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Method Article

Ligand binding studies by high speed centrifugal precipitation and linear spectral summation using ultraviolet–visible absorption spectroscopy

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A B S T R A C T

In ligand-protein binding experiments the major challenge is to separate bound from free ligand. Equilibrium and gel filtration separation techniques are often hampered by competition for the ligand and non-specific binding. Biophysical assays have attempted to circumvent this problem using titration calorimetry and spectroscopic methods. However, insoluble ligands require solvents that can overwhelm the discernible enthalpic changes of the protein and ligands. Spectroscopic methods are effective but may suffer from insensitivity (NMR) or the need for a lipid analog e.g., fluorescence and electron paramagnetic resonance. Our purpose is to compare the standard fluorescence assay to a technique we call high speed centrifugal precipitation. High speed centrifugal precipitation is suited to ligands that are insoluble in aqueous. The method permits separation of insoluble free ligand from that bound to the protein. The concentration of the each fraction can be precisely measured by absorbance spectrophotometry.

A second technique, linear spectral summation has been published for protein-ligand associations using fluorescence of labeled ligands [1]. Here, the method is altered for use with ultraviolet-visible (UV–Vis) absorption spectroscopy. If the ligand complex shows a shift in the peak absorption of >8 nm, the bound and free concentrations can be measured simultaneously. The composite spectra of the samples are fit by linearly scaling UV–Vis absorption spectra of pure bound and free components at each point.

- Ligand- protein binding kinetics is accessible with an ordinary spectrophotometer.
- Concentrations are accurately measured from molar extinction coefficients.
- The methods are ideal for lipid ligands that show absorption spectral peaks shifts in the bound and free states and/or are insoluble.

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Specifications Table

Subject area	• *Biochemistry, Genetics and Molecular Biology*
	• *Pharmacology, Toxicology and Pharmaceutical Science*
More specific subject area	*Ligand interactions*
Method name	*Linear Spectral Summation and High Speed Centrifugal Separation for UV–Vis Absorption Spectroscopy*
Name and reference of original method	*Linear spectra summation adapted from* [1] *Centrifugal Separation adapted from* [2]
Resource availability	*Deconvolution formulas are provided* [1]

Method details – high speed centrifugal precipitation

Ligand binding assays require separation of bound and free components either by physical or spectroscopic means. Assays with insoluble ligands present unique challenges with regard to non-specific binding, matrix interactions, and sensitivity [3]. High speed centrifugal precipitation capitalizes on the insoluble property of some lipid ligands in order to physically separate and measure bound and unbound portions of a complex mixture. High speed centrifugal precipitation is suitable for ligands that are insoluble in aqueous but are solvated when bound to protein. UV–Vis absorption and/ or fluorescence spectra of the bound and unbound portions can be measured directly. High speed centrifugal precipitation and fluorescence were performed on separate samples in our companion article [4]. Here, measurements from the same samples are compared by the different techniques.

Protocol background

High speed centrifugal precipitation requires that at least a portion of the ligand's spectral signature in (UV–Vis) absorption spectroscopy and/or fluorescence) is distinguishable from that of the protein. Also, the insoluble (free) ligand should be completely precipitated by high speed centrifugation. This can be tested with suspensions of the ligand in buffer. For example C6-NBD ceramide is suspended in 10 mM sodium phosphate buffer at 2 M excess above that used in the binding assay. After centrifugation at high speed $\sim$196,000 $\times$ g for 1 h, the absorbance of the supernatant matched that of the blank. All lipid was recovered in the precipitate as measured in an appropriate solvent such as methanol for which the molar extinction coefficient is known. Unlike other methods, no assumptions need to be made regarding spectral shifts during binding. All bound and free ligand can be accounted.

