e. non-AUC) methodology, since as we noted earlier (2.1), alternative solution methods do not extend into this region of ultra-weak interaction. A degree of support for the validity of the ultra-weak K_a estimates comes from the fact that the estimates for the second virial terms which are yielded, and which are in line with expectation for 'globular' soluble proteins. Nonetheless, it would be very instructive to be able to compare an interaction in the $K_a = 1\text{-}10 \text{ mM}$ region detected by both AUC-SE using the INVEQ algorithm and NMR Spectroscopy.

4. Sedimentation velocity approach

4.1 Historical background

The earliest generally recognised approach to characterising reversible protein-protein interaction in the AUC came with the derivation by Gilbert and Jenkins [60] of a theory describing the radial distribution of a self-interacting macromolecular solute in a SV system, showing in particular that despite both monomeric and dimeric species being present, only a single 'boundary' would be discerned. That boundary would show slight asymmetry, but in the presence of appreciable translational diffusion, it would not be expected that this would be easily seen. The practical implications of this theory were explored by Gilbert & Gilbert [61], and a method suggested whereby K_a values could be estimated for suitable systems by plotting out isotherms of s vs c for a range of assumed K_a values, and locating an optimal fit to experimental data [62, 63]. This original method took no account of the inherent s - c dependence for both monomer and dimer species.

A particular issue which arises in the SV method is that the kinetics of the interaction must be considered. In the SE mode, one can (at least in principle) run the experiment until data scans become time-invariant, which means, by definition, that an equilibrium state has been reached. In an SV experiment the duration of the

experiment may be of the same order as the kinetics of the interaction, which makes simple isotherm fitting difficult or even impossible.

Modern usage has been focussed either upon modifications of the isotherms method, or on direct fitting of the data,, incorporating Gilbert-Jenkins theory into Lamm equation modelling (4.2 – 4.3). Although almost no results have been published in which K_d values in the ultra-weak range have been identified, the method does have potential in that area, and one example of an ultra-weak interaction is described below (4.2.1).

4.2 Analysis via c-regression of s_w values (isotherms approach)

Analysis of a set of SV data via $c(s)$ yields a weight-averaged s value, and providing the concentration dependence of s can be taken into account then it is simple to fit the data set (s_w vs c) to yield a K_a value. The c -dependence of the s value for each species in the interaction is characterised by the coefficient k_s in the equation

$$(s_c)^{-1} = (s^0)^{-1}(1 + k_s c) \quad [10]$$

As is noted below (4.3), a more exact formulation for concentration dependence is available, but eqn [10], in which k_s is treated simply as an operational parameter to be either fixed at some ‘average’ value or floated, is adequate for most purposes.

A detailed set of instructions for carrying out this procedure in SEDPHAT are available [63]. This procedure is also implemented in other software such as SEDANAL [19,64]. An elegant application of this software is provided in a recent publication by Zhao & Beckett [65].

4.2.1 Use of c-dependence theory

For solute particles where net charge is either absent or suppressed by the presence of neutral salt (~50+ mM), a general theory exists which describes the c -dependence of s in terms of the limiting slope of s vs c (k_s in eqn [10]), the swollen specific volume of the particle (V_s) and the maximal packing fraction of the particles ϕ_p – the latter being in the region of 0.4 for proteins

$$s_c = s^0(1 - (k_s * c - ((cV_s)^2 V_s (2\phi_p - 1)) / (\phi_p^2)) / (k_s c - 2cV_s + 1)) \quad [11]$$

where s^0 is the limiting s value at infinite dilution. This equation is exact over the full range of c up to 40% by volume – the maximum packing fraction, and the values given are in full agreement with rigorous fluid mechanics analysis for spherical particles [66]. It also predicts that eqn [10] above will hold to a good approximation over a moderate range in c .

It is thus very simple to write a program/function which will fit for the weight-averaged $s_{w,c}$ value as a function of c (our function is called MONOMER-DIMER). To illustrate its use, we offer an example which includes what is (so far as we can ascertain) the only published example to date of an ultra-weak K_d defined by an SV method. For purposes of fitting, we routinely fix the maximum packing fraction ϕ_p – the precise value is almost irrelevant – and float other parameters.

