ORIGINAL PAPER

Iminobiotin Binding Induces Large Fluorescent Enhancements in Avidin and Streptavidin Fluorescent Conjugates and Exhibits Diverging pH-Dependent Binding Affinities

Marc P. Raphael · Catherine A. Rappole · Lynn K. Kurihara · Joseph A. Christodoulides · Syed N. Qadri · Jeff M. Byers

Abstract The pH-dependent binding affinity of either avidin or streptavidin for iminobiotin has been utilized in studies ranging from affinity binding chromatography to dynamic force spectroscopy. Regardless of which protein is used, the logarithmic dependence of the equilibrium dissociation constant (K_d) on pH is assumed conserved. However a discrepancy has emerged from a number of studies which have shown the binding affinity of streptavidin for iminobiotin in solution to be unexpectedly low, with the K_d at values usually associated with non-specific binding even at strongly basic pH levels. In this work we have utilized a Bodipy fluorescent conjugate of avidin and an Oregon Green fluorescent conjugate of streptavidin to determine the K_d of the complexes in solution in the pH range of 7.0 to 10.7. The study was made possible by the remarkable fluorescent enhancement of the two fluorescent conjugates (greater than 10 fold) upon saturation with iminobiotin. The streptavidin-iminobiotin interaction exhibited almost no pH dependence over the range studied, with K_d consistently on the order of 10^{-5} M. In contrast, under identical experimental conditions the avidin-iminobiotin interaction exhibited the expected logarithmic dependence on pH. We discuss the possible origins for why these two closely related proteins would diverge in their binding affinities for iminobiotin as a function of pH.

M. P. Raphael (✉) · C. A. Rappole · L. K. Kurihara ·
J. A. Christodoulides · S. N. Qadri · J. M. Byers
Materials and Sensors Branch, Naval Research Laboratory,
4555 Overlook Ave SW,
Washington, DC 20375-5320, USA
e-mail: raphael@anvil.nrl.navy.mil

Introduction

The collection of techniques that have utilized the pH-dependent binding affinity of avidin or streptavidin for iminobiotin is long and includes affinity binding chromatography, Raman spectroscopy, X-ray diffraction and mass spectrometry to name a few. Throughout, the logarithmic dependence of the equilibrium dissociation constant (K_d) on pH is assumed to be conserved whether using avidin or streptavidin. This pH dependence has been demonstrated repeatedly for avidin and iminobiotin regardless of whether both constituents were in solution or one was immobilized onto a matrix [1–7]. The same cannot be said for the pH dependence of streptavidin and iminobiotin. On the one hand, techniques which immobilize iminobiotin to either a polymer matrix or magnetic microspheres for streptavidin-based protein purification have been applied successfully since 1980 and are currently commercially available [8–10]. On the other, in the few published studies that have measured the binding affinity of iminobiotin for streptavidin in solution the resulting K_d was on the order of 10^{-5} M; a value far too high for efficient protein purification [11, 12].

We have addressed this discrepancy by measuring the dependence of K_d on pH for iminobiotin and a fluorescent conjugate of streptavidin in solution. The sensitivity of the fluorometric technique used allowed for measurements to be made at nanomolar concentrations of the protein, thus ensuring that the experiments were conducted in the dilute

limit. The unexpected result was that over the pH range studied, the binding affinity was nearly independent of pH. In contrast, when the study was repeated with an avidin fluorescent conjugate under identical conditions, a logarithmic dependence of K_d on pH was observed. Thus, unlike avidin and iminobiotin, the streptavidin-iminobiotin interaction does not appear to have an intrinsic pH dependence meaning that the observed pH dependence in affinity chromatography applications might be induced. This insight could have implications both for improved purification techniques based upon this receptor-ligand pair and, at a more fundamental level, for understanding how structural differences between streptavidin and avidin can lead to markedly different binding kinetics.

Materials and Methods

Oregon Green 514 streptavidin conjugate (OGSA) and BODIPY FL avidin conjugate (BA) were obtained from Invitrogen, Inc. (Carlsbad, CA, USA) as lyophilized powders and solutions were prepared according to the manufacturer's instructions. The average number of Oregon Green fluorophores per streptavidin protein was 5.0 while the average number of BODIPY FL fluorophores per avidin protein was 2.5. D-biotin and 2-iminobiotin were obtained from Thermo Fisher Inc. (Rockville, IL, USA) and Sigma-Aldrich Corp. (St. Louis, MO, USA) respectively as lyophilized powders and prepared according to the manufacturer's instructions. The buffers used were: phosphate buffered saline (0.1 M sodium phosphate, 0.15 M NaCl) for pH 7.0; Borate buffered saline (0.05 M sodium borate, 0.15 M NaCl) for pH 8.3; Carbonate-bicarbonate buffered saline for pH 9.3 (0.2 M sodium carbonate-bicarbonate, 0.15 M NaCl); Carbonate buffered saline (0.1 M sodium carbonate, 0.15 M NaCl) for pH 9.9 and 10.7.

