[9] Crystal Forms of Avidin

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Although many varied and sophisticated applications have been developed[1] for the tight binding of biotin to avidin, the nature and mechanism of the interaction between them are not yet fully understood. High-resolution X-ray crystallographic studies could give us a direct answer to the nature of this strong interaction. It may also be possible to establish a correlation between the structure of bound biotin analogs and their differential affinities toward avidin.

Crystal Forms of Egg-White Avidin

Type I

The first single crystals suitable for X-ray diffraction studies were reported by Green and Joyson in 1970.[2] These crystals were obtained using the dialysis method[3] at room temperature by equilibrating a solution containing 10–20 mg/ml of protein versus a sodium phosphate buffer at pH 5.2. Crystals appeared when the buffer concentration was increased to about 3.0 *M*. Two crystal forms were obtained: prisms with a maximum dimension of 0.2 mm and needles 0.5 × 0.05 × 0.05 mm^3 in size. The prism-shaped crystals were not studied crystallographically.

The space group and cell dimensions of the needle-shaped crystals were determined, via precession photographs, to be *C*222 with cell dimensions a = 62 Å, b = 107 Å, c = 43 Å. The crystals diffracted up to 2.5 Å and did not appear to be particularly sensitive to prolonged radiation. On soaking biotin into the crystals, there appeared to be no morphological changes in the crystals; however, no crystallographic studies were reported for the complex.

Assuming the molecular mass of the avidin subunit as 15,600 daltons, if there were one subunit per asymmetric unit, then V_M = 2.28 $Å^3$/dalton,[4] whereas if there were two subunits per asymmetric unit, V_M = 1.14 $Å^3$/

[1] M. Wilchek and E. A. Bayer, *Anal. Biochem.* **171,** 1 (1988).

[2] N. M. Green and M. A. Joyson, *Biochem. J.* **118,** 71 (1970).

[3] A. McPherson, "Preparation and Analysis of Protein Crystals," Chap. 4. Wiley, New York, 1982.

[4] V_M is defined as the volume of the asymmetric unit/molecular weight of protein per asymmetric unit.

dalton. Matthews[5] has shown that V_M normally lies within the range 1.68–3.53 for proteins up to 70,000 daltons, so that the asymmetric subunit most probably consists of a single subunit.

Another crystal form was obtained[2] via the dialysis method at room temperature using 3 *M* ammonium sulfate (pH 5.1) as the precipitating reagent. Thin square plates up to 0.4 mm were obtained but not examined crystallographically.

Type II

A different crystal form was obtained[6] from polyethylene glycol (PEG) 6000. These crystals were grown using the batch method[3] at room temperature. The protein solution contained 20–45 mg/ml protein, 10 m*M* sodium phosphate (pH 6.0), and 150 m*M* NaCl. It was reported[6] that to 100 μl of the protein solution PEG 6000 was added until a faint precipitate appeared. The precipitate was then redissolved in 5–15 μl of the buffer. Oil drops developed in the solution and crystals grew out of them after 16–17 days, reaching a final size of $0.8 \times 0.2 \times 0.2$ mm^3 within 1 week. These crystals diffracted to 1.6 Å on still photographs and were stable to irradiation for more than 150 hr at room temperature. Although the crystals diffracted well, the structure determination of avidin could not be advanced owing to a failure to reproduce this crystal form.

The space group was determined by precession photographs, and the cell constants were determined by diffractometer measurements to be $P2_12_12$ with $a = 71.6$ Å, $b = 80.0$ Å, $c = 43.1$ Å. On soaking biotin into the crystals, the *a* axis undergoes a significant alteration[7,8] to $a = 74.7$ Å, whereas the *b* and *c* axes are essentially unchanged. Again, taking 15,600 as the molecular mass of the avidin monomer, and assuming two avidin molecules in the unit cell, one obtains a value of $V_M = 2.06$ Å^3/dalton, implying that the crystals contain two subunits in the asymmetric unit.

Type III

A tetragonal crystal form was obtained from ammonium sulfate.[9] The crystals were grown using microdialysis buttons[3] at room temperature.

[5] B. W. Matthews, *J. Mol. Biol.* **33,** 491 (1968).

[6] E. Pinn, A. Pahler, W. Saenger, G. A. Petsko, and N. M. Green *Eur. J. Biochem.* **123,** 545 (1982).

[7] Note that Ref. 6 incorrectly attributes the published value for the *a* axis to native avidin instead of the avidin–biotin complex (see Ref. 8).

[8] A. Pahler, W. A. Hendrickson, M. A. Gawinowicz-Kolks, C. E. Argarana, and C. R. Cantor, *J. Biol. Chem.* **262,** 13933 (1987).

[9] G. Gatti, M. Bolognesi, A. Coda, F. Chiolerio, E. Filippini, and M. Malcovati, *J. Mol. Biol.* **178,** 787 (1984).

