Construction of a Dual Chain Pseudotetrameric Chicken Avidin by Combining Two Circularly Permutated Avidins*

Received for publication, March 30, 2004, and in revised form, May 3, 2004
Published, JBC Papers in Press, May 6, 2004, DOI 10.1074/jbc.M403496200

Henri R. Nordlund‡§, Olli H. Laitinen‡§, Vesa P. Hytönen‡, Sanna T. H. Uotila‡, Evaleena Porkka‡, and Markku S. Kulomaa‡

From the ‡NanoScience Center (NSC), Department of Biological and Environmental Science, P. O. Box 35, FIN-40014 University of Jyväskylä, Finland and the §A. I. Virtanen Institute, Department of Molecular Medicine, University of Kuopio, P. O. Box 1627, FIN-70211 Kuopio, Finland

Two distinct circularly permuted forms of chicken avidin were designed with the aim of constructing a fusion avidin containing two biotin-binding sites in one polypeptide. The old N and C termini of wild-type avidin were connected to each other via a glycine-serine-rich linker, and the new termini were introduced into two different loops. This enabled the creation of the desired fusion construct using a short linker peptide between the two different circularly permuted subunits. The circularly permuted avidins (circularly permuted avidin 5 → 4 and circularly permuted avidin 6 → 5) and their fusion, pseudotetrameric dual chain avidin, were biologically active, i.e. showed biotin binding, and also displayed structural characteristics similar to those of wild-type avidin. Dual chain avidin facilitates the development of dual affinity avidins by allowing adjustment of the ligand-binding properties in half of the binding sites independent of the other half. In addition, the subunit fusion strategy described in this study can be used, where applicable, to modify oligomeric proteins in general.

Avidin, a glycoprotein found in chicken egg white as well as its distant relative, streptavidin, from Streptomyces bacteria, are nanoscale devices that perform one particular task, the harvesting of free biotin, extremely well. The avidin-biotin interaction is so strong that even the harsh conditions in the digestive tract are insufficient to break the ingested avidin-biotin complexes (1). It is understandable, therefore, that this highly stable interaction has been widely applied in numerous fields of the life sciences to probe, label, affinity separate, and highly stable interaction has been widely applied in numerous fields of the life sciences to probe, label, affinity separate, and high power of the target (1). This technology has been on the adjustment of the physicochemical properties of (strept)avidin, whereas in other studies the target has been the fine-tuning of the protein. Nevertheless, because these mutant protein subunits are single gene products, the desired changes, produced by mutations, take effect simultaneously in all (strept)avidin subunits.

In several cases, however, it would be of benefit to be able to alter, for example, the binding affinity in only some subunits of the tetramer while conserving the tight binding in the remaining binding sites. Chilkoti et al. (17) developed a partial solution to this problem by producing two separate streptavidin forms, one with natural high affinity biotin-binding capacity and the other with reduced affinity. They denatured and mixed these two forms, after which the mixture was renatured. Nevertheless, the refolding led to a wide variety of alternative forms: some contained four high affinity binding sites, whereas other forms had an ascending series of lower affinity binding sites, finally ending in the form that contained four lower affinity binding sites. It is arguable that genetic fusion of the subunits might be a more straightforward and effective strategy to create (strept)avidin molecules with divergent affinity properties. The N and C termini of the distinct (strept)avidin subunits are, however, located far away from each other in the quaternary structure; therefore, any simple fusion strategy would presumably fail.

A common approach to the study of protein folding and the significance of secondary structure topology is the creation of circularly permuted forms of the proteins in question (26). Usually in this approach the original N and C termini are brought together with a linker peptide, whereas the new termini are typically introduced into a loop region. In most cases proteins withstand these modifications rather well, exhibiting no radical alterations in their structure or function. Chu et al. (23) have described a circularly permuted streptavidin that...
displayed a three-dimensional structure almost identical to that of the native protein. In their study, the circular permutation strategy was used as a tool to delete the loop between β-strands 3 and 4 in streptavidin. This loop is functionally important because it undergoes an open-to-closed conformational change upon biotin binding (7, 9). Consequently, when the new termini were introduced into this loop, the affinity of the resultant mutant for biotin collapsed by six orders of magnitude in comparison to that of wild-type (wt)1 streptavidin.

