Rapid estimation of avidin and streptavidin by fluorescence quenching or fluorescence polarization

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Abstract

A new biotin–carboxyfluorescein conjugate has been presented in the accompanying study (G. Kada et al., Biochim. Biophys. Acta 000 (1999) 000–000) which contains ethylene diamine as a 4-atom spacer. This so-called biotin-4-fluorescein showed exceptionally fast and tight binding to avidin and streptavidin, and binding was accompanied by strong quenching. In the present study the specific quenching of ‘biotin-4-fluorescein’ was utilized to measure (strept)avidin concentrations (0.2–2 nM) by the extent of fluorescence quenching at 8 nM ligand concentration. Adsorption of (strept)avidin to the assay tubes was suppressed by inclusion of bovine serum albumin (0.1 mg/ml). Virtually the same specific response to avidin and streptavidin was also observed with commercial ‘fluorescein–biotin’, except that >10 h incubation times were required. The slow association of ‘fluorescein–biotin’ was attributed to the anti-cooperative binding which is due to the much longer spacer as compared to ‘biotin-4-fluorescein’. The third ligand tested in this study was ‘biotin-4-FITC’ which was analogous to ‘biotin-4-fluorescein’ except that carboxyfluorescein was replaced by the fluorescein isothiocyanate residue. Surprisingly, this probe was much less quenched by avidin but this was compensated by an exceptionally high fluorescence polarization in the avidin-bound state. In conclusion, the new ligand ‘biotin-4-fluorescein’ appeared to be the most general and convenient probe: quenching was most pronounced and linearly dependent on (strept)avidin concentrations, the dose response for streptavidin was almost the same as for avidin, and the association kinetics were fast enough to reach equilibrium within 30 min incubation time. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Avidin and streptavidin are widely used in bioscience and technology [1,2], thus specific measurement of (strept)avidin concentrations is frequently needed. Until recently, only radioligand binding methods [3,4] or a solid phase assay with a biotinylated marker enzyme [5] were sensitive enough to quantitate (strept)avidin in the low nanomolar and picomolar range. However, the good performance of
these assays is associated with laborious protocols and long incubation times.

In principle, a simple, fast, and sensitive fluorimetric assay for avidin was proposed a decade ago [6]. Biotin-4-FITC\(^1\) was synthesized as a specific fluorescent ligand, and at 4 nM biotin-4-FITC the change in fluorescence polarization could be correlated with 0.1–1 nM avidin concentrations. Unfortunately, the precision of the published assay was at best semiquantitative which explains why this method has never been used since.

Recently it has been shown that commercial ‘fluorescein–biotin’ is a valuable probe for the specific titration of biotin-binding sites in avidin and streptavidin [7], the major drawback being long assay times and moderate sensitivity (≥2 nM).

In the accompanying study [11] these limitations were broken with the new ligand biotin-4-fluorescein: it is the smallest of all biotin–fluorescein conjugates, quenching in the (strept)avidin-bound state is even more pronounced than seen with ‘fluorescein–biotin’, and ≥200 pM (strept)avidin could accurately be measured when titrating each unknown with this new ligand.

The present study aimed at replacing the titration method [7,11] by a single point assay in which (strept)avidin concentrations could be deduced from the fluorescence change at constant ligand concentrations. All three biotin–fluorophore conjugates named above were tested in this respect. The new ligand biotin-4-fluorescein emerged as the most general, sensitive, and convenient probe for both avidin and streptavidin. Moreover, inclusion of 0.1 mg/ml BSA was found not to perturb the new assay while solving the problem of adsorption losses at such low (strept)avidin concentrations.

2. Materials and methods

2.1. Reagents, buffers and stock solutions

P.a. grade materials were used as far as commercially available. Affinity-purified avidin, streptavidin, and D-biotin were obtained from Sigma. ‘Fluorescein–biotin’ was obtained from Molecular Probes. Biotin-4-fluorescein and biotin-4-FITC were synthesized as described in the accompanying paper [11]. All other materials were purchased from Merck. Buffer A (100 mM NaCl, 50 mM Na\(_2\)HPO\(_4\), 1 mM EDTA, pH adjusted to 7.5 with NaOH) was used throughout.

Stock solutions of avidin, streptavidin, and of the three fluorescent biotin derivatives were prepared and standardized as described before [7,11]. The effective concentrations of (strept)avidin tetramers in the stock solutions were defined as one fourth of the measured D-biotin-binding sites. Stock solutions with >300 nM (strept)avidin were stable at room temperature for 1 day. Any more dilute solution was prepared immediately prior to use.