The protein solution contained 20 mg/ml protein and 50 m*M* phosphate buffer (pH 5.2). The protein solution was equilibrated versus a solution of 2.7 *M* ammonium sulfate in 50 m*M* phosphate buffer (pH 5.7). Under these conditions crystals grew to the size of $0.4 \times 0.15 \times 0.15$ mm^3 within 2–4 weeks.

It was established via X-ray analysis precession photographs that this crystal form of avidin belongs to tetragonal space group $P4_22_12$ with cell constants $a = b = 79.6$ Å, $c = 84.3$ Å. Reflections were observed to a resolution of 2 Å on still photographs. Considering the molecular mass of the avidin monomer and the V_M values of the other crystal forms, the tetragonal crystal form should contain two avidin subunits per asymmetric unit, giving rise to a V_M value of 2.14 Å/dalton.

Crystal Form of Nonglycosylated Avidin

The carbohydrate moiety of the avidin is not essential for its biotin-binding activity.[10] The biotin-binding properties of the nonglycosylated avidin are equivalent to those obtained for the native (glycosylated) avidin molecule. Owing to the unsuccessful attempts at reproducing some avidin crystal forms, we felt that the avidin with no carbohydrate moiety (thus deleting a flexible part from protein surface) might crystallize more readily than the glycosylated avidin, and well-diffracting crystals would be obtained.

Crystals are grown using the hanging drop method[3] at a constant temperature of 19°. The protein is dissolved at 4 mg/ml[11] into a solution containing 10 m*M* phosphate buffer (pH 6.0) and 18 m*M* sodium azide. Crystals are obtained using PEG 6000 as the precipitating agent. The 8-μl drops contain 2 mg/ml protein, 5.6% PEG 6000, 55 m*M* phosphate buffer (pH 5.4), and 5 m*M* sodium azide. The 1-ml reservoir contains 20% PEG 6000. Crystals grew after 4–5 days and reached the final size of $0.18 \times 0.14 \times 0.04$ mm^3 within 8 days. To obtain larger crystals we applied macroseeding techniques.[12] By reseeding the crystals at least 5 times, they reached a size of $0.4 \times 0.3 \times 0.18$ mm^3.

Our first attempt to determine the unit cell of the nonglycosylated avidin crystals via X-ray crystallography failed within a few hours as the resolution decreased drastically after 6 hr on X-ray irradiation. Using

[10] Y. Hiller, J. M. Gershoni, E. A. Bayer, and M. Wilchek, *Biochem. J.* **248,** 167 (1987); Y. Hiller, E. A. Bayer, and M. Wilchek, this volume [6].

[11] It should be noted that the solubility of nonglycosylated avidin is decreased by factor of 5 compared to avidin, apparently owing to the loss of the carbohydrate chain.

[12] C. Thaller, G. Eichele, L. H. Weaver, E. Wilson, R. Karlson, and J. N. Jansonius, this series, **114,** p. 132.

techniques of Hope[13] for extreme low-temperature X-ray data collection, we were able to preserve the lifetime of the crystal in the X-ray beam practically indefinitely. The method consists of first coating the crystal with a viscous oil in the crystallization droplet and removing all traces of mother liquor. The crystal is then picked up with a thin glass spatula, putting it directly under a stream of boiled liquid N_2 at a temperature of approximately 90 K.

The cell constants and space group were determined by measurements on a Rigaku AFC5-R rotating anode diffractometer operated at 10 kW. The crystal was orthorhombic, space group $P2_12_12$, with cell constants $a = 71.24$ Å, $b = 79.21$ Å, $c = 43.12$ Å, and contained two subunits in the asymmetric unit. On soaking biotin into the crystals the cell constants changed to $a = 73.62$ Å, $b = 79.84$ Å, $c = 43.27$ Å.

When comparing these results with those obtained from the glycosylated avidin orthorhombic crystal,[6] one can see that the crystals appear to be isomorphous, i.e., with a unit cell volume change of about 1%. It may be concluded that the absence of the carbohydrate chain appears not to affect the molecular packing in the crystal.

[13] H. Hope, *Acta Crystalogr.* **B44,** 22 (1988).

[10] Nonavidin Biotin-Binding Proteins

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Introduction

The role of biotin as the prosthetic group of the carboxylases and transcarboxylases in various organisms is well established.[1] Apart from these proteins, in which biotin is attached covalently, there are a group of proteins that bind to biotin noncovalently. Avidin, the biotin-binding protein of raw egg white of birds, and streptavidin, the bacterial analog, have an exceedingly high affinity for biotin, with a K_d of about 10^{-15} M, the strongest noncovalent binding known between a protein and a low molecular weight ligand. In addition to these two examples of exceptionally strong affinity, there are other biotin-binding proteins [such as the egg-yolk biotin-binding proteins, biotinidase (EC 3.5.1.12), biotin holocar-

[1] H. G. Wood and R. E. Barden, *Annu. Rev. Biochem.* **46,** 385 (1977).