In the present study, our objective was to create an avidin with four binding sites that would consist of only two polypeptide chains. At first, we constructed two circularly permuted forms of avidin in which the new termini were in an ideal position to allow the building of the desired subunit fusion. We describe the construction of this dual chain avidin (dcAvd) through the fusing of the monomers of the structural dimer and show that it formed a wt-like pseudotetrameric quaternary structure and that its four biotin-binding sites exhibited high affinity biotin binding. This construct can be used in the future as a structural scaffold to change the affinity parameters in some subunits while preserving the high biotin binding affinity in the remaining binding sites. This study also sheds light on the factors that should be considered when other multisubunit proteins are engineered by combining separate subunits into genetically continuous units.

**EXPERIMENTAL PROCEDURES**

Production and Purification of the Mutant Avidins—The circularly permuted avidins and dcAvd were produced in baculovirus-infected insect cells. They were purified from the cell extracts by 2-iminobiotinagarose affinity chromatography essentially as described in detail elsewhere (11, 27).

Biotin-Binding Assays—An IAxsys biosensor was used to determine the affinity of the proteins toward 2-iminobiotin and the reversibility of biotin binding, essentially as previously described in detail (10). In the reversibility assay, the sample proteins were allowed to bind to a biotinylated cuvette surface in phosphate-buffered saline containing 1 mM NaCl. After equilibrium was reached, the cuvette was washed and filled with phosphate-buffered saline, 1 mM NaCl containing biotin (423 μg/ml), and the dissociation of the proteins was monitored for 1 h. Colorimetric 2-(4-hydroxyazobenzene)benzoic acid assay for the determination of the free biotin-binding sites was performed essentially as described by Green (28) using a Beckman DU640 spectrophotometer.

Get 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 Biosciences) connected to a Shimadzu HPLC system with a SCL-10A VP system controller, RF-10A XL fluorescence detector, and SPD-M10A VP diode array detector. The data obtained were processed with the Class VP 5.03 program. As a running buffer we used 50 mM sodium phosphate, 650 mM NaCl, pH 7.2. All runs were performed with a flow rate of 0.5 ml/min. The molecular mass markers were bovine serum albumin (68 kDa), ovalbumin (43 kDa), and cytochrome c (12.4 kDa).

Stability Analyses—Protein samples were acetylated and temperature-dependent dissociation of the subunits after heat treatment for 20 min at temperatures between 25 and 100 °C were monitored from Coomassie-stained SDS-PAGE gels essentially as described in detail by Bayer et al. (29). Proteinase K assay was performed essentially as described by Laitinen et al. (11).

**RESULTS**

Planning of the Constructs—The three-dimensional structure of the avidin tetramer (coordinates kindly given by Dr. Oded Livnah) was examined to identify the β-strands of the barrels, along which polypeptide transition from one subunit to the other would create as little structural disturbance as possible. The study revealed that in the quaternary structure β-strand 4 in one subunit and β-strand 6 in neighboring subunit of the structural dimer are juxtaposed. Moreover, when β-strand 4 goes up in one barrel, β-strand 6 goes down in the neighboring subunit. Therefore, we decided to use the loops 4 → 5 and 5 → 6 of the neighboring subunits as the monomer-monomer transition point in dcAvd (Fig. 1). Determination of the transition point automatically determined the positions of the termini of the two circularly permuted avidins, which were as follows: the N terminus of the first circularly permuted avidin (cpAvd5 → 4) is located before β-strand 5 in the wild-type sequence, and the new C terminus follows β-strand 4. Likewise, the second circularly permuted avidin (cpAvd6 → 5) started just before β-strand 6 and ended after β-strand 5 (Fig. 1A).

In cpAvd5 → 4 and cpAvd6 → 5, the original N and C termini were brought together with an artificial hexapeptide loop (GGSGGS). Both constructs started with the normal signal peptide of avidin, followed by the first three N-terminal amino acid residues of wt avidin to ensure appropriate cleavage by signal peptidase. Finally, the desired subunit fusion was constructed by combining the two circularly permuted avidins described above (Fig. 1). A tripeptide linker (SGG) was used to connect the C terminus of cpAvd5 → 4 to the N terminus of cpAvd6 → 5, and the resultant subunit fusion protein (GenBank™ accession number A6167862) was designated dual chain avidin (dcAvd).

Protein Production and Purification—Production of the proteins was done in baculovirus-infected insect cells essentially as previously reported (27). All constructs were soluble and were efficiently purified in a single step on 2-iminobiotin-agarose column (data not shown).