This study [67] was conducted on samples of a biologically active, narrow fraction heteroxylan. This highlights one immediate advantage of the SV method, inasmuch as it is simple to define and estimate $s_{w,c}$ values over a concentration range. The precise nature of the distribution is not even relevant. There is no way in which SE could sensibly be employed for this system. Selected isotherms are shown in Figure 2 for sample PO-5, at three different temperatures. At 30°C the interaction is ultra-weak, but even so, the regression line fitted is clearly distinguishable from the pure ‘monomer’ curve (Figure 2). The K_d values from the three temperatures enable an interpretation to be made concerning the nature of the interaction, which was considered to be most likely to be hydrophobic in nature [67].

4.3 Analysis via direct fitting of data sets

The consideration that direct fitting of data sets, either singly or globally, is unbiased and information-rich is a persuasive one [64]. Clearly fitting in this manner can be stable, well documented [65] and potentially yield information as to kinetic parameters, which is not attainable using isotherm-based methods. What is worrying however is that what we may call the “first order information” in a single data set relates to radially translational movement, with K_d effects making a fairly major impact on the dispersion of data points within the set, but c -dispersion effect being very much “second order”. Obviously this problem can be addressed by (global) analysis of multiple data sets, but this will not address effects which may be encountered arising from hydrostatic pressure. The excess pressure arising in the SE mode is essentially negligible, thanks to short columns of fluid and relatively low speeds. But in SV mode excess pressures of up to 200+bar can arise, and this brings us into a range where equilibria can be displaced, and oligomers can be depolymerised. Even granting that the total depolymerisation of myosin polymers at rotor speeds exceeding 30,000 rpm [68] is unusual, these effects cannot be neglected. Taking also into account that there are pressure effects on the solvent properties, not

universally simple to account for, and on the structure of individual proteins leading to changes in sedimentation rate, at least above ~30,000 rpm [69], and the danger of over-interpretation becomes obvious.

A genetic algorithm is incorporated in the ULTRASCAN software, and it has recently been claimed [70] that this mode of analysis is superior to that obtainable via SE. This is not obvious on the basis of the data presented. In particular, the levels of precision (in terms of confidence intervals at 95% confidence) are very high indeed for any SE estimates. Two K_d values are quoted, for two proteins, of 54.0 (29.1, 166.1) $\times 10^{-6}$ M and 22.7 (8.64, 63.0) $\times 10^{-6}$ M (confidence limits in brackets). For comparison, the precision of the estimate of K_d for the simulated system shown in Figure 1 at the same confidence limits would be 5.76 (5.28, 6.32) $\times 10^{-3}$ M, assuming the solute to be RNase A. Similar levels of precision were found for experimental data on this protein [50]. These levels of precision are comparable in magnitude to the limits quoted [66] for the SV method.

5. SE or SV: which approach is optimal?

Each method has its advantages, and the ultimate precision attainable in estimates for K_d are closely matched, as noted above. Thus in many cases the method of choice will be determined by the type of additional parameters which can be retrieved: thermodynamic parameters for SE, kinetic parameters for SV. Perhaps if all other things are equal, then SE would be the method of choice, as there are none of the pressure-related uncertainties which are present for SV analysis.

However, there are extraneous considerations which may well sway the decision. The SV method calls for more instrument time and very much more sample than does SE. The latter indeed can be semi-high-throughput, with up to 24 samples per day analysed, using multi-channel cells. But with the proviso that the samples should be free from oligomers, or an extrapolation to infinite time will have to be carried out.

SV on the other hand can cope with two situations which are outside of the scope of the SE method: the presence of modest amounts of higher s value species (oligomers, other proteins) need not affect the analysis, other than requiring a correction for actual concentration being lower than cell-loaded concentration; and as outlined above, narrow-fraction polymers can be analysed successfully. However, the situation with the heteroxylan polymers is in some way more favourable to SV analysis than would be an ultra-weakly interacting protein system. The intrinsically high k_s value provides a good 'baseline' against which small changes can be measured, i.e. it is always easier

to measure a change in a large base signal than in a small base signal. And the modest degree of polydispersity actually help to avoid problems arising from the extreme gradients in c which would be found with a monodisperse protein-based system.