Materials and methods

High speed centrifugal precipitation can be done as multiple steps in a single tube or in multiple tubes in a single step. The former method saves reagents and the latter saves time. In our example the ligand, C6-NBD ceramide, is dissolved in an appropriate solvent, (e.g., dimethyl sulfoxide (DMSO), and added to the protein in separate tubes. The tubes contain progressively increasing concentrations (1 μM increments) of ligand but the protein solution is the same in each tube (e.g., 10 μM tear lipocalin). The total concentration of solvent should never exceed 2% of the total volume of the binding solution so that the properties of the buffer are not altered by the solvent to any appreciable extent. This is accomplished by adding small volumes of a highly concentrated stock solution (e.g., 1 mM C6-NBD ceramide in DMSO. The mixture of ligand and protein is allowed to equilibrate (e.g., 16 h at 34 °C in this example) before centrifuging the mixture. High speed centrifugation was performed in a Sorvall Discovery M150 (Thermo Fisher Scientific, Fairlawn, NJ, USA), with an S150AT rotor at $\sim$196,000 $\times$ g for 1 h at 34 °C. This rotor is hermetically sealed to vitiate evaporative losses. The concentration of the supernatant containing the protein-ligand complex, is then measured by UV–Vis absorbance

spectrophotometry and/or fluorescence. For the method employing separate tubes with multiple concentration additional advantages are accrued. First, the concentration of the precipitant (unbound) can be directly measured with UV–Vis absorption spectrophotometric measurement by redissolving in an appropriate solvent for which the molar extinction coefficient is known (e.g., methanol). Often, the molar extinction coefficient for a particular protein-ligand complex may not be known, initially. This method is particularly advantageous where the molar extinction coefficient of the ligand is known only for a physiologically incompatible solvent that is not suitable for the binding analysis. Second, the use of separate tubes permits cross-checking the concentrations of bound and free ligand with the total amount used at each point. Third, the method provides multiple data points for the calculation of the molar extinction coefficients of ligand complexed to the protein. The precise amount of ligand bound can be determined by subtracting the precipitated (free) ligand from the total amount of ligand added. In our case, experiments were repeated in triplicate and the means calculated. The total solvent concentration did not exceed 0.6%. The bound ligand (B) and free ligand (L) concentrations at each point were plotted and fit to appropriate binding models, (e.g. hyperbolic function or the Hill equation, $B/P = nL/(K_d + L)$ or $= L^x/(L^x + K_d)$ by standard nonlinear regression techniques (Microcal Origin, Northhampton, MA, USA), where P is the concentration of total protein to determine apparent number of binding sites (n), apparent dissociation constant K_d and Hill coefficient (x) for tear lipocalin and ligand.

Method validation

For method validation, high speed centrifugal precipitation assays using UV–Vis absorbance could be directly compared to those using fluorescence of C6-NBD ceramide. The apparent Kd's and n's obtained were further compared to a standard fluorescence assay prior to centrifugation. The same samples were used for all 3 assays. After equilibration, both UV–Vis absorbance spectra as well as fluorescence spectra were obtained on each sample before and after centrifugation. UV–Vis absorbance spectra were obtained in range of 300–600 nm with a Shimadzu UV-2401PC spectrophotometer, (Kyoto, Japan). Fluorescence spectra of C6-NBD ceramide were obtained with a Jobin Yvon-SPEX Fluorolog-3 spectrofluorimeter; at $\lambda_{ex} = 420$ nm, bandwidth 2 nm, and for emission, 4 nm. Raman, Raleigh, and background scattering by the solvent and protein were corrected using appropriate blank solutions. The relative intensities at 522 nm, were used to compute each bound label. An example of the binding curve generated by this method is shown in Fig. 1.
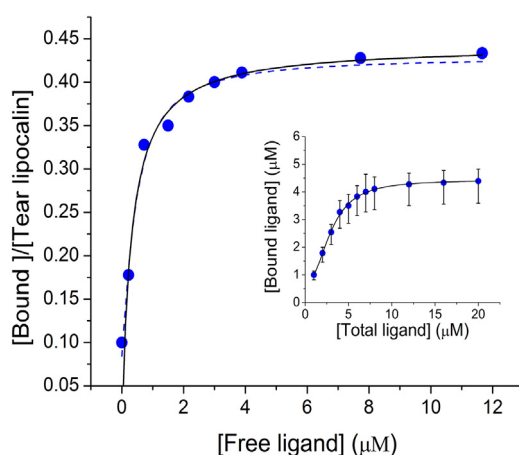

Fig. 1. High speed centrifugal precipitation binding assay of C6-NBD ceramide (●) incubated with 10 μM tear lipocalin at 25 °C for 30 min. Concentration of bound ligand-protein complex (supernatant) was determined by absorption spectra after centrifugal separation from unbound insoluble precipitant. Curve fit to a hyperbola (—) $K_d = 0.32$ μM, n = 0.44 and the Hill equation (- - -) $K_d = 0.29$ μM, n = 0.45. Inset: concentration of total versus bound ligand concentration.