Fluorometry experiments were carried out with the Horiba Jobin Yvon Fluoromax-3 spectrofluorometer using 4 mL quartz cuvettes. A temperature controlled stage ensured a constant temperature of 23 °C and a magnetic stirrer was utilized to mix the solutions during titration. To ensure that the measured equilibrium dissociation constants were independent of protein concentration, experiments were repeated for protein concentrations ranging from 0.16 µg/mL to 3.2 µg/mL and found to be consistent. A concentration of 0.32 µg/mL was determined as optimal as it was a high enough concentration to give an acceptable signal to noise ratio in the fluorometry experiments but low enough so that only small volumes of (imino)biotin solutions were required for titration (less than 30 µL), thus avoiding dilution effects on the measured fluorescent intensities. The pH dependence of the binding kinetics of (imino)biotin with BA and OGSA were studied within the

pH range of 7.0 to 10.7 with no evidence of photobleaching. In attempts to go to lower pH levels OGSA exhibited pronounced photobleaching and thus the more acidic pH levels were not deemed reliable for that protein conjugate.

Results

Figure 1a and b show the enhanced fluorescence of OGSA and BA upon the addition of excess biotin at pH 8.3 and pH 9.3, respectively. For both figures the (strept)avidin concentration was 5 nM and the final biotin concentration was 3 µM. The BA fluorescence intensity is enhanced by a factor of 8.8 upon saturation while the OGSA is enhanced by a factor of 10.8. The pH dependence of this enhancement is shown in Fig. 2a for OGSA (triangles) and BA (squares) saturated with biotin and in Fig. 2b for OGSA and BA saturated with iminobiotin. The figure plots I/I_o versus pH where I and I_o are the measured peak intensities with and without (imino) biotin present in solution. OGSA saturated with iminobiotin exhibited a monotonic decline in its fluorescent enhancement as a function of increasing pH while the fluorescent enhancement of BA by iminobiotin was especially pronounced above pH 9. Unconjugated Oregon Green 514 and BODIPY FL were found to have less than a 10% variation in their fluorescent intensity over the same pH range thus confirming that the primary contribution to the pH
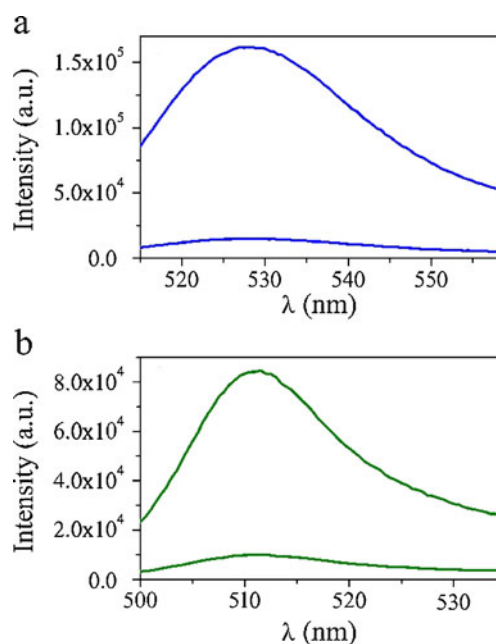