6. NMR vs AUC – which approach is preferable?

NMR Spectroscopy has the clear edge when it comes to the ability to determine not only K_a values, but to investigate specific inter-domain interaction, and to offer the possibility of structural information regards the complex. Relatively high solute concentrations (up to 1 mM or more, equivalent to 30 mg/ml for a 30 kDa protein) are routinely called for and employed in NMR technologies, so no additional concentration of solvent is called for. Furthermore, new developments in the application of NMR methods continue to evolve, and show high promise for the precise definition of ultra-weak association in formation of complexes [71, 72]; and for ubiquitin-binding proteins a whole range of interactions reaching into the ultra-weak category have been defined [73]. The strength of AUC methods lies in their ease of use with only a routine, non-dedicated instrument being required; in a slightly enhanced sensitivity to interaction; in yielding information on thermodynamic interaction parameters; and in an ability to cope with proteins of molecular weight above ~30 kDa and with non-protein polymers. Furthermore, up to 24 samples (in multi-channel cells in an 8-hole rotor) can be analysed simultaneously. Clearly these two methodologies are complementary. Both can estimate K_a values, albeit with size limits for NMR. Neither can ever do what the other does in the way of yielding ancillary information. As regards ultra-weak interactions, these are (only) two widely available approaches which can yield results. As yet there does not seem to be a case where a K_a value has been estimated by both technologies: that will be of interest when it happens, but it will be very surprising indeed if there is not satisfactory agreement.

- [1] Macromolecular Bioscience Special Issue: Analytical Ultracentrifugation (ed Helmut Cölfen) 2010 Wiley Interscience
- [2] Svedberg, T., Pedersen, K.O. (1940) The Ultracentrifuge, Oxford University Press, London
- [3] Yphantis, D.A. (1964) Equilibrium ultracentrifugation of dilute solutions. *Biochemistry* 3 297-317
- [4] Schachman, H.K. (1992) Is there a future for the Analytical Ultracentrifuge? in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S.E, Rowe, A.J. and Horton, J.C. eds), Royal Society of Chemistry, Cambridge.
- [5] Schuck, P. (2007) Sedimentation Equilibrium Analytical Ultracentrifugation for Multicomponent Protein Interactions in *Protein Interactions* 289-316 DOI 10.1007/978-0-387-35966-3_10
- [6] Fu, H. (2004) Protein-Protein Interactions: Methods and Applications: Methods in Molecular Biology, 261
- [7] Lebowitz J, Lewis, M.S., Schuck, P. (2002) Modern analytical ultracentrifugation in protein science: a tutorial review. *Protein Science* 11 2067-2079
- [8] Kuroda, K, Kato, M, Mima, J, Ueda, M (2006) Systems for the detection and analysis of protein-protein interactions *Appl Microbiol Biotechnol.* 71 127-136
- [9] Brown, P.H., Balbo, A., Schuck, P. (2008) Characterizing protein-protein interactions by sedimentation velocity analytical ultracentrifugation. *Curr Protoc Immunol.* 2008 18:Unit 18.15.
- [10] Jones, S., Thornton, J.M. (1996) Principles of protein-protein interactions *Proc. Natl. Acad. Sci. USA* 93 13-20
- [11] Mommaerts, W.F.H.M., Parrish, R.G. J. (1950) Studies on Myosin I. Preparation and Criteria of Purity *Biol. Chem.* 188 545-552
- [12] Schuck P.(1998) Sedimentation analysis of noninteracting and self-associating solutes using numerical solutions to the Lamm equation. *Biophys J.* 75 1503–1512

