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Structural investigation of the interactions of biotinylruthenocene with avidin

Paweł Strzelczyk [a], Anna Bujacz [a,*], Damian Plażuk [b], Janusz Zakrzewski [b], Grzegorz Bujacz [a]

[a] Institute of Technical Biochemistry, Lodz University of Technology, 90-924 Lodz, Stefanowskiego 4/10, Poland
[b] Department of Organic Chemistry, University of Lodz, 91-403 Lodz, Tamka 12, Poland

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ABSTRACT

The crystal structure of avidin, a protein from hen egg white, was determined in the form of a complex with biotinylruthenocene. This biotin-derived organometallic ligand is a potential anticancer agent for targeted therapy based upon avidin–biotin technology. Isothermal titration calorimetry experiments, involving avidin complexes with biotin (vitamin H or B_7) derivatives, show differences in their affinity to the protein in comparison to its avidin–biotin complex, the strongest known biochemical interaction in Nature. The crystal structure of the first complex of avidin with biotinylruthenocene, determined at 2.5 Å resolution (PDB: 4I60), shows unique interactions of the ruthenocene moiety with avidin. Biotin derivatives exhibit weaker affinity to avidin then biotin, which allows their wider use in biotechnology. The specific properties of biotinylruthenocene and the knowledge of its interactions with avidin may be useful in biochemical, medical, and nanotechnological applications.

1. Introduction

Avidin, a tetrameric glycoprotein capable of binding biotin with exceptionally high affinity is present in the egg-white of birds, reptiles, and amphibians. A homotetramer of avidin binds up to four molecules of biotin (vitamin H or B_7) through non-covalent interactions with $K_d \approx 10^{-13}$–10^{-15} M [1]. The strength of the avidin–biotin interaction is several orders of magnitude higher than the strength of typical antigen–antibody complexes [2,3]. In Nature the biotin binding to avidin is practically irreversible and it is needed to protect the growing embryo from microbial infections. For biochemical applications such strong interactions are sometimes very useful. For almost four decades the interactions between avidin and biotin, as well its conjugates, have been exploited as powerful and essential tools in a variety of biochemical and biotechnological applications [4–6]. Nowadays, such complexes are commonly used in numerous laboratory techniques, including immunoprecipitation, Western blotting, affinity purification, enzyme linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), as well as labeling and sophisticated drug pre-targeting [7,8].

In parallel to biochemical methodology, various conjugates and derivatives of biotin were synthesized for usage in specific applications. Most of such conjugates are based on amide bond creation between the biotin carboxyl group with the amine group of a linker or the external part of a conjugate. This group contains biotin–protein and biotin–nucleoside conjugates. Biotinylated proteins can be synthesized biochemically using enzymes called biotin ligases [9]. The chemically synthesized biotin derivatives with ester linkage [10] can be unstable in some specific *in vivo* experiments. It is also possible to create biotin derivatives possessing a C–C linkage obtained via a Friedel–Crafts reaction. Such an approach allows synthesis of biotin conjugates with various aromatic systems including metallocene and fluorescent markers [11]. The interactions of a biotin derivative, biotinylruthenocene, with avidin are described here.

Ruthenocene, as a moiety of a biologically active molecule, was incorporated into the inhibitors of human carbonic anhydrase II and its crystal structure with the target enzyme was determined [12]. The organometallic drugs based on osmium, ruthenium, and platinum have lately received increasing attention due to their anticancer activity [13,14]. It is worth noting that streptavidin has already been used as a delivery system for specific drugs containing a piano-stool ruthenium moiety attached to biotin, capable of recognizing telomeric DNA [15]. Various applications of biotin conjugates and the lack of detailed knowledge regarding protein ligand recognition in such systems justify crystallographic research on the avidin–biotinylruthenocene complex. The crystal structure of avidin–biotinylruthenocene complex has been determined by us with very good statistics and shows details of biotin conjugate interactions with protein. Thermodynamic parameters of biotinylruthenocene binding to avidin were measured by ITC and results of that experiment has proved the lower ligand affinity to

* Corresponding author. Tel.: +48 42 631 34 94; fax: +48 42 636 66 18.
E-mail address: anna.bujacz@p.lodz.pl (A. Bujacz).