Fig. 1 Emission scans of the (*strept*)avidin conjugates before (*lower data*) and after (upper data) adding a saturating amount of biotin. **a** OGSA at pH 8.3 and **b** BA at pH 9.3
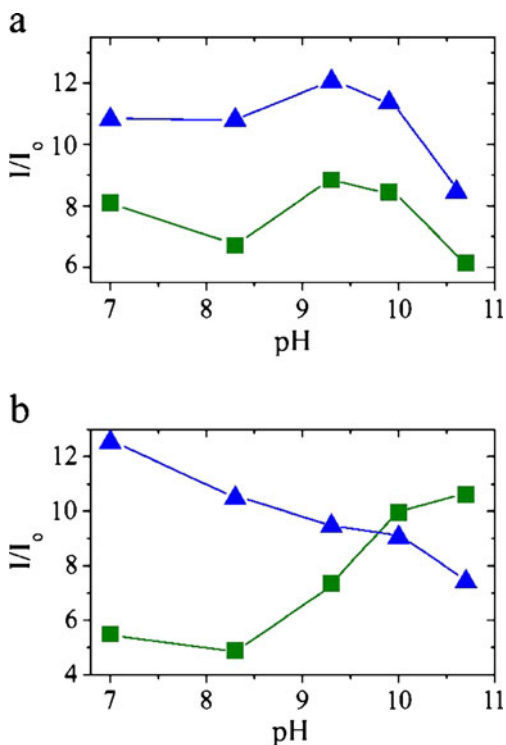
Fig. 2 The pH dependence of the fluorescent enhancement upon addition of saturating amounts of (imino)biotin. I is the intensity of OGSA (*triangles*) or BA (*squares*) in the presence of a saturating amount of biotin or iminobiotin. I_o is the intensity without biotin or iminobiotin present. **a** biotin **b** iminobiotin

dependence shown in Fig. 2b comes from the association of iminobiotin with (strept)avidin.

For each pH studied, a standard curve was constructed by titrating the OGSA or BA solution with biotin and plotting the fraction of maximum fluorescence enhancement, θ, versus biotin concentration. Here θ is defined by:

$$\theta = \left(F_{528(obs)} - F_{528(initial)}\right) / \left(F_{528(final)} - F_{528(initial)}\right)$$

where $F_{528(initial)}$, $F_{528(final)}$, and $F_{528(obs)}$ are the measured fluorescent intensities of the OGSA at 528 nm with no biotin present, fully saturated and at intermediate concentrations, respectively. The same equation was applied to the BA data but with the measured intensities taken at 510 nm.

Figure 3a and b show the results of titrating OGSA and BA with biotin at pH 9.3. As denoted by the guidelines in the figures, the saturation of the (strept)avidin solution occurred when the biotin concentration equaled four times that of the protein—consistent with four binding sites per (strept)avidin molecule. The observed 4:1 ratio confirmed that the amount of free biotin in solution was negligible until saturation of the (strept)avidin binding sites occurred. Also evident from Fig. 3 is the nonlinear dependence of the fluorescent enhancement as a function of bound biotin. As

shown by the guidelines, a biotin:(strept)avidin ratio of 2:1, or half-occupancy, occurred at a θ value lower than 0.5, typically between 0.3 and 0.4.

Figure 4a and b show the titration of OGSA and BA with iminobiotin at pH 10.0. For each pH, the θ value for half-occupancy was determined from the standard curve as shown in Fig. 3. This θ value was then matched with its free iminobiotin concentration on the iminobiotin titration curve to determine K_d. The iminobiotin-BA interaction exhibited a logarithmic dependence on pH with a K_d of 9.6 μM at pH 7.0 and 120 nM at pH 10.7. The measured pH dependence of the iminobiotin-OGSA interaction was much weaker with a K_d of 28 μM at pH 7.0 and 12 μM at pH 10.7. The values of $\log(K_d)$ are plotted versus pH in Fig. 5. Error bars were based on the propagation of error from the uncertainty in the θ value at half-occupancy as determined from the standard curve. Also, included in Fig. 5 are two streptavidin-iminobiotin K_d values as measured by Melko et al. [11] at pH 11.1 using isothermal titration calorimetry (open circle) and Reznik et al. [12] at pH 7.5 using radioactively-labeled iminobiotin (closed circle). These data points combined with our fluorometric-based data support our conclusion that the K_d of streptavidin-iminobiotin remains on the order of 10^{-5} M even at the most basic pH values. In contrast, over the same pH range the K_d for BA-iminobiotin drops by two orders of magnitude.
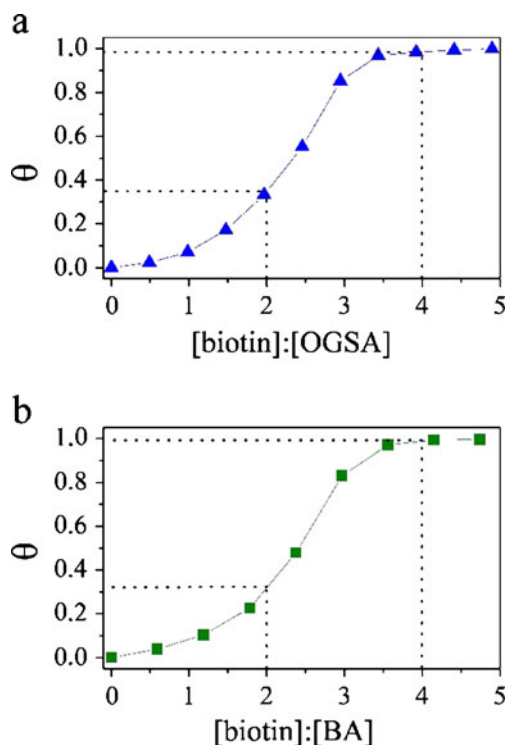