- [13] Vistica, J., Dam, J., Balbo, A., Yikilmaz, E., Mariuzza, R.A., rouault, T.A., Schuck, P. (2004) Sedimentation Equilibrium Analysis of Protein Interactions with Global Impact Mass Conservatgin Constgraints and systematic Noise Decomposition. [www.analyticalultracentrifugation.com/.../SedimentationEquilibriumAnalysis. pdf](http://www.analyticalultracentrifugation.com/.../SedimentationEquilibriumAnalysis.pdf)
- [14] Demeler, B. (2004) Ultrascan - A Comprehensive Data Analysis Software Package for Analytical Ultracentrifugation Experiments in Analytical Ultracentrifugation: Techniques and Methods (eds, Scott, D.J., Harding, S.E., Rowe, A.J. RSC Publishing, London pp 210-230
- [15] Demeler, B. (2009) Ultrascan Version 9.9, release 1016, The University of Texas Health Science Center, Department of Biochemistry, <http://www.ultrascan.uthscsa.edu>
- [16] Philo, J.S. (1997) An improved function for fitting sedimentation velocity data for low molecular weight solutes. *Biophys. J.* 72 435-444
- [17] Stafford, W.F. (1992) "Boundary Analysis in Sedimentation Transport Experiments- A Procedure for Obtaining Sedimentation Coefficient Distributions Using the Time Derivative of the Concentration Profile." *Analytical Biochem.* 203 295-301
- [18] Philo, J.S. (2006) Improved methods for fitting sedimentation coefficient distributions derived by time-derivative techniques. *Anal. Biochem.* 354 238-246
- [19] Stafford, W.F., Sherwood, P.J. (2004) Analysis of heterologous interacting systems by sedimentation velocity: curve fitting algorithms for estimation of sedimentation coefficients, equilibrium and kinetic constants. *Biophys Chem.* 108:231-243.
- [20] Behlke, J., Ristau, O. (2002) A new approximate whole boundary solution of the Lamm differential equation for the analysis of sedimentation velocity experiments. *Biophys. Chem.* 95 59-68
- [21] The Jena Protein-Protein Interaction Website <http://ppi.fli-leibniz.de/>
- [22] Harding, S.E., Rowe, A.J. (2010) Insight into protein-protein interactions from analytical ultracentrifugation, *Biochem. Soc. Trans* 38 901-907
- [23] Arkin, M.R. Wells, J.A. (2004) Small-molecule Inhibitors of Protein Interactions, *Nature Reviews: Drug Discovery* 3 301-317
- [24] Laue, T.M., Anderson, A.L., Weber, B.W. (1997) in *Ultrasensitive Clinical Laboratory Daignostics*, Cohn E.J. Soper, S.A. eds, SPIE Proceedings, 2985 196-204

- [25] Fields, S., Song, O-K. (1989) A Novel Genetic System to Detect Protein Protein Interactions *Nature* 340 245-246
- [26] Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.L., Millar, A., Taylor, A., Bennett, K., Boutilier, K. (2002) Systematic identification of protein complexes in *Saccharomyces cerevisiae* *Nature* 415 180-183
- [27] Vaynberg, J. Fukuda, T., Chen, K, Vinogradova, O., Velyvis, A., Tu, Y. Ng, L., W. W, Qin, J (2005) Structure of an Ultraweak Protein-Protein Complex and its Crucial role in Regulation of Cell Morphology and Motility *Molecular Cell* 17 513-523
- [28] Nooren, I.M., Thornton, J.M. (2003) Diversity of Protein-Protein Interactions, *EMBO J.* 22 3486-3492
- [29] Truong, K., Ikura, M. (2001) The use of FRET imaging microscopy to detect protein-protein interactions and protein conformational changes in vivo. *Current Opinion in Structural Biology* 11 573-578
- [30] van der Merwe, P.A. Surface Plasmon Resonance
<http://users.path.ox.ac.uk/~vdmerwe/Internal/spr.PDF>
- [31] James, J.R., Oliviera, M.I., Carmo, A.M., Iaboni, A. David, S.J. (2006) A rigorous experimental framework for detecting protein oligomerisation using bioluminescence resonance energy transfer. *Nature Methods* 3 1001-1006
- [32] ITC XXX Reviews: <http://www.microcal.com/documents/reference-list/January%202009%20ITC%20XXX%20-%20ITC%20%20Reviews.pdf>
- [33] <http://www.endocytosis.org.techniqs/ITC.html>
- [34] Phizicky, E.M., Fields, S. (1995) Protein-protein interactions: methods for detection and analysis. *Microbiol. Rev.* 59 94-123
- [35] Vaynberg, J, & Qin, J. (2006) Weak protein-protein interactions as probed by NMR Spectroscopy. *Trends in Biotechnology* 24 22-27
- [36] Van der Merwe, P.A., Davis, S.J. (2003) Molecular Interactions mediating T cell antigen antigen recognition. *Annu Rev. Immunol.* 21 659-684
- [37] Schornburg, D., Schornburg, I. (2001) Springer Handbook of Enzymes, Edition 2 (New York: Springer)
- [38] Huxford, T., Mishler, D., Phelps, C.B., Huang, D.B., Sengchantha-langsy, L.L., Reeves, R., Hughes, C.A. Kornives, E.A., Ghosh, G. (2002) Solvent exposed non-contacting amino acids play a critical role in NK-kappaB/kappaBalpha complex formation. *J. Mol. Biol.* 324 587-597