Biotin Binding Experiments—Reversibility of biotin binding and affinity toward 2-iminobiotin were determined by surface plasmon resonance using an IAxsys biosensor (Table I). CpAvd5 → 4 showed binding characteristics similar to those of wt avidin in both assays, whereas cpAvd6 → 5 was more reversible and exhibited reduced affinity when compared with that of wt avidin. Interestingly, dcAvd showed similar reversibility to that of wt avidin, and its affinity toward 2-iminobiotin showed only a negligible decrease. The number of functional biotin-binding sites per dcAvd pseudotetramer ("pseudo" because, in fact, it is a dimer with four binding sites) was determined by a colorimetric 2-(4-hydroxyazobenzene)benzoic acid assay according to Green (28). The results from two independent experiments gave an approximation of 3.3 free biotin-binding sites/molecule.

Structure Analyses—According to gel filtration chromatography, the deduced molecular masses (Table II) indicated that all constructs had folded correctly and formed quaternary structures resembling that of wt avidin; circularly permuted forms were tetramers and dcAvd was a dimer (pseudotetramer). In the denaturing SDS-PAGE both circularly permuted avidins had apparent molecular masses corresponding to a monomeric form (Fig. 2), whereas dcAvd had a molecular mass of ~32 kDa, which corresponds well to its theoretical molecular mass. Moreover, we also observed that cpAvd6 → 5 was more glycosylated than cpAvd5 → 4.

Stability Analyses—All the avidin proteins were subjected to a heat/SDS-PAGE stability analysis (29). The results (Table II) revealed that the mutants were somewhat less stable than wt avidin. The durability of the avidins in the presence of proteinase K was also tested (Fig. 3), and the mutated avidin forms were found to be more prone to cleavage than wt avidin, both in the absence and presence of biotin.

**DISCUSSION**

There are two main reasons for the plethora of studies describing different chemical modifications and genetically engineered forms of avidin and streptavidin. The first is naturally

1 The abbreviations used are: wt, wild-type; dcAvd, dual chain avidin; cpAvd, circularly permuted Avd.
connected to the widespread utilization of these proteins across a multitude of applications. This has led to regulation of their physicochemical properties and biotin-binding properties both to broaden the spectrum of their potential applications and conditions of use and to overcome some of the drawbacks inherent in the (strept)avidin-biotin system. Secondly, the (strept)avidin-biotin pair serves as an interesting model system of an oligomeric protein that displays extremely high affinity toward a small ligand.

In the present study, we used avidin as a model by engineer-
Dual Chain Avidin

The association ($k_a$) and dissociation ($k_d$) rate constants and the affinity toward 2-iminobiotin for different avidins were determined with an IAsys optical biosensor. $K_d$ (equilibrium) was determined experimentally from the binding curves, whereas the $K_d$ (rel) was calculated from the rate constants. The reversibility value corresponds to reversibility from the biotin surface.

| Protein | $k_a$ | $k_d$ | $K_d$ (equilibrium) | $K_d$ (rel) | Reversibility |
|---------|-------|-------|---------------------|-------------|---------------|
| wt Avd  | $(5.5 \pm 0.5) \times 10^5$ | $(1.9 \pm 1.4) \times 10^{-3}$ | $(2.2 \pm 1.0) \times 10^{-8}$ | $(3.4 \pm 2.5) \times 10^{-8}$ | 9             |
| cpAvd5 → 4 | $(2.6 \pm 0.3) \times 10^5$ | $(1.6 \pm 0.7) \times 10^{-3}$ | $(1.5 \pm 0.5) \times 10^{-8}$ | $(5.9 \pm 2.6) \times 10^{-8}$ | 9             |
| cpAvd6 → 5 | $(3.9 \pm 1.2) \times 10^4$ | $(3.9 \pm 2.2) \times 10^{-3}$ | $(5.5 \pm 2.3) \times 10^{-7}$ | $(9.9 \pm 7.6) \times 10^{-7}$ | 16            |
| dcAvd   | $(9.8 \pm 1.3) \times 10^4$ | $(1.1 \pm 0.5) \times 10^{-3}$ | $(4.1 \pm 1.2) \times 10^{-8}$ | $(1.1 \pm 0.5) \times 10^{-7}$ | 8             |

Table II

Characterization of the proteins

The molecular mass (MW) of different avidins deduced from the gel filtration experiments and the stability parameters obtained from heat/SDS-PAGE analysis. Transition temperature ($T_r$) is a temperature in which half of the tetramer/pseudotetramer was broken into monomers in the presence (+btn) or absence of biotin.

| Protein | MW | $T_r$ | $T_r$ + btn |
|---------|----|-------|------------|
| wt Avd  | 62 | 60    | 95         |
| cpAvd5 → 4 | 52 | 45    | 75         |
| cpAvd6 → 5 | 56 | 40    | 70         |
| dcAvd   | 56 | 40    | 75         |

Fig. 2. Denaturing SDS-PAGE analysis. The molecular mass of the dcAvd (dc) monomer was expectedly twice that observed for cpAvd5 → 4 (cp54) and cpAvd6 → 5 (cp65). The unit of the molecular mass markers (MW) is kDa. The three major bands present in the samples result from differences in the glycosylation of the monomers in insect cells.