Fig. 3 Fraction of maximum fluorescent enhancement, θ, versus the ratio of biotin concentration to that of **a** OGSA and **b** BA. The pH value was 9.3 for (a) and (b)
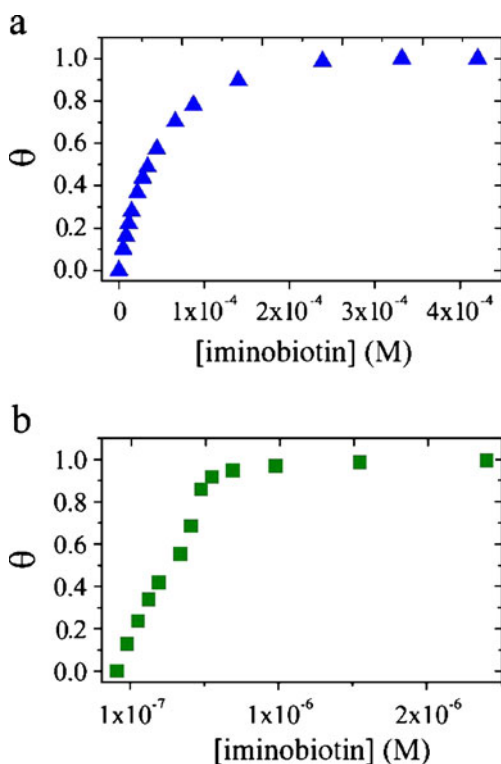
Fig. 4 Fraction of maximum fluorescent enhancement, θ, versus the concentration of iminobiotin at pH 10.0 for **a** OGSA and **b** BA

Discussion

Fluorescent conjugates of avidin and streptavidin have long been in use for a diverse range of applications including cell labeling, determination of biotin concentration and kinetics studies. The discovery that a number of avidin's fluorescent conjugates showed a marked increase in fluorescence upon binding with biotin has served to widen the scope of such applications. The first such conjugate to obtain widespread use was FITC-avidin which showed a
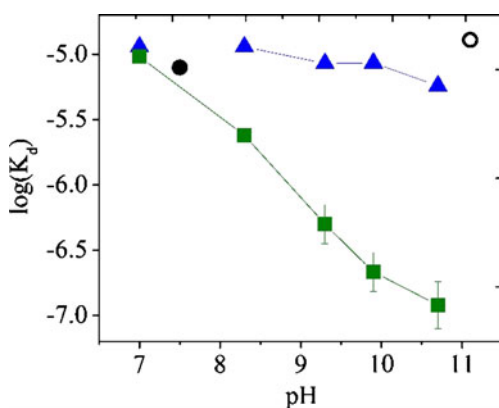

Fig. 5 $\log(K_d)$ versus pH for OGSA-iminobiotin (triangles) and BA-iminobiotin (*squares*). Also shown are streptavidin-iminobiotin K_d values from Reference [11] (*open circle*) and Reference [12] (*closed circle*)

factor of two increase in fluorescent intensity upon saturation with biotin [13]. Later, Emmans et al. reported a remarkable ten-fold increase in the fluorescence of a BODIPY-avidin conjugate upon saturation with biotin and utilized this effect to study the kinetics of endosome fusion in baby hamster kidney fibroblasts [14]. Here we have shown that like biotin, iminobiotin is capable of inducing large fluorescent enhancements in certain avidin and streptavidin fluorescent conjugates. In particular, we found fluorescence enhancements as high as 12-fold in OGSA conjugate and 11-fold in BA conjugate which enabled the fluorometry-based binding kinetics studies at nanomolar concentrations.

