Introduction of histidine residues into avidin subunit interfaces allows pH-dependent regulation of quaternary structure and biotin binding

Henri R. Nordlund[a], Vesa P. Hytönen[a], Olli H. Laitinen[b], Sanna T.H. Uotila[b], Einari A. Niskanen[a], Janne Savolainen[c], Eevaleena Porkka[a], Markku S. Kulomaa[a,*]

[a] *Department of Biological and Environmental Science, P.O. Box 35, FIN-40014 University of Jyväskylä, Finland*
[b] *A.I. Virtanen Institute, University of Kuopio, FIN-70211 Kuopio, Finland*
[c] *Department of Chemistry, P.O. Box 35, FIN-40014 University of Jyväskylä, Finland*

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Abstract In order to turn the subunit association and biotin binding of avidin into pH-sensitive phenomena, we have replaced individually three amino acid residues in avidin (Met96, Val115 and Ile117) with histidines in the 1–3 interface, and in combination with a histidine conversion in the 1–2 interface (Trp110). The single replacements Met96His and Val115His in the 1–3 interface were found to have a clear effect on the quaternary structure of avidin, since subunit associations of these mutants became pH-dependent. The histidine replacement in the 1–2 interface affected the biotin-binding properties of the mutants, in particular reversibility of binding and protein–ligand complex formation were pH-sensitive, as measured by IAsys biosensor and fluorescence correlation spectroscopy, respectively. The possibility of regulating the quaternary structure and function of avidin in a controlled and predictable manner, due to introduced interface histidines, will expand even further the range and versatility of the avidin–biotin technology.

Key words: Avidin; pH dependence; Protein engineering

1. Introduction

Chicken avidin and bacterial streptavidin are tetrameric proteins which exhibit exceptionally high affinity toward the water-soluble vitamin biotin ($K_d \approx 10^{-15}$ M [1]). This extraordinarily tight interaction is the foundation for the well known and widely used (strept)avidin–biotin technology, which includes numerous applications in life sciences for analyzing, probing, labeling, isolation and targeting of desired materials both in vitro and in vivo (reviewed in [2,3]).

Avidin and streptavidin tetramers are composed of identical monomers. Their quaternary structures are highly homologous and contain three different types of monomer–monomer interface contacts [4–6]. Subunits 1 and 4 (numbered according to Livnah et al. [6]) share the largest interface 1–4, in the sense of buried surface area and number of intermonomeric contacts, and their complex is therefore often referred to as the structural dimer (Fig. 1A) – and the same with the equivalent subunit 2 and 3 pair (2–3 interface). Consequently, the (strept)avidin tetramer can be considered a dimer of dimers. The combined dimer–dimer interface includes the rest of the monomer–monomer contacts being the 1–2 and 1–3 (and their equivalents 3–4 and 2–4, respectively) interfaces. The 1 and 2 (and the identical 3 and 4) pair is usually referred to as the functional dimer, because W110 in avidin (and W120 in streptavidin) from subunit 1 participates in biotin binding at the biotin-binding site of the neighboring subunit 2 and vice versa (Fig. 1C).

Both avidin and streptavidin tetramers are extremely stable and tolerant of many conditions such as extreme pH and temperature, and the presence of proteases and denaturants [7–9]. However, it has previously been observed that the tetrameric assembly of both proteins can readily be modified by site-directed mutagenesis [10–13]. One of the most important amino acid residues for the stability of the tetrameric quaternary structure in the 1–2 interface was found to be W110 in avidin, and its equivalent residue W120 in streptavidin. Mutation of this tryptophan to lysine [11] has been described to turn the mutant into a dimeric form with reversible biotin-binding characteristics. On the other hand, mutation of M96, V115 and I117 (in the 1–3 interface) and N54 (in the 1–4 interface) to alanines has been described to convert avidin into a monomeric form, which however formed tetramers upon addition of biotin [12]. Furthermore, it was shown to be possible to make avidin–biotin interaction partially pH-dependent by mutating the ligand-binding tyrosine (Y33) to histidine [14].