- [39] Velyvis, A., Vinogradova, O., Wu, C. Qin, J. (2003) Structural and functional insights into PINCH LIM4 domain-mediated integrin signalling. *Nat. Struct. Biol* 10 558-564
- [40] Tu, Y., Kucik, F. Wu, C. (2001) Identification and kinetic analysis of the interaction between Nck-2 and DOCK180. *FEBS Lett.* 491 193-199
- [41] Scott, D.J., Schuck, P. (2005). A Brief Introduction to the Analytical Ultracentrifugation of Proteins for Beginners in *Analytical Ultracentrifugation: Techniques and Methods* RSC Publishing 1-23
- [42] Johnson, M.L., Correia, J.J., Yphantis, D.A., Halvorson, H.R. (1981) Analysis of data from the analytical ultracentrifuge by nonlinear least-squares techniques. *Biophys. J.* 36, 575-588
- [43] Taylor, W.R., Thornton, J.M. and Turnell, R.J. (1983) An ellipsoidal approximation of protein shape. *J. Mol. Graphics* **1**, 30-38
- [44] Silkowski, H., Davis, S.J., Barclay, A.N., Rowe, A.J., Harding, S.E. and Byron, O. (1997) Characterisation of the low affinity interaction between rat cell adhesion molecules CD2 and CD48 by analytical ultracentrifugation. *Eur. Biophys. J.*, **25**, 455-462
- [45] McRorie, D.K., Voelker, P.J. (1993) Self-Associating Systems in the Analytical Ultracentrifuge. Beckman Instruments, Inc.
- [46] Rowe, A.J. (1992) The concentration dependence of sedimentation. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S.E, Rowe, A.J. and Horton, J.C. eds), pp394-406, Royal Society of Chemistry, Cambridge.
- [47] Adams, E.F. Jr, Fujita, H. in J.W. Williams (Editor) *Ultracentrifugal Analysis*, Academic Press, New York, 1963, p.119
- [48] Winzor, D.R., Wells, P.R. Characterisation of weak protein dimerization by direct analysis of sedimentation equilibrium distributions: the INVEQ approach, *Analytical Biochemistry* (2007) **368**, 168
- [49] Rowe, A.J. (2005), Weak Interactions: Optimal Algorithms for Their Study in the AUC, in *The Analytical Ultracentrifuge: Techniques & Methods*, (eds, D J Scott, S E Harding, A J Rowe), The Royal Society of Chemistry, London pp 484-490
- [50] Ang, S, Rowe, A J (2010) Evaluation of the Information Content of Sedimentation Equilibrium Data in Self-Interacting Systems. *Macromolecular Bioscience* 10 798-807

- [51] R. Luo, Beth Mann, William S. Lewis, Arthur Rowe, Richard Heath, Michael L. Stewart, Agnes E. Hamburger, Siva Sivakolundu, Eilyn Lacy, Pamela J. Bjorkman, Elaine Tuomanen, Richard W. Kriwacki (2005) "Solution structure of choline binding protein A, the major adhesin of *Streptococcus pneumoniae*" EMBO Journal 24, 1
- [52] C.L.Gee, A.Nourse, A-Y Hsin, Q.Wu, J.D.Tyndall, G.G.Grunwald, M.J.McLeish, J.L.Martin, Disulphide-linked dimers of human adrenaline synthesising enzyme PNMT are catalytically active, Biochim. Biophys. Acta (2005) 1750 82-92
- [53] W.Zeng, H.E. Seward, A.Malnasi-Csizmadia, S.Wakelin, R.J.Woolley, G.S.Cheema, J.Basran, T.R.Patel, A.J.Rowe, C.R.Bagshaw, Resonance Energy Transfer between Green Fluorescent Protein Variants: complexities revealed with Myosin Fusion Proteins, Biochemistry (2006) 45, 10482-10491
- [54] A.Nyarko, K.Moshabi, A.J.Rowe, A.Leech, M.Botter, K.Shirasu, C.Kleanthous, TPR-Mediated Self-Association of Plant SGT1. Biochemistry (2007) 46, 11331
- [55] N.P.Mullin, P.Nicholas, A.Yates, A.J.Rowe, B.Mijmeijer, D.Colby, P.N.Barlow, M.Walkinshaw, I.Chambers, The pluripotency rheostat Nanog functions as a dimer. Biochem J (2008) 4, 227
- [56] S.Zorilla, M.Jiménez, P.Lillo, G.Rivas, A.P.Minton, Sedimentation equilibrium in a solution containing an arbitrary number of solute species at arbitrary concentrations: theory and application to concentrated solutions of ribonuclease, Biophysical Chemistry (2004) 108 89-100
- [57] Lloyd, P.H. (1974) Optical Methods in Ultracentrifugation, Electrophoresis and Diffusion, Clarendon Press, Oxford pp 86-89
- [58] Rowe, A.J. (2001) Probing Hydration and the Stability of Protein Solutions: a Colloid Science Approach. Biophys Chem 93 93-102
- [59] D.X.Liu, H.W.Xiang, Corresponding-States Correlation and Prediction of Third Virial Coefficients for a Wide Range of Substances, Int.J.Thermophysics (2003) 24 1667-1680
- [60] Gilbert, G.A. and Jenkins, R.C. 1959. Sedimentation and electrophoresis of interacting substances. II. Asymptotic boundary shape for two substances interacting reversibly. Proc. Royal Soc. A 253 420-437
- [61] Gilbert, L.M. and Gilbert, G.A. 1978. Molecular transport of reversibly reacting systems: Asymptotic boundary profiles in sedimentation, electrophoresis, and chromatography. Meth. Enzymol. 48 195-211