While the details of the mechanism which underlies the large increase in fluorescent emission exhibited by OGSA and BA upon binding biotin and iminobiotin have yet to be fully explained there are clues as to its origins. Emans et al. measured an enhancement in the intrinsic tryptophan fluorescence of BODIPY avidin (which was not present in the unconjugated form of avidin) and hypothesized that the enhancement was due to conformational changes upon binding of biotin which alters the non-radiative interaction between tryptophan and BODIPY [14]. Our measurements showed evidence that self-quenching of the Oregon Green 514 fluorophores may also play a role in OGSA. In addition to the OGSA conjugate reported here which had on average 5.0 dye molecules/protein, we have also studied a conjugate with an average of 4.2 dye molecules/protein. The conjugate with less dye was brighter, having a quantum yield of 0.09 versus 0.05. However the dimmer conjugate showed a larger fluorescence enhancement upon saturation with biotin. This effect would be consistent with the binding of (imino)biotin inducing a reduction in the self-quenching of the Oregon Green dye by means of conformational changes in the protein.

For the streptavidin-iminobiotin interaction to have an equilibrium dissociation constant on the order of 10^{-5} M at the most basic pH levels is surprising considering that this is a well established receptor-ligand pair for use in affinity binding chromatography and as such is available commercially. A value of K_d in this range is more typically associated with non-specific binding events than with the high affinities necessary for protein purification applications. In addition, such applications rely on the binding affinity varying strongly with pH for efficient protein retrieval, yet we have shown here that the K_d is nearly independent of pH. It is interesting to note that while nearly every avidin-iminobiotin related study references Green's original solution-based experiments [1], until now there has been no equivalent study published for streptavidin and iminobiotin in solution. Instead, the assumption of a pH dependent binding affinity in streptavidin-iminobiotin studies is typically based upon the work of Hoffman et al. [9]

which utilized immobilized iminobiotin in a column and on the facts that avidin and streptavidin are similar structurally as well as in their affinity for biotin.

Here we have shown that the pH dependent binding affinity of streptavidin for iminobiotin utilized in affinity chromatography applications does not appear to be intrinsic and presently we can only speculate as to its physical origins. One clue may come from the work of Melkko et al. [11] who showed that by pairing two iminobiotin molecules with the appropriate molecular scaffolding, K_d can be reduced by three orders of magnitude at pH 11.1. However, it still remains to be shown that such a chelate effect will also induce a strongly pH dependent binding affinity. In addition, because affinity chromatography applications currently immobilize monomeric iminobiotin on to a matrix of polymers or microspheres, it would need to be demonstrated that the density of this immobilized imino-biotin was high enough to mimic the chelate effect of a carefully designed scaffold.

Over the years a number of techniques including X-ray diffraction [15] and mass spectrometry have been applied to the study of streptavidin-iminobiotin kinetics in solution [12, 16–19]. Many of these studies required excessively large concentrations of iminobiotin or saw no evidence of a pH dependence in the crystallized costructures, which agrees with our findings that the binding affinity is actually at levels usually associated with non-specific binding and the pH dependence is minimal (see Fig. 5). The pH dependence of the avidin-iminobiotin interaction is attrib-uted to the degree to which the protonated form of iminobiotin is present in solution [1, 9, 20]. Our results, however, show that the protonation of iminobiotin as the pH is lowered plays a minor role at best for the streptavidin-iminobiotin interaction and that the divergent kinetic behavior between avidin-iminobiotin and streptavidin-iminobiotin is more likely due to the subtle structural differences between the two proteins. The divergent binding affinities are especially interesting in light of the fact that streptavidin and avidin have similar affinities for biotin ($K_d \sim 10^{-15}$ M) [1, 21, 22]. A better understanding of streptavidin-iminobiotin kinetics from such a fundamental perspective has the potential to promote new design approaches for improved purification techni-ques based on this receptor-ligand pair.

Finally, an interesting topic for future study will be the implications that the divergent pH dependencies of avidin and streptavidin for iminobiotin in solution have on measurements made in the single and few molecule regimes. Complexes of (strept)avidin and (imino)biotin have been measured exten-sively in these regimes using techniques such as atomic force spectroscopy and the bioforce probe which map the complex energy landscape of receptor-ligand binding [20, 23–25]. Both avidin-iminobiotin and streptavidin-iminobiotin com-plexes exhibited pH-independent rupture forces, however, the streptavidin-iminobiotin complex showed evidence of cooperative binding at pH 10.