Thus, in the present study, it was of interest to investigate whether introduction of histidine residues into the subunit interfaces of avidin might also lead to pH-dependent quaternary structure formation: the three 1–3 (and identically 2–4) interface amino acid residues, which interact in the tetramer with the same residue from the neighboring subunit [4–6], were replaced one at a time with a histidine residue (Fig. 1B, Table 1). Therefore, we expected that these replacements could lead, due to protonation of the histidine side chains, to repulsion and subunit disintegration by positive charges on the respective histidines at low pH. On the other hand, at higher pH the effect of these mutations was expected to be inconsequential. Similarly, tryptophan (Trp110) in the functionally important 1–2 interface (and identical 3–4 interface) was also converted to histidine (Fig. 1C) in combination with the described 1–3 interface mutants, in order to regulate the biotin binding in an adjustable way. The introduced substitu-

*Corresponding author. Fax: (358)-14-2602221.
E-mail address: kulomaa@csc.fi (M.S. Kulomaa).

Abbreviations: FCS, fluorescence correlation spectroscopy; wt, wild-type; Avm, avidin mutant

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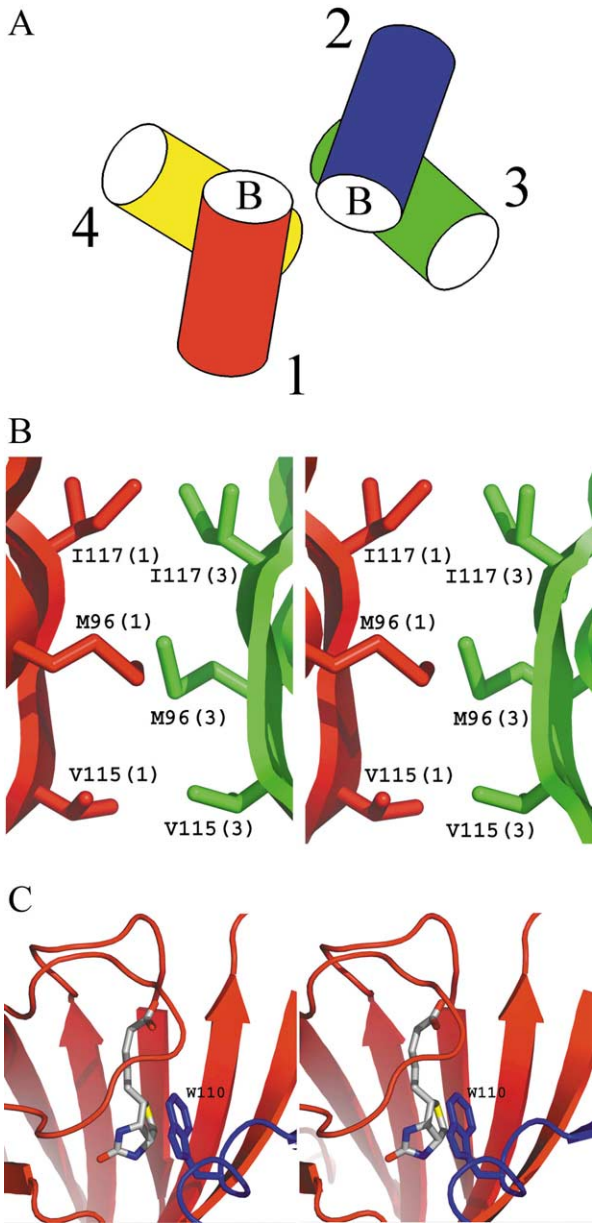

Fig. 1. Schematic representation of avidin tetramer and description of the mutated amino acid residues. A: The subunits of avidin are shown as barrels, and numbered according to Livnah et al. [6]. The same colors of the subunits are used throughout the figure. The biotin-binding sites of subunits 1 and 2, indicated with B, point toward the viewer. B: Amino acid residues M96, V115 and I117 in the 1–3 monomer–monomer interface interact with the equivalent residue from the neighboring subunit. These residues are replaced with histidines in the avidin mutants Avm(M96H), Avm(V115H) and Avm (I117H). The numbers in parentheses indicate the subunit number. C: The important biotin-binding and 1–2 interface residue W110 from the blue subunit (number 2) participates in biotin binding at the binding site of the red subunit (number 1). This tryptophan is converted into a histidine in the double mutants Avm(M96H, W110H), Avm(V115H,W110H) and Avm(I117H, W110H). Biotin is also shown in the binding site.

tions allowed us to change the quaternary structure assembly and biotin-binding characteristics, in some cases, by simply adjusting the solution pH. In addition to better understanding of protein subunit interactions, development of (strept)avidin–biotin techniques will benefit from avidins that can change their quaternary structure assembly and/or biotin-binding characteristics due to directed changes in their solution environment.