Fig. 3. Sensitivity of the mutants to proteinase K treatment. The values indicate the percentage of intact monomer present in the sample after 30 (1) and 60 min (2) and 16 h (3) treatment. The samples treated in the absence of biotin are indicated with gray triangles, whereas the biotin-containing samples are indicated with black squares. The samples are: cpAvd54 (A), cpAvd6 → 5 (B), dcAvd (C), and wt avidin (D).
served. Furthermore, in cpAvd5 → 4, where the new termini were introduced into the loop between β-strands 4 and 5, which is at the non-binding end of the barrel, no major changes were detected in its biotin-binding properties.

Interestingly, dcAvd exhibited binding properties that were somewhere between the two circularly permuted avidins. One reason for this could be that the dcAvd pseudotetramer has two rather well preserved biotin-binding sites exhibiting strong affinity toward biotin originating from cpAvd5 → 4 and two biotin-binding sites with reduced affinity originating from cpAvd6 → 5. On the other hand, in dcAvd two of the new termini are fused with the SGG linker, which provides the transition from one monomer to the other, thereby possibly rescuing part of the structural rigidity of the wt loop (5 → 6) and therefore also retaining part of the binding affinity.

The thermal stability and proteinase K durability of the circularly permuted avidins and dcAvd were somewhat lower than in the case of wt avidin. Proteinase K seemed to digest the new loops faster (not shown) than loop 3–4, which it is able to break in wt avidin (31). This indicates that the artificial hexapeptide loops that connect the old termini were probably not optimal in terms of protein stability. Furthermore, for some unknown reason cpAvd6 → 5 seemed to be more glycosylated than cpAvd5 → 4. However, this may explain why the molecular mass of the former mutant, according to high pressure liquid chromatography, was slightly higher than that of the latter one.

There is an interesting uncertainty concerning the quaternary structure of dcAvd. Because of its structural symmetry, it may have two different quaternary structures. Depending on the outcome of this quaternary structure assembly, the termini of both dcAvd monomers may be oriented to the same or to the opposite face of the pseudotetramer. At present, we do not know what the precise orientation of the dcAvd subunits is. They may form a mixture of two different structural forms; alternatively, some unknown structural phenomenon may favor one over the other. However, it might be possible to introduce a non-symmetrical disulfide bridge between the dcAvd monomers in order to fix their quaternary structure to the desired assembly.

Although in this study we utilized avidin as a model of a multisubunit protein whose topology was extensively transformed, dcAvd also provides a potential structural scaffold for avidins with mixed affinity properties. Such forthcoming dcAvd variants may have enormous value in avidin-biotin technology applications. The rationale for these high expectations derives from the fact that dcAvd is a genetically fused entity, which enables the avidin subunit properties to be engineered separately. It should be possible to maintain the high affinity toward biotin in two of the binding sites while modifying the affinity in the other two sites as desired. One option could be to make two of the binding sites reversible, which would enable bound materials to be mildly detached by free biotin (11, 14, 32, 33). Another procedure might be to modify half of the binding sites with smart polymer conjugates able to respond to changes in pH, light intensity, and temperature (34, 35). It might then be possible to alter the binding characteristics of the modified sites by adjusting these physical/chemical parameters. In theory, it is even possible to adjust the binding characteristics so that the resultant protein is able to bind something totally different from biotin with half of the binding sites.