- [62] Schuck P. (2003) On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation. *Analytical Biochemistry*, 320 104-124
- [63] http://www.analyticalultracentrifugation.com/sedphat/isotherm_analysis.htm
- [64] Stafford, W.F. and Sherwood, P.J. (2004) Analysis of heterologous interacting systems by sedimentation velocity: Curve fitting algorithms for estimation of sedimentation coefficients, equilibrium and rate constants. *Biophys. Chem.* 108 231-243
- [65] Zhao, H. and Beckett, D. (2008) Kinetic Partitioning Between Alternative Protein-Protein Interactions Controls a Transcriptional Switch. *J. Mol. Biol.* **380**, 223-226
- [66] Rowe, A.J. (1992) The concentration dependence of sedimentation in "Analytical Ultracentrifugation in Biochemistry & Polymer Science" (ed. S E Harding, J C Horton & A J Rowe), The Royal Society of Chemistry, London
- [67] Patel, T.R., Harding, S.E., Ebringerova, A., Deszczynski, M., Hromadkova, Z., Togola, A., Paulsen, B.S., Morris, G.A. and Rowe, A.J. (2007) Weak Self-Association in a Carbohydrate System *Biophysical Journal* 93 741-749
- [68] Emes, C.H., Rowe, A.J. (1978) Frictional properties and molecular weight of native and synthetic myosin filaments from vertebrate skeletal muscle. *Biochim. Biophys. Acta* 537 125-144
- [69] Errington, N., Mistry, P., Rowe, A.J. (2002) Protein hydration varies with protein crowding and with applied pressure: a sedimentation velocity study *Progr Colloid Polym Sci* 119 58-63
- [70] Demeler, B., Brookes, E., Wang, R. Schirf, V., Kim, C.A. (2010) Characterisation of Reversible Associations by Sedimentation Velocity with Ultrascan. *Macromol. Biosci.* 10 775-782
- [71] Yu D, Volkov AN, Tang C. (2009) Characterizing dynamic protein-protein interactions using differentially scaled paramagnetic relaxation enhancement. *J. Am. Chem. Soc.* 131:17291-1729
- [72] Tang C, Ghirlando R, Clore GM. (2008) Visualization of transient ultra-weak protein self-association in solution using paramagnetic relaxation enhancement. *J. Am. Chem. Soc.* 130:4048-4056.
- [73] Hurley, H.H., Lee, S., Prag, G. (2006) Ubiquitin-binding domains. *Biochem. J.* 399 361-372