2. Materials and methods

2.1. Production and purification of the mutant avidins

The avidin mutants (Avm) were produced in baculovirus-infected insect cells as previously described in detail [15]. Mutations to the coding sequence of avidin were introduced with the QuikChange mutagenesis system according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Purification of the proteins by affinity chromatography on a 2-iminobiotin agarose or biotin agarose column (Affiland, Liege, Belgium) was performed from the cell extracts as described earlier [11].

2.2. Stability analysis by SDS–PAGE

Thermal stability of the mutants was studied by using a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)-based method [16]. Briefly, the protein sample in the absence or presence of biotin was diluted to a final concentration of 0.2 mg/ml in 50 mM Na-carbonate buffer, and the proteins were acetylated by adding acetyl NHS ester. An equal amount of SDS–PAGE sample buffer was added to each sample, which were incubated at different temperatures (between 25 and 100°C) for 20 min. After that the samples were subjected to SDS–PAGE analysis and the gel was finally stained with Coomassie brilliant blue.

2.3. Gel filtration chromatography

Quaternary status of the avidin mutants was determined by high performance liquid chromatography (HPLC), using a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) connected to a Shimadzu HPLC SCL-10A VP system controller with RF-10A XL fluorescence detector and SPD-M10A VP diode array detector. The data obtained were processed with the Class VP 5.03 program. We used as a high pH buffer (pH 11) 50 mM Na-carbonate, 650 mM NaCl, as a neutral pH buffer (pH 7.2) 50 mM Na-phosphate, 650 mM NaCl, and as a low pH buffer (pH 4) 50 mM Na-acetate, 650 mM NaCl. Each injection contained 10–20 μg of the sample protein. Specimens preincubated with biotin had a biotin:avidin (monomer) ratio of 3:1. All analyses were performed with a flow rate of 0.5 ml/min, and the elution data were collected with both the absorbance and fluorescence detectors. With the latter, 280 nm excitation and 350 nm emission wavelengths were used to obtain data via the intrinsic fluorescence of the aromatic, particularly tryptophan, residues. Molecular weight markers were bovine serum albumin (68 kDa), ovalbumin (43 kDa) and cytochrome *c* (12.4 kDa).

2.4. IAsys optical biosensor studies

Interaction analyses were performed using 50 mM Na-acetate buffer (pH 4), and 50 mM borate buffer (pH 9.5). In order to reduce the non-specific binding, the buffers contained 1 M NaCl [17]. Reversibility of biotin binding was measured for avidin and the mutants essentially as previously reported [18]. Briefly, protein samples were allowed to bind onto the biotinylated surface of an aminosilane cuvette. After reaching equilibrium, biotin-containing buffer (50 mM Na-acetate or borate buffer, 1 M NaCl, biotin 0.4 mg/ml) was applied and the dissociation of the proteins was monitored for 60 min.

2.5. Fluorescence correlation spectroscopy (FCS)

Measurements with FCS were performed at 23–25°C using a ConfoCor 2 instrument (Carl Zeiss, Jena, Germany) with the following instrument settings: He/Ne laser (633 nm) for excitation, a HFT 633 beam-splitter filter as a dichroic mirror, and an LP 650 long pass filter to collect the fluorescence signal detected by the avalanche photodiode. The correct pinhole was obtained by optimizing the structure parameter in the *x*, *y* and *z* coordinates with the automated pinhole adjustment using Dy630-Biotin label (Dyomics, Jena, Germany). The ConfoCor 2 instrument was operated in the autocorrelation mode and the software was used to calculate the autocorrelation function, diffusion time(s) and the number of particles per observation volume according to the manufacturer's instructions. All FCS experiments, with a measuring time of 10 or 20 s, were repeated 10 times, using

LabTek® II eight-well chambered borosilicate glass plates (Nalge Nunc International, Naperville, IL, USA).