Known avidin samples were titrated with the stock solutions of the biotin–fluorescein conjugates, and the effective concentrations of the latter were calculated from the consumption of fluorescent ligand up to the abrupt rise in fluorescence which was due to the appearance of free ligand [7–9,11]. Aqueous stock solutions of the fluorescent biotins were stable for one workday at room temperature and for one week at −25°C. At longer storage times under these conditions their effective concentrations were gradually lowered and recalibration with a known avidin sample was required. Concentrated DMSO stock solutions, however, could be stored at −70°C for 1 year without detectable change in the effective ligand concentration.

2.2. Measurement of (strept)avidin concentration by fluorescence quenching or fluorescence polarization

For a typical calibration curve, samples with a variable (strept)avidin content were mixed with invariant amounts of a biotin–fluorescein conjugate, resulting in 1 ml assay volume with 8 nM total ligand concentration. Samples were incubated at 25°C for the times given in the figure legends. For all experiments with ‘fluorescein–biotin’ and biotin-4-FITC, polystyrene assay tubes (4 ml) had been precoated with BSA as described before [11]. In the experiments with biotin-4-fluorescein the BSA precoating was

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\(^1\) See Fig. 2 in the accompanying study [11] for the molecular formulae of all biotin–fluorescein conjugates used in this study.
omitted but BSA (0.1 mg/ml) was included in the assay buffer.

Fluorimeter settings are given in the figure legends. Fluorescence polarization was defined in the usual way, i.e., $(I_{\text{par}} - I_{\text{orth}})/(I_{\text{par}} + I_{\text{orth}})$. In the case of biotin-4-FITC and ‘fluorescein–biotin’ the fluorescence intensities were calculated from the same data set simply by adding $I_{\text{par}}$ and $I_{\text{orth}}$.

3. Results

3.1. Measurement of avidin and streptavidin concentrations by fluorescence quenching of biotin-4-fluorescein

As shown in the accompanying study [11], quenching by avidin and streptavidin was most pronounced with the new ligand biotin-4-fluorescein. The same conclusion was drawn from a comparison of Fig. 1 with Fig. 2A and 3A. Moreover, quenching in Fig. 1 was linearly dependent on the concentration of (strept)avidin up to the distinct breakpoint which indicated stoichiometric binding of 8 nM ligand by 2 nM of (strept)avidin tetramers. The distinct minimum at 2 nM (strept)avidin was predicted by our previous findings that quenching was maximal at 4:1 ratios of ligand/(strept)avidin [7–9,11], whereas quenching was weaker at lower ligand/(strept)avidin ratios which explained the minor increase at >2 nM (strept)avidin seen in Fig. 1. In practical application the slight recovery of fluorescence at >2 nM (strept)avidin concentration makes it necessary to analyze several dilutions of an unknown sample to make sure that the (strept)avidin concentration is below 2 nM.

No attempt was made to evaluate the fluorescence

![Figure 1. Fluorescence quenching of biotin-4-fluorescein in the presence of avidin or streptavidin. Samples (1 ml) containing 8 nM biotin-4-fluorescein, 0.1 mg/ml of BSA, and the indicated concentrations of avidin (□) or streptavidin (○) were incubated for 30 min, and fluorescence was measured at 485 nm excitation (5 nm slit) and 525 nm emission wavelength (5 nm slit). The error bars were consistently smaller than symbol height and are omitted for clarity. Identical results were also obtained when using BSA precoating rather than inclusion of BSA in the assay buffer (data not shown).](image1)

![Figure 2. Fluorescence quenching (A) and fluorescence polarization increase (B) of ‘fluorescein–biotin’ in the presence of avidin or streptavidin. Samples (1 ml) with effectively 8 nM ‘fluorescein–biotin’ and the indicated concentrations of avidin (□) or streptavidin (○) were incubated in BSA-coated tubes for 13 h, and fluorescence was measured at 490 nm excitation (10 nm slit) and 525 nm emission wavelength (10 nm slit). In A, the error bars were consistently smaller than symbol height and are omitted for clarity.](image2)
polarization of biotin-4-FITC (compare Figs. 2B and 3B) because the polarization of such strongly quenched signals was not easily evaluated.

When working with biotin-4-FITC we discovered that the precoating of assay tubes (see Figs. 2 and 3) could be replaced by inclusion of 0.1 mg/ml of BSA in the assay buffer, with the same effect of suppressing (strept)avidin adsorption to the tube walls while not perturbing the assay shown in Fig. 1 (see legend to Fig. 1).