Table 1

Results of Fitting of Various Assays for Binding of Tear Lipocalin and C6-NBD ceramide.

Assay Method, Temperature and Incubation Duration			Fitting Equation	Kd (μM)	n
High speed centrifugal precipitation	25 °C, 30 min		Hyperbola	0.32	0.44
			Hill	0.29	0.45
	34 °C, 16 h		Hyperbola	0.99	0.44
			Hill	0.81	0.40
Linear spectral summation	25 °C, 30 min		Hyperbola	0.06	0.45
			Hill	0.07	0.47
	34 °C, 16 h	Before centrifugation	Hyperbola	0.24	0.58
			Hill	0.19	0.51
		After centrifugation	Hyperbola	0.53	0.65
Fluorescence	25 °C, 20 min		Hyperbola	0.08	0.32
			Hill	0.08	0.32
	34 °C, 16 h	Before centrifugation	Hyperbola	0.05	0.45
			Hill	0.07	0.50
		After centrifugation	Hyperbola	0.17	0.50
			Hill	0.31	0.61

High speed centrifugal separation assay results are included in Table 1 and can be compared to results from linear spectral summation as well as those from standard fluorescence assays.

Table 1 shows consistent apparent stoichiometry(n) for the various methods. More variation is evident for apparent Kd measurements but differ at most by about one order of magnitude. This magnitude of variation for the same binding components is often reported between methods [3,5]. High speed centrifugal precipitation yields a higher apparent Kd (0.29–0.99) than the linear spectral summation or fluorescene without centrifugation. Centrifugation of samples prior to analysis by fluorescence or linear spectral summation also resulted in a higher apparent Kd than their counterparts with centrifugation (Table 1). It is possible that the suspension of ligands may have somehow contributed to the appearance of greater binding in some assays. Preliminary experiments showed that the unbound ligand showed exiguous fluorescence in aqueous, which was subtracted. Other interactions of ligand and protein at high concentrations in suspension may have been possible. Temperature did not seem to have an impact on the results from centrifugal precipitation.

Theoretical considerations

A common misconception is that centrifugation of unbound insoluble ligand somehow shifts equilibrium to drive lipid out of the complexed state with protein. However, the key principle to consider is that of a supersaturated solution of salt. The fraction of insoluble compound is governed by the solubility product of the substance (salt or lipid) at equilibrium. The location of excess insoluble substance, whether at the bottom of the tube or in suspension is irrelevant. This was easily demonstrated by performing repetitive high speed centrifugation of a suspension of protein with

Table 2

Effect of Duration of Centrifugation on Tear Lipocalin-C6-NBD ceramide Concentration.

Centrifugation time (hour)	[Bound C6-NBD ceramide] μM
1	4.0
2	4.4
3	4.8
4	4.7
5	4.5
6	4.2

excess ligand and measuring the UV–Vis absorbance at each time point. Extended centrifugation did not result in a consistent lowering of the absorbance of the supernatant containing the complex Table 2.

Method details – linear spectral summation

This method capitalizes on the common phenomenon that UV–Vis absorption spectral peaks of many ligands are often shifted when complexed to a protein. The change results in unique spectra for bound versus free ligand. The spectra of pure free and bound ligand can be scaled computationally and summed to fit the composite spectra that contains bound and free components. The scaled factors that result in the best fit reveal the amounts of free and bound ligand in any composite mixture.