REFERENCES

1. Green, N. M. (1975) Adv. Protein Chem. 29, 85–133
2. Wilchek, M., and Bayer, E. A. (1999) Biomol. Eng. 16, 1–4
3. Ahlroth, M. K., Kola, E. H., Ewald, D., Masabanda, J., Sazanov, A., Fries, R., and Kulomaa, M. S. (2000) Annu. Rev. Biophy. 29, 367–375
4. Wallén, M. J., Laukkonen, M. O., and Kulomaa, M. S. (1995) Gene 161, 256–269
5. Argarana, C. E., Kuntz, I. D., Birken, S., Axel, R., and Cantor, C. R. (1986) Nucleic Acids Res. 14, 1871–1882
6. Pugliese, L., Coda, A., Malcovati, M., and Bolognesi, M. (1993) J. Mol. Biol. 231, 689–710
7. Hendrickson, W. A., Pahler, A., Smith, J. L., Satow, Y., Merritt, E. A., and Phizackerley, R. P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2190–2194
8. Livnah, O., Bayer, E. A., Wilchek, M., and Sussman, J. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5076–5080
9. Weber, P. C., Ohlendorf, D. H., Wendoloski, J. J., and Salenmne, F. R. (1989) Science 243, 85–88
10. Marttila, A. T., Airenne, K. J., Laitinen, O. H., Kulik, T., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (1998) FEBS Lett. 441, 313–317
11. Laitinen, O. H., Airenne, K. J., Marttila, A. T., Kulik, T., Parkka, E., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (1999) FEBS Lett. 461, 52–56
12. Marttila, A. T., Laitinen, O. H., Airenne, K. J., Kulik, T., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (2000) FEBS Lett. 467, 31–36
13. Laitinen, O. H., Marttila, A. T., Airenne, K. J., Kulik, T., Livnah, O., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (2001) J. Biol. Chem. 276, 8219–8224
14. Marttila, A. T., Hytönen, V. P., Laitinen, O. H., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (2003) Biochem. J. 369, 249–254
15. Nordlund, H. R., Laitinen, O. H., Urtila, S. T., Nyholm, T., Hytönen, V. P., Slotte, J. P., and Kulomaa, M. S. (2003) J. Biol. Chem. 278, 2479–2485
16. Laitinen, O. H., Nordlund, H. R., Hytönen, V. P., Urtila, S. T., Marttila, A. T., Savolainen, J., Airenne, K. J., Livnah, O., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (2003) J. Biol. Chem. 278, 4010–4014
17. Chilkoti, A., Schwartz, B. L., Smith, R. D., Long, C. J., and Stayton, P. S. (1995) Bio/Technology 13, 1198–1204
18. Chilkoti, A., Tan, P. H., and Stayton, P. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1754–1758
19. Sano, T., and Cantor, C. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3180–3184
20. Reznik, G. O., Vajda, S., Smith, C. L., Cantor, C. R., and Sano, T. (1996) Nat. Biotechnol. 14, 1007–1011
21. Sano, T., Vajda, S., Smith, C. L., and Cantor, C. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6153–6158
22. Reznik, G. O., Vajda, S., and Cantor, C. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13525–13530
23. Chu, V., Freitag, S., Le Trong, I., Stenkamp, R. E., and Stayton, P. S. (1998) Protein Sci. 7, 848–859
24. Freitag, S., Chu, V., Benetti, J., Klumb, L. T., Hyre, D., Trong, I., Lybrand, T., Stenkamp, R., and Stayton, P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8384–8389
25. McDevitt, T. C., Nelson, K. E., and Stayton, P. S. (1999) Biotechnol. Prog. 15, 391–396
26. Uziel, S., Floss, A., and Unger, R. (2001) Protein Eng. 14, 523–542
27. Airenne, K. J., Oker-Blom, C., Marjomaki, V. S., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (1997) Protein Expression Purif. 9, 100–108
28. Green, N. M. (1970) Methods Enzymol. 18, 414–424
29. Bayer, E. A., Ehrlisch-Rogunzinski, S., and Wilchek, M. (1996) Electrophoresis 17, 1319–1324
30. Sanders, K. E., Lo, J., and Sligar, S. G. (2002) Blood 100, 299–305
31. Ellison, D., Hinton, J., Hubbard, S. J., and Beynon, R. J. (1995) Protein Sci. 4, 1337–1345
32. Qureshi, M. H., Yeung, J. C., Wu, S. C., and Wong, S. L. (2001) J. Biol. Chem. 276, 46424–46428
33. Qureshi, M. H., and Wong, S. L. (2002) Protein Expression Purif. 25, 409–415
34. Ding, Z., Fong, R. B., Long, C. J., Stayton, P. S., and Hoffman, A. S. (2001) Nature 411, 59–62
35. Shioseboji, T., Ding, Z., Stayton, P. S., and Hoffman, A. S. (2001) Bioconjugate Chem. 12, 314–319
Construction of a Dual Chain Pseudotetrameric Chicken Avidin by Combining Two Circularly Permuted Avidins
Henri R. Nordlund, Olli H. Laitinen, Vesa P. Hytönen, Sanna T. H. Uotila, Eevaleena Porkka and Markku S. Kulomaa

J. Biol. Chem. 2004, 279:36715-36719.
doi: 10.1074/jbc.M403496200 originally published online May 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403496200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 13 of which can be accessed free at http://www.jbc.org/content/279/35/36715.full.html#ref-list-1