The FCS measurements were performed using three different buffers: 50 mM Na-carbonate (pH 11), 50 mM Na-phosphate (pH 7.2) and 50 mM Na-acetate (pH 4), all containing 150 mM NaCl. Concentration of the label was 12.5 nM in all experiments and the protein sample was applied after the measurement of the free label to a final (monomeric) concentration of 68 nM. For some avidin mutants, protein–label complex formation was rather low. In these cases higher protein concentrations were used in order to obtain a reliable diffusion time for the complex. The protein samples were mixed with a buffer in the cuvette and the measurements continued after incubation for 5 s.

The FCS data were analyzed using the ConfoCor 2 software version 2.8. Single component model was used for measurements with the free Dy630-Biotin label. The diffusion time for the free label was fixed in the two-component model used to analyze the actual protein samples. In the case of the mutants that displayed low affinity toward the Dy630-Biotin, a one-component model was used to determine the diffusion time for protein–label complex at a higher protein concentration. All other parameters were allowed to fit to the data. In order to get the counts per molecule (cpm) ratio, the value of the measured average cpm for protein–label complex was divided with that of the free label.

2.6. Fluorescence emission spectroscopy

Measurements were performed for the Dy630-Biotin label diluted in the same buffers that were used in the FCS studies. Wild-type (wt) avidin was used to measure the fluorescence emission spectrum of the avidin–label complex (avidin monomer/label molar ratio was 10:1). In addition, wt avidin presaturated with excess biotin was used as a control. A Perkin Elmer LS50B luminescence spectrometer (Wellesley, MA, USA) was used with an excitation wavelength of 600 nm and a 5 nm slit. The emission spectrum from 650 to 700 nm was collected with a scan speed of 120 nm/min with a 5 nm slit.

3. Results

3.1. Stability of the avidin mutants by SDS–PAGE-based analysis

In the SDS–PAGE-based thermal stability analysis (Table 1, Fig. 2), estimated half-point transition (tetramer→monomer) temperatures (T_r) indicate the temperature where half of the avidin or Avm is tetrameric and half is monomeric [16]. The single mutants in the 1–3 interface, Avm(I117H) and Avm(M96H), behaved similarly to wt avidin in the absence of biotin. The same was also observed for the Avm(I117H, W110H) double mutant with one additional histidine residue in the 1–3 and another in the 1–2 interface. Interestingly, the V115H mutation alone had an effect strong enough to disassemble the tetrameric structure of Avm(V115H) at room temperature. Addition of biotin had a tetramer-stabilizing effect for all mutants, except Avm(V115H,W110H). However, a significant decrease in thermal stability was observed with the other two double mutants, Avm(M96H,W110H) and Avm (V115H,W110H), both in the absence and in the presence of biotin.

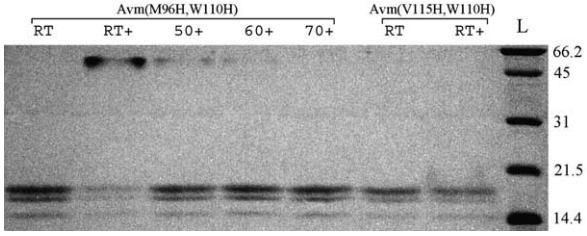

Fig. 2. The principle of the SDS–PAGE assay. Protein samples were acetylated and incubated at different temperatures in SDS–PAGE sample buffer for 20 min before they were subjected to electrophoresis. The RT marking indicates that the sample was incubated at room temperature, whereas other temperatures are indicated with numbers (°C), and the presence of biotin is shown with the + sign. The half-point transition (tetramer to monomer) temperatures (T_r) were estimated from several gels. The tetramer band is around 60 kDa, and the differentially glycosylated monomers are seen in the three lower molecular weight bands (15–18 kDa). In this gel, the Avm(M96H,W110H) and Avm(V115H,W110H) samples are shown. The unit of molecular weight markers indicated with L is kDa.

3.2. Gel filtration chromatography

The quaternary structure assembly of the mutant avidins was determined by HPLC analysis in the absence and presence of biotin at three different pH values (Table 2, Fig. 3). Wt avidin and the mutants Avm(I117H) and Avm(I117H, W110H) showed a tetrameric quaternary structure throughout the gel filtration experiments regardless of biotin, and different pH conditions.