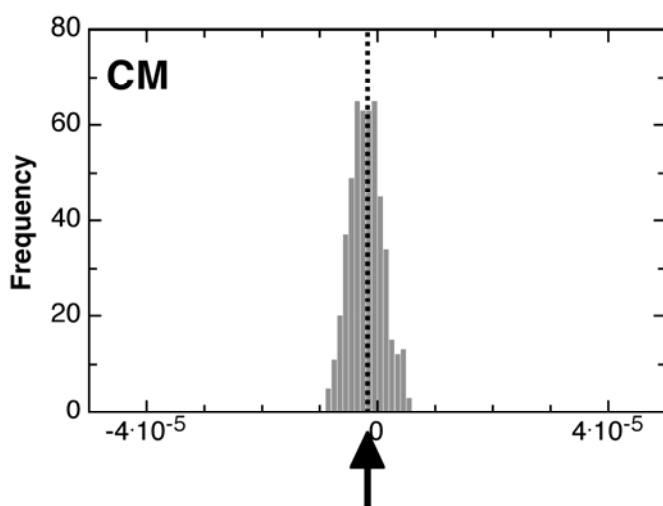
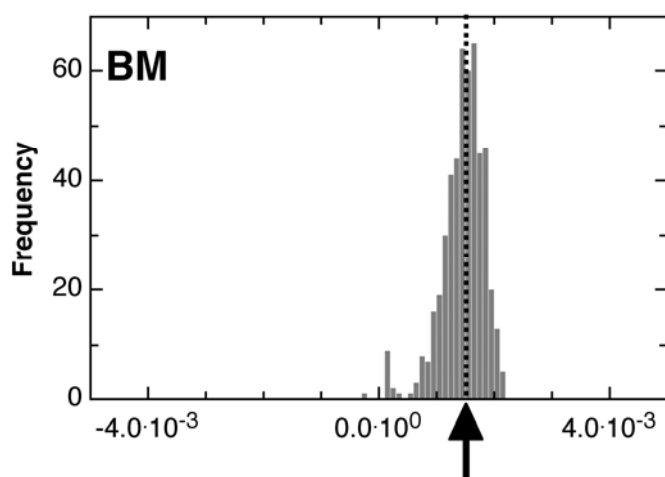
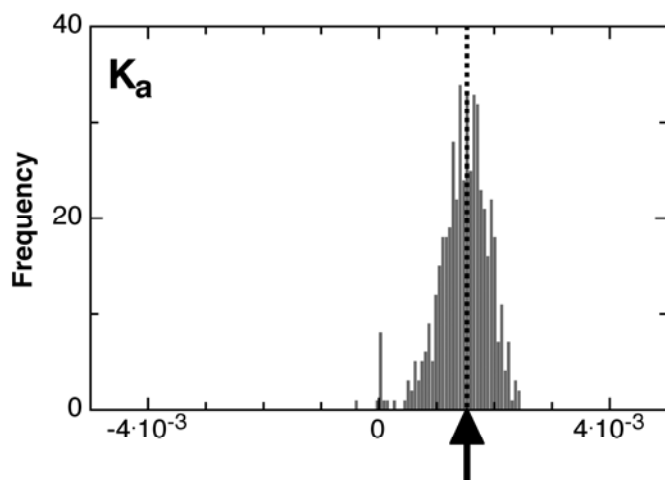
Legends to Figures

Figure 1

Illustrative plots of the distribution of estimated parameters (K_a , BM, CM) for a fit via the INVEQ algorithm to a simulated data set of fringe numbers vs radial position generated via the basic equation for sedimentation equilibrium. The fringe increment at mid-cell position was 30 fringes, equivalent to ~ 10 mg/ml for a ‘typical protein’, reduced floatational molecular weight (σ) value was 1.5, and the solution column extended from 6.9 cm to 7.1 cm. Normal random error (± 0.007 fringe) was added to the simulated fringe increment values. The fitting was carried out as described (see text). For error analysis, 500 fits were generated, with a 68.4% confidence interval. Vertical dotted lines denote the initial value of the parameter: vertical arrows below the x-axis denote the final estimate yielded. It is seen that in all cases these estimates agreed closely with the initial values used, though the parameter distribution for the third virial term (CM) indicates that resolution of the estimate from zero value is limited.

Figure 2

Plots of absolute and reduced sedimentation coefficient as a function of concentration for a sample (PO-5) of a narrow-fraction heteroxylan [67] at three different temperatures, 5°C, 20°C and 30°C. In panel A the ‘isotherms’ fitted to the data of absolute sedimentation coefficient ($s_{20,w}$) vs concentration are shown. In panel B the values used are given relative to the extrapolated s value. In both cases fitted ‘isotherms’ are generated by the function MONOMER-DIMER in pro Fit™. The dashed lines in panel B represent the regression expected for the monomer (small dashes) and for the dimer (larger dashes) by themselves. It is seen that both the extrapolated s value and its c -dependence are functions of the temperature. The interaction is seen to be weakest at 30°C, where a value of $K_d = \sim 600$ μ M approaches a limit at which the c -regression can be confidently distinguished from the line of zero interaction (i.e. $K_a = 0$).