Protocol background

Linear spectral summation is suitable as an analytic tool for a ligand binding reaction when the free ligand can be spectrally separated from the bound ligand. To avoid the need to normalize the protein contribution to spectra, only the portion of the bound spectra contributed by the ligand is analyzed. The advantage of this method is that no physical separation is required of the components of equilibrium mixture. Further no assumptions are made regarding the amount of free ligand at any concentration.

Validation of spectral shift

Ligands often show shifts in peaks in UV–Vis absorbance or fluorescence spectra with binding. Linear spectral summation is suitable when the shift of the spectral peak is greater than about 8 nm between bound and free ligand [1]. In our case, C6 ceramide complexed to tear lipocalin showed such a shift and was amenable to the technique but not C12 ceramide (Fig. 2).

The spectrum of the unbound ligand is obtained in the absence of protein. If the solubility is low the signal strength of dissolved ligand may be too weak to be useful. In this case, a suspension of the ligand can be used. Absorbance at varying concentrations of ligand in suspension must obey Beer's law. To obtain a pure bound spectra the protein is added in excess concentration compared to that of the ligand. In the current work both ceramides are insoluble in aqueous. Therefore, suspensions of C6-NBD ceramide at various concentrations were measured with UV–Vis absorbance to find the range where Beer's law was obeyed. The range of concentrations where absorbance and concentration were linear was adequate for the binding assay. In the case of C12-NBD ceramide the peak was too broad at the concentrations chosen to give a clear separation between free and bound ligand.
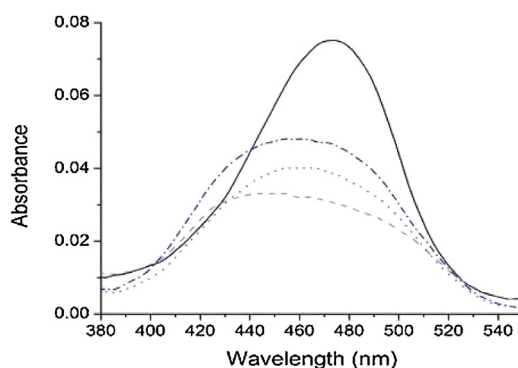

Fig. 2. Spectral features for linear spectral summation. Absorption spectral changes with C6-NBD ceramide and tear lipocalin complex verus insufficient spectral separation of bound and unbound C12-NBD ceramide. Unbound C6-NBD ceramide (3 μM) (− −), C6-NBD ceramide (3.4 μM) complexed with tear lipocalin (10 μM) (——). Unbound C12-NBD ceramide (3 μM) (● ● ●), C12-NBD ceramide (2 μM complexed with tear lipocalin (10 μM) (− ● −).

Materials and methods for linear spectral summation

Tear lipocalin, 10 uM, in 10 mM sodium phosphate at pH 7.3 was titrated by successive addition of C6-NBD ceramide such that the final concentration was incrementally increased by 1 μM with each addition. The absorbance spectra were measured. Following each addition of C6-NBD ceramide, the solution was mixed and allowed to equilibrate for 5 min. At the end of the titration experiment, the DMSO concentration did not exceed 2%. Bound C6-NBD ceramide spectra were obtained from spectra of the mixture of 2 μM C6-NBD ceramide and 10 μM tear lipocalin in 10 mM sodium phosphate at pH 7.3. The molar excess of tear lipocalin ensures that essentially all C6-NBD ceramide molecules are bound to the protein. The unbound spectra were obtained from 2 μM C6-NBD ceramide (without any protein) suspended in buffer. Composite spectra were deconvolved by fitting the scaled spectra of bound (2 μM) and unbound (2 μM) C6-NBD ceramide using a program created in LabView (National Instruments, Austin, TX). The program uses the "General linear Fit VI" subVI to fit each experimental spectrum to the linear summation of the component spectra. The fitting algorithm uses iterative reconvolution to minimize error. The goodness of the fit is judged by the normalized root mean square deviation [1]. The appropriate scaled factors for the bound and free spectra, respectively are calculated that result in the best fit. The bound and unbound lipid concentrations can then be determined from the product of the respective scaling factors and the known concentration of the pure bound and free input spectra [1]. The experiment was repeated 3 times and the means were calculated. An example of a composite spectra analysed in this manner is shown in Fig. 3.