At the highest pH of 11 used in this study, the double mutants Avm(M96H,W110H) and Avm(V115H,W110H) with an additional histidine residue in both 1–3 and 1–2 interfaces were solely monomeric in the absence of biotin (Table 2, Fig. 3). In addition to monomers, small amounts of tetramers were also seen with Avm(M96H) and Avm(V115H) in the absence of biotin. In the presence of biotin all of the mutants were mainly or completely tetrameric, except the double mutant Avm(V115H,W110H), which was a monomer irrespective of biotin presence.

Table 1
Description of the proteins used in this study and their thermal stability characteristics

Protein	Mutation	Interface[a]	T_r (°C)[b]	T_r (°C +btn)[b]
Avidin	none		60	95
Avm(I117H)	I117H	1–3	65	95
Avm(M96H)	M96H	1–3	55	90
Avm(V115H)	V115H	1–3	NA[c]	75
Avm(I117H,W110H)	I117H	1–3	55	80
	W110H	1–2		
Avm(M96H,W110H)	M96H	1–3	NA[c]	45
	W110H	1–2		
Avm(V115H,W110H)	V115H	1–3	NA[c]	NA[c]
	W110H	1–2		

The +btn indicates that the sample was preincubated with biotin.
[a]Indicates the interface between subunits where the mutations reside.
[b]Indicates the estimated temperature where half of the protein is in tetrameric and half in monomeric form after 20 min incubation in SDS–PAGE sample buffer [16].
[c]Not applicable, already monomeric at room temperature.

Table 2
Deduced molecular weights of avidin and the avidin mutants from gel filtration experiments and measured values from FCS experiments

pH	Sample	Gel filtration		FCS		
		MW (kDa)	MW +btn (kDa)	Diffusion time (μs)	Protein–label complex (%)[a]	Cpm ratio[a,b]
11	Avidin	54	54	315 ± 7	100	4.1
11	I117H	44	43	299 ± 14	84	4.1
11	M96H	20 (44)[c]	45	309 ± 15	86	4.3
11	V115H	16 (45)[c]	45	314 ± 21	75	3.4
11	I117H,W110H	46	44	303 ± 22	87	4.3
11	M96H,W110H	14	44 (16)[c]	320 ± 15	80	3.4
11	V115H,W110H	15	16	197 ± 22[d]	38	1.0
7.2	Avidin	63	65	305 ± 23	99	3.8
7.2	I117H	49	49	322 ± 64	93	4.3
7.2	M96H	50	51	312 ± 31	85	3.3
7.2	V115H	10 (49)[c]	51	301 ± 51	87	2.5
7.2	I117H,W110H	50	49	320 ± 27	69	3.5
7.2	M96H,W110H	13 (48)[c]	49 (13)[c]	324 ± 12	62	1.6
7.2	V115H,W110H	11	25	199 ± 5[d]	17	0.8
4	Avidin	62	68	305 ± 13	100	4.0
4	I117H	53	54	298 ± 11	90	3.9
4	M96H	12	51	338 ± 7	99	4.4
4	V115H	9	56	301 ± 24	26	1.0
4	I117H,W110H	52	54	337 ± 23[d]	47	1.3
4	M96H,W110H	13	14	169 ± 10[d]	12	0.9
4	V115H,W110H	10	9	150 ± 12[d]	3	0.8

The +btn indicates that the sample was preincubated with biotin. The standard deviations for the diffusion times are indicated after the ± symbol. Dy630-conjugated avidin (Dyomics) was used as a control protein and the measured diffusion time was 240 ± 7 μs (pH 11), 259 ± 12 μs (pH 7.2) and 282 ± 20 μs (pH 4). The diffusion time of the free biotin-Dy630 label was 58 ± 5 μs in all buffers.
[a]Calculated using a two-component model.
[b]Ratio of the measured average value for cpm at protein concentration 68 nM to measured average value for free biotin-Dy630 label.
[c]Value in parentheses indicates the apparent molecular weight of a smaller secondary peak.
[d]Obtained from titration experiments. The monomeric concentrations related to these values are: Avm(V115H,W110H), pH 7.2, (4.8–8.8)×10^{-7} M; Avm(I117H,W110H), pH 4, (4.0–5.2)×10^{-7} M; Avm(M96H,W110H), pH 4, (1.3–1.9)×10^{-6} M; Avm(V115H,W110H), pH 4, (2.6–3.5)×10^{-6} M.