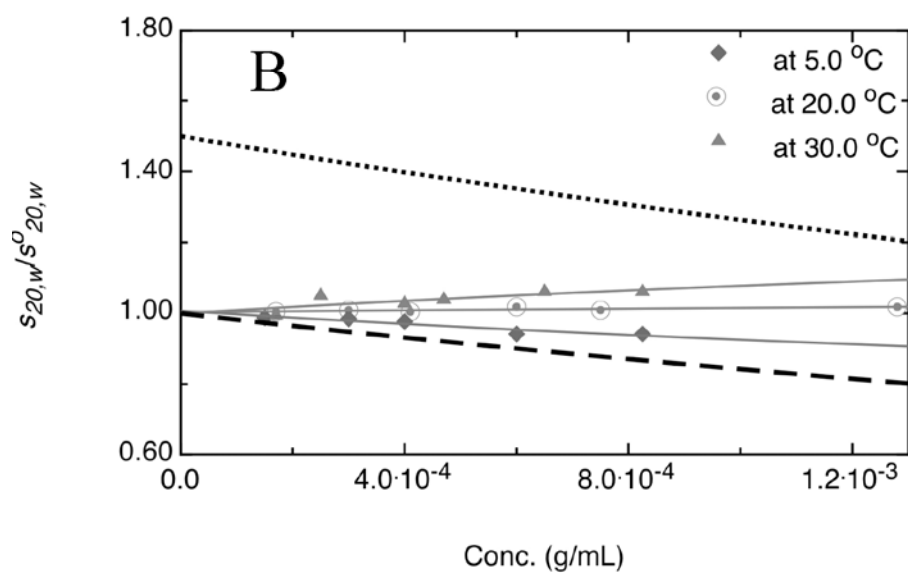
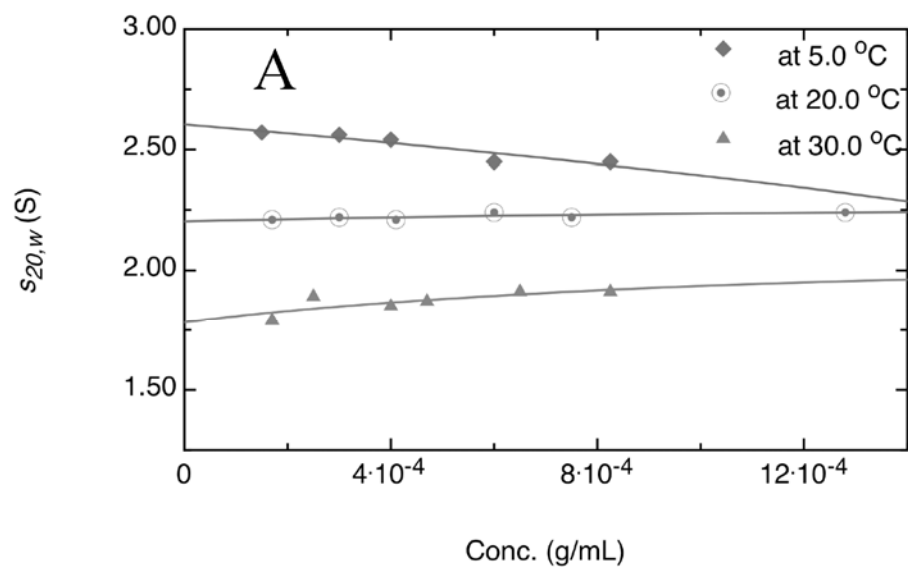


Table 1 Parameters retrieved from INVEQ fits to data from solutions of RNase A, at a concentration of 10 mg.ml, floating K_d , BM and CM, over a range of ionic strengths. In the absence of conventionally accepted units for the third virial term, (ml.fringe)² units are quoted, enabling comparison to be made with results from simulated data. No stable fit could be found for CM at I = 150 mM

Ionic Strength (mM)	K_d (mM)	2BM (ml.g)	CM*1e5 (fringe)⁻²
0.0	12.2	44.4	-9.00
3.0	15.7	21.1	-6.35
5.0	15.1	32.0	-6.76
9.0	15.7	21.1	-5.40
17.0	2.9	27.4	-0.77
150	4.7	6.6	ND

Table 2 Parameters retrieved from INVEQ fits to data from solutions of RNase A in PBS (pH = 7.4) at various solute concentrations, floating the two parameters K_d and BM

Concn (mg.ml)	K_d (mM)	2BM (ml.g)
3.0	3.34	6.20
6.0	5.63	5.31
10.0	4.70	6.64
20.0	7.83	6.64
40.0	8.69	13.28