Method validation and data analysis

The binding curves and parameters derived there in are shown in Fig. 4 and Table 1, respectively.

Linear spectra summation appears closely aligned to a standard fluorescence assay at 25 °C but appears discrepant from high speed centrifugal precipitation at the same temperature. Further, the data before and after centrifugation for linear spectral summation are also consistent and similar to that of high speed centrifugal precipitation. The variation in results did not seem correlated to method. The differences may constitute error often encountered in various binding assays performed with the same proteins and ligands. The apparent stoichiometry values (n) appear similar for each. For example, various methods gave apparent Kd's on the order of both 0.07 or 0.17 despite variation at both incubation temperatures and with both 20 min and 16 h incubation.

As mentioned above the apparent n is consistent by all methods but always lower than one. The appearance of a low n might imply a polymeric complex that includes multiple tear lipocalin molecules bound to the lipid. Past experience and molecular docking experiments (see companion article) have shown that tear lipocalin possesses a single intracavitary binding site. While both alkyl
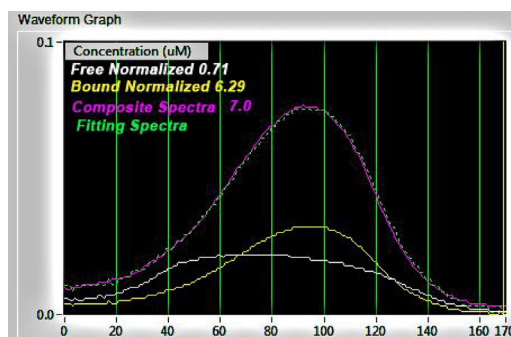

Fig. 3. Linear spectral summation for one concentration of total ligand (7 μM). The spectra for pure free ligand (white line) and pure bound ligand to tear lipocalin (yellow line) are scaled to fit the composite spectrum (purple line). The fitting spectrum is the green dashed line. The concentrations of the free and bound ligand are calculated from the scaling parameters and are normalized to the total ligand used.
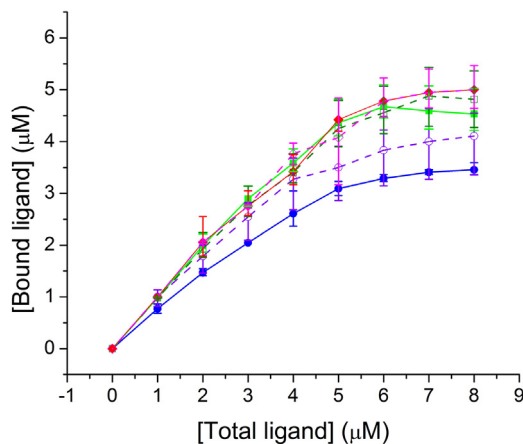
Fig. 4. Binding of C6-NBD ceramide to 10 μM tear lipocalin by different assay types. (-●-) high speed centrifugal precipitation assay at 34 °C; (-○-) high speed centrifugal precipitation assay at 25 °C; (-■-) fluorescent assay before centrifugation; (-□-) fluorescent assay after centrifugation; (-▪-) linear spectral summation before centrifugation; (-▪-) linear spectral summation assay after centrifugation.

chains of ceramide may bind to tear lipocalin, an n less than one could also be due to the presence of native ligands that occupy the binding sites and are not fully displaced by the tested lipid.

Discussion of theoretical considerations

Generally, UV–Vis absorbance spectroscopy is valid for solvated ligands that obey Beer's law. However, it is well known that suspensions of insoluble materials can be used if there is uniform dispersion and the particles are quite small [6]. Even solvated molecules at high concentrations may interact such that Beer's law is not obeyed [7]. To avoid these interactions, low concentrations of ligand were purposely used for linear spectral summation assays performed with UV–Vis absorbance spectroscopy. The resulting spectra are somewhat broad and test the limits of linear spectral summation. Higher concentrations generally produced sharper peaks and facilitate separation of free and bound spectra.

Acknowledgements

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