When the analysis was performed at pH 7.2, Avm(V115H, W110H) was the only mutant that remained solely monomeric in the absence of biotin (Table 2). The mutants Avm(V115H) and Avm(M96H,W110H) were mainly monomers although some tetramers were detected, and Avm(M96H) was completely tetrameric. In the presence of biotin, all were tetramers, except Avm(V115H,W110H) which appeared to be dimeric.

At the lowest pH of 4 used in this study, Avm(M96H), Avm(V115H), Avm(M96H,W110H) and Avm(V115H, W110H) appeared to be monomers in the absence of biotin. The best example of pH-dependent subunit association was Avm(M96H) which in the absence of biotin was a tetramer at pH 7.2 and a monomer at pH 4. However, upon addition of biotin the single mutants in the 1–3 interface Avm(M96H) and Avm(V115H) exhibited tetrameric quaternary structure (Table 2).

3.3. *Fluorescence correlation and fluorescence emission spectroscopy*

The diffusion time for free Dy630-Biotin label (MW 997 Da) was 58 ± 5 μs in all the buffers which is in good agreement with the reported diffusion time of 52 μs for the free Dy630 label (MW 650 Da) [19]. The diffusion times for most of the samples could be determined using a single protein concentration and a two-component model. In the case of a few samples (indicated with [d] in Table 2), only a negligible amount of protein–label complexes was detected at an avidin concentration (monomeric) of 68 nM, and therefore, a higher protein concentration was used to determine their diffusion times. All of the avidins examined, except Avm(V115H,W110H), showed strong label binding at pH 11 (Table 2). Almost all (75–100%) of the Dy630-Biotin was seen in protein complexes, which displayed diffusion times between 299 ± 14 and 320 ± 15 μs. This indicated that the avidin mutants formed tetrameric quaternary structures similar to that of wt avidin, which showed a diffusion time of 315 ± 7 μs. In the case of Avm(V115H,W110H), only 38% of the label was seen in complex form, and the smaller diffusion time (197 ± 22 μs) indicated that the quaternary structure assembly was not a tetramer.

At pH 7.2 the proteins showed almost identical behavior when compared with that observed at pH 11, except Avm(V115H,W110H) which displayed a clear decrease in the binding efficiency at neutral pH.

At pH 4 only Avm(I117H) and Avm(M96H) showed strong complex formation with values of 90% and 99%, respectively, which were close to the value of 100% obtained for wt avidin. Moreover, Avm(V115H), Avm(I117H,W110H) and Avm(M96H,W110H) formed low quantities of complexes with the Dy630-Biotin label, whereas Avm(V115H,W110H) displayed an almost negligible amount of label-bound form (Table 2).

In fluorescence emission spectroscopy, an about two-fold increase in the fluorescence intensity (quantum yield) was observed in all the buffers used, when excess of avidin was added to a Dy630-Biotin label solution. Avidin control, which was presaturated with biotin, did not cause an increase in the intensity (data not shown). Due to this, in FCS measurements a cpm ratio around 4 obtained for wt avidin corresponds to a situation where on average two labels are bound tightly to each measured avidin tetramer.

At pH 11, all of the proteins examined, except Avm (V115H,W110H), showed a cpm ratio around 4, suggesting that there were also on average two labels per observed molecule, and that the mutants were tetrameric and displayed high affinity toward the labeled biotin (Table 2). At pH 7.2 Avm(I117H), Avm(M96H) and the double mutant Avm (I117H,W110H) showed comparable cpm ratios with wt avidin. At the acidic pH 4 only the 1–3 interface mutants Avm(M96H) and Avm(I117H) showed similar cpm ratios to that of wt avidin.