References

1. Green NM (1966) Thermodynamics of binding of biotin and some analogues by avidin. Biochem J 101:774–780
2. Heney G, Orr GA (1981) The purification of avidin and its derivatives on 2-iminobiotin-6-aminohexyl-sepharose 4B. Anal Biochem 114:92–96
3. Hofmann K, Titus G, Montibeller JA, Finn FM (1982) Avidin binding of carboxyl-substituted biotin and analogs. Biochemistry 21:978–984
4. Orr GA (1981) The use of the 2-iminobiotin-avidin interaction for the selective retrieval of labeled plasma-membrane components. J Biol Chem 256:761–766
5. von Leoprechting A, Horth P, Haehnel W, Schilz E, Muhlenhoff U (1998) Identification of biotinylation sites on proteins by selective retrieval of 2-iminobiotinylated peptides from proteolytic peptide mixtures: localization of the accessible lysine residues on the photosystem I subunits PsaD and PsaE. Anal Biochem 262:110–121
6. Zeheb R, Chang V, Orr GA (1983) An analytical method for the selective retrieval of iminobiotin-derivatized plasma-membrane proteins. Anal Biochem 129:156–161
7. Garret-Flaudy F, Freitag R (2001) Use of the avidin (imino)biotin system as a general approach to affinity precipitation. Biotechnol Bioeng 71:223–234
8. Cao Y, Bai G, Zhang L, Bai F, Yang WB (2006) Immobilized iminobiotin on magnetic poly(vinyl alcohol) microspheres for single-step purification of streptavidin. Artif Cells Blood Substit Biotechnol 34:487–500
9. Hofmann K, Wood SW, Brinton CC, Montibeller JA, Finn FM (1980) Imino-biotin affinity columns and their application to retrieval of streptavidin. Proc Nat Acad Sci USA 77:4666–4668
10. Hylarides MD, Mallett RW, Meyer DL (2001) A robust method for the preparation and purification of antibody/streptavidin conjugates. Bioconjug Chem 12:421–427
11. Melkko S, Dumelin CE, Scheuermann J, Neri D (2006) On the magnitude of the chelate effect for the recognition of proteins by pharmacophores scaffolded by self-assembling oligonucleotides. Chem Biol 13:225–231
12. Reznik GO, Vajda S, Sano T, Cantor CR (1998) A streptavidin mutant with altered ligand-binding specificity. Proc Nat Acad Sci USA 95:13525–13530
13. Alhakiem MHH, Landon J, Smith DS, Nargessi RD (1981) Fluorimetric assays for avidin and biotin based on biotin-induced fluorescence enhancement of the fluorescein-labeled avidin. Anal Biochem 116:264–267
14. Emans N, Biwersi J, Verkman AS (1995) Imaging of endosome fusion in BHK fibroblasts based on a novel fluorometric avidin-biotin binding assay. Biophys J 69:716–728
15. Athappilly FK, Hendrickson WA (1997) Crystallographic analysis of the pH-dependent binding of iminobiotin by streptavidin. Protein Sci 6:1338–1342
16. Schwartz BL, Gale DC, Smith RD, Chilkoti A, Stayton PS (1995) Investigation of noncovalent ligand-binding to the intact strepta-vidin tetramer by electrospray-ionization mass-spectrometry. J Mass Spectrom 30:1095–1102
17. Wortmann A, Jecklin MC, Touboul D, Badertscher M, Zenobi R (2008) Binding constant determination of high-affinity protein-ligand complexes by electrospray ionization mass spectrometry and ligand competition. J Mass Spectrom 43:600–608

18. Clarkson J, Batchelder DN, Smith DA (2001) UV resonance Raman study of streptavidin binding of biotin and 2-iminobiotin: comparison with avidin. Biopolymers 62:307–314
19. Miyamoto S, Kollman PA (1993) Absolute and relative binding free-energy calculations of the interaction of biotin and its analogs with streptavidin using molecular-dynamics free-energy perturbation approaches. Proteins Struct Funct Bioinform 16:226–245
20. Moy VT, Florin EL, Gaub HE (1994) Intermolecular forces and energies between ligands and receptors. Science 266:257–259
21. Green NM (1990) Avidin and Streptavidin. Meth Enzymol 184:51–67
22. Weber PC, Wendoloski JJ, Pantoliano MW, Salemme FR (1992) Crystallographic and thermodynamic comparison of natural and synthetic ligands bound to streptavidin. J Am Chem Soc 114:3197–3200
23. Florin EL, Moy VT, Gaub HE (1994) Adhesion forces between individual ligand-receptor pairs. Science 264:415–417
24. Merkel R, Nassoy P, Leung A, Ritchie K, Evans E (1999) Energy landscapes of receptor-ligand bonds explored with dynamic force spectroscopy. Nature 397:50–53
25. Zhang XH, Moy VT (2003) Cooperative adhesion of ligand-receptor bonds. Biophys Chem 104:271–278