3.2. Measurement of (strept)avidin with commercial ‘fluorescein–biotin’

As can be seen from Fig. 2A, ‘fluorescein–biotin’ can serve as substitute for the new ligand biotin-4-FITC when correlating the extent of fluorescence quenching with (strept)avidin concentration. Measurement of streptavidin with ‘fluorescein–biotin’ had the additional advantage that fluorescence intensity continued to decrease above 2 nM streptavidin (Fig. 2, open circles), thus any fluorescence value corresponded to a single, unequivocal streptavidin concentration. The only (major) drawback of ‘fluorescein–biotin’ derived from its unusually slow association kinetics [11] which made it necessary to incubate > 10 h before analyzing fluorescence.

3.3. Measurement of avidin by fluorescence polarization of biotin-4-FITC

Biotin-4-FITC was only moderately quenched when binding to avidin (Fig. 3A, closed circles). As a consequence, it was easy to analyze the fluorescence polarization in the bound state (Fig. 3B, closed circles), in spite of significant light absorption by the polarizer unit. Moreover, the rise in fluorescence polarization did not halt at 2 nM avidin (when 1:4 complexes were formed with 8 nM ligand) but it continued to rise up until 8 nM avidin. This means that fluorescence polarization was much higher when ≪4 ligands were bound per avidin tetramer. The data scattering in the avidin-dependent polarization increase (Fig. 3B, closed circles) was much smaller than in the original publication of the same experiment [6], the most obvious explanation being prevention of avidin adsorption by BSA in the present study.

Surprisingly, the strong polarization effect is unique for the combination of biotin-4-FITC with avidin (Fig. 3B, closed circles). Streptavidin produced a much weaker response (Fig. 3B, open circles), and the fluorescence polarization of commercial ‘fluorescein–biotin’ was hardly affected by avidin or streptavidin in the interesting concentration range below 2 nM (see Fig. 2B).

In conclusion, biotin-4-FITC was suitable for measurement of avidin (Fig. 3B, closed circles), as well as of streptavidin (see Fig. 3A, open circles) when using fluorescence polarization for avidin and fluorescence quenching for streptavidin, respectively. In terms of kinetics, biotin-4-FITC showed the same favorable properties as its close structural analogue...
biotin-4-fluorescein, thus only short incubation times were sufficient to achieve equilibrium and stable fluorescence readings.

4. Discussion

4.1. Comparison of the three tested biotin–fluorescein conjugates

In principle, both biotin-4-fluorescein and ‘fluorescein–biotin’ were useful probes for quantitation of both avidin and streptavidin by fluorescence quenching (compare Figs. 1 and 2A), and the same applied to measurement of streptavidin with biotin-4-FITC (Fig. 3A, open circles). Practical application of ‘fluorescein–biotin’, however, was hampered by the need for > 10 h incubation time, whereas the other two probes required 15–30 min incubation times only.

Measurement of avidin with biotin-4-FITC, however, was only possible when monitoring fluorescence polarization (Fig. 3B, closed circles), rather than fluorescence quenching (Fig. 3A, closed circles). The differential response of biotin-4-FITC to avidin as compared to streptavidin seems to derive from specific details in protein surface structure. Thus the behaviour cannot be rationalized by a simple geometric model [10,11].

4.2. Advantages of the new ligand biotin-4-fluorescein

In the accompanying study [11] biotin-4-fluorescein proved to be the best ligand for specific fluorescence titration of biotin-binding sites in avidin and streptavidin samples. In this study the same biotin–fluorescein conjugate emerged as the most convenient fluorescent probe for ‘single point assays’.

Two criteria are relevant for selection of titration method [11] or single point method (this study). The titration method is indispensable for (strept)avidin samples with significant absorbance/fluorescence background or with impurities that modify fluorescence of biotin-4-fluorescein by weak, non-specific interaction. The second criterion is sample number. For very few unknowns it may be less effort to use the titration method: no calibration is necessary when using a freshly thawed aliquot of a standardized biotin-4-fluorescein stock solution. For large sample numbers, however, the single point assay represents the most convenient of all (strept)avidin assays, as long as interference by sample impurities is negligible.

In conclusion, the new ligand biotin-4-fluorescein has three general advantages. (i) The same ligand can be used when switching between titration method and single point assay. (ii) In each of the two methods, assay protocols and assay performance are equal for avidin as compared to streptavidin. (iii) The mechanism of biotin-4-fluorescein binding to (strept)avidin is much simpler than of any other known fluorescent biotin derivative: each ligand exclusively interacts with a single binding site region [11], in close analogy to the natural ligand D-biotin.

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References