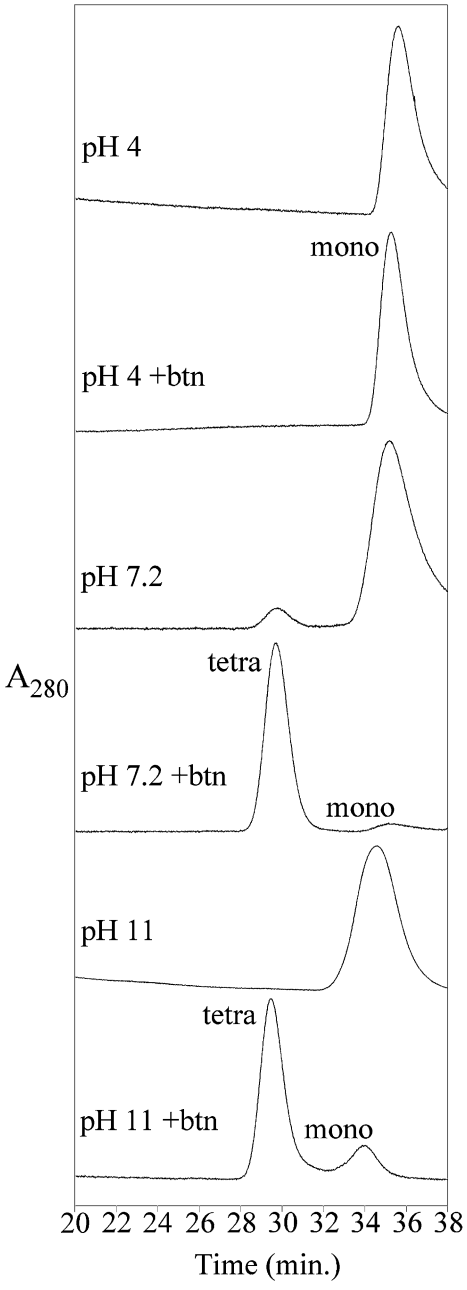

Fig. 3. Gel filtration chromatograms of Avm(M96H,W110H) at different pH and in the presence (+btn) and absence of biotin. The label mono indicates the position of a peak for a monomeric form, whereas the position for a tetrameric form is indicated with the tetra label.

Table 3
Biotin-binding reversibility by IAsys optical biosensor

Protein	Reversibility %	
	pH 9.5	pH 4
Avidin	22	17
I117H	27	27
M96H	32	29
V115H	42	55
V115H,W110H	80	86
M96H,W110H	40	93
I117H,W110H	43	95

Reversibility % indicates the measured dissociation of a protein from biotin surface during 60 min incubation with excess biotin in the buffer.

3.4. Optical biosensor studies

An IAsys optical biosensor with biotin surface was used to analyze the binding characteristics of the avidin mutants. Avm(V115H) was the only single mutation-bearing avidin, which displayed clearly more reversible binding toward the biotin surface than wt avidin (Table 3). The other two 1–3 interface mutants, Avm(I117H) and Avm(M96H), displayed almost irreversible binding to biotin surface regardless of pH, which was comparable to that of wt avidin. The double mutants Avm(M96H,W110H) and Avm(I117H,W110H) showed a remarkable pH-dependent difference in binding to the biotin surface, with mediocre reversibility of 40% and 43% at pH 9.5, respectively. Lowering of the pH to 4 turned their binding almost fully reversible (Table 3). The third double mutant, Avm(V115,W110H), showed relatively reversible binding of 80% and 86% at both pH 9.5 and 4.0, respectively.

4. Discussion

In order to investigate whether it would be possible to turn avidin subunit association into a pH-dependent event, the 1–3 interface of chicken avidin was scanned by changing separately all three interface residues [6] into histidines. For the same reason, the important biotin-binding and 1–2 interface residue Trp110 [6] was chosen to be converted to histidine. This W110H mutation was combined with the 1–3 interface histidine mutants to enhance the possible effects that changes in pH might have on the quaternary structure and biotin-binding properties of the resultant mutants.

Out of the single 1–3 interface mutants, quaternary structure assembly of Avm(M96H) showed the clearest pH dependence. This could be explained by the distance of about 11 Å between the methionine 96 α-carbons from the opposite monomers [6]. Histidine side chains, in that position, may not form extensive ring stacking, but reject each other, due to like charges, in acidic conditions. On the other hand, as seen also from a modeled structure (data not shown) this mutation was relatively ineffectual at pH 7.2, where normal monomer–monomer interactions, in particular the contacts involving V115 and I117, were probably well preserved in the tetrameric quaternary structure assembly. This was confirmed by the SDS–PAGE stability analysis (performed at pH 6.8–8.8) wherein Avm(M96H) proved to be only slightly less stable than wt avidin. Interestingly, this mutant was partially dimeric at the pH of 11 in the absence of biotin, reflecting that the mutant was more sensitive than wt avidin to changes in overall charge interactions also at high pH. This indicates that even though this histidine substitution produced the desired

outcome at other pH values, full conservation of the extreme stability, characteristic of wt avidin, is not a simple task.

The single 1–3 interface mutant Avm(V115H) was exclusively monomeric at acidic pH in the absence of biotin, whereas at higher pH values small amounts of tetrameric forms were also observed. The distance between the opposite valine 115 α-carbons is around 9 Å, and more importantly, all four residues are also in close proximity to each other in the quaternary structure of avidin [6]. The mutants bearing the V115H substitution were mainly monomeric at all pH values in the absence of biotin, suggesting that this mutation caused relatively fundamental changes in the interface architecture. Most probably, as suggested by molecular modeling, the histidine side chain is simply too bulky in that position and environment, and the tetrameric quaternary structure is therefore compromised.

The replacement of isoleucine 117 with histidine in avidin had virtually no effect on tetramerization even at low pH. Moreover, the additional W110H mutation in another interface did not change this situation. This may be explained by the fact that in the 3D structure of avidin the α-carbons of isoleucine 117 from the opposite monomers are only about 7 Å apart from each other. This could allow extensive ring stacking between the histidines, possibly resembling that between the analogous H127 residues present in wt streptavidin, which was also supported by a model structure of the mutant (data not shown) [4,5]. In addition, the interface region seemed to tolerate a histidine in that position remarkably well, because Avm(I117H) behaved almost identically in all assays when compared to wt avidin.

Addition of the 1–2 interface mutation W110H enhanced markedly the structural consequences of the single 1–3 interface mutations in Avm(M96H) and Avm(V115H). From the double mutants, Avm(I117H,W110H) was an exception, since it was a tetramer at all pH values used, regardless of the absence or presence of biotin. FCS results showed that binding of the labeled biotin was pH-dependent for the W110H replacement-bearing mutants, and they displayed reversible biotin-binding characteristics in the biosensor studies, being practically completely reversible at pH 4. Actually Avm (V115H,W110H) was highly reversible even at higher pH, but this was obvious because of the absence of tetrameric quaternary structure, which is essential for high-affinity biotin binding of avidin.

Concerning potential applications, the most interesting mutants are Avm(M96H,W110H), Avm(M96H) and Avm (I117H,W110H). Their behavior seemed to be consistently predictable. In the case of Avm(M96H,W110H), biotin was able to induce formation of tetramers at pH 7.2 and 11, but lowering the pH to 4 caused its dissociation into monomers regardless of biotin presence. This should allow easy detachment of bound biotinylated materials from immobilized Avm(M96H,W110H) just by lowering the pH, and presumably addition of free biotin. Avm(M96H) was a tetramer both in the absence and in the presence of biotin at pH 7.2. It was, however, a monomer at pH 4 in the absence of biotin, but addition of biotin induced its tetramerization at this pH. Therefore, it is possible to regulate its quaternary structure assembly alternatively by changing pH in the absence of biotin, or by adding biotin at a low pH. The mutant Avm (I117H,W110H) exhibited quaternary structure characteristics similar to wt avidin, being a stable tetramer at all pH values studied. Therefore, its full biotin-binding reversibility at low pH might be a valuable property in some applications.

In conclusion, the series of mutants constructed in this study proved the power of using the pH-sensitive histidine as a tool in rational protein engineering. By replacing critical interface and/or ligand-binding residues with histidine residues, the extraordinarily stable avidin tetramer was transformed, so that its quaternary structure assembly could be adjusted by changing the pH of the solution. Moreover, replacement of tryptophan 110 with histidine in the interface between the functional subunits enabled the adjustment of biotin-binding reversibility in a pH-dependent fashion. Therefore, histidine-based engineering should be a strategy worthy of consideration even in more general approaches, when it is desirable to adjust the quaternary structure and/or function of an oligomeric protein in a pH-dependent manner.

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