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avidin than biotin, what makes it a promising candidate for the use in targeted cancer therapy based on avidin–biotin technology [16] as well as in wide range of other applications.

2. Materials and methods

2.1. Protein and ligand preparation

Avidin, supplied by USBiological (Boston, MS, United States), was additionally purified by gel filtration chromatography on an AKTA FPLC system to remove trace contaminations by other proteins and DNA. Size exclusion chromatography was performed on an XK 16/100 column (Amersham Biosciences, Uppsala, Sweden) filled with Superdex 200 pg; a mixture of 100 mM NaCl and 10 mM Tris pH 7.3 was used as a buffer. The main fraction, containing the tetramer of avidin (Fig. 1), was concentrated on Vivaspin filters with a 10 kDa cutoff (Sartorius, Goettingen, Germany) on a centrifuge under 7000 rcf (Eppendorf, Hamburg, Germany). During concentration the elution buffer was exchanged with 10 mM HEPES pH 7.5. The biotinylruthenocene was obtained by chemical synthesis based on the Friedel–Crafts acylation of an electron-rich aromatic metallocene–ruthenocene with biotinyl trifluoroacetate generated *in situ* from biotin and trifluoroacetic anhydride according to a published procedure [11].

2.2. Complex formation and crystallization

The complex of avidin with biotinylruthenocene was obtained by mixing avidin at 15 mg/ml concentration with 25 mM of biotinylruthenocene in 25% methanol in the molar ratio 1:3, followed by overnight incubation. Crystallization of the complex was conducted using the vapour diffusion method in a hanging drop. The crystals were grown at 19 °C in a drop consisting of 1 μl of protein solution (15 mg/ml) and 1 μl of the reservoir mixture, which contained 35% pentaerythritol propoxylate (5/4 PO/OH), 0.05 M HEPES pH 7.5 and 0.2 M potassium chloride.

2.3. Diffraction data collection

X-ray diffraction data were collected on the X-13 beam line at the DESY synchrotron in Hamburg. The crystal was flash frozen directly in the nitrogen cooling stream at 100 K. Due to the cryoprotective properties of the crystallization buffer no additional cryoprotectant was needed. A total number of 120 images with 1 deg oscillation were collected. Diffraction data were processed up to 2.5 Å resolution. The crystal belongs to the space group $P4_22_12$ with the unit cell parameters: $a = 60.22$, $b = 60.22$, $c = 62.47$ Å. Data collection and processing statistics are shown in Table 1.

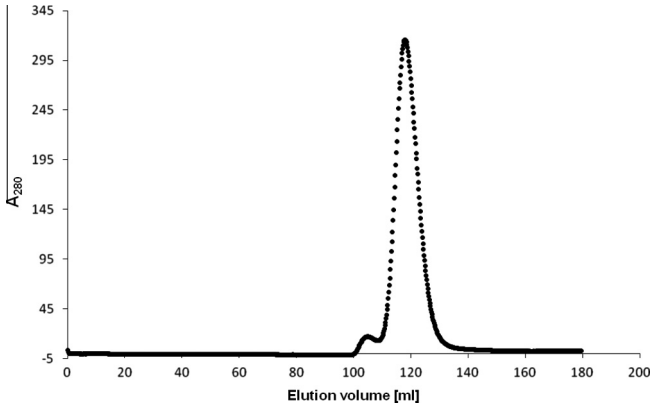

Fig. 1. Size exclusion chromatography of avidin.

2.4. Structure determination and refinement

Diffraction data were indexed, integrated and scaled using *HKL2000* [17], and then reprocessed by XDS [18,19], which resulted in better statistical parameters such as completeness, I/sigma(I) but slightly worse R-merge. The structure behaved much better during refinement using XDS processed data. The Matthews coefficient of 1.98 Å^3/Da suggested a single monomer in the asymmetric unit. The structure was phased by molecular replacement in *Phaser* [20] using an avidin (PDB: 1VYO) [21] as a search model and refined in *REFMAC5* from the *CCP4* package [22] followed by *Coot* [23]. The ligand library was prepared in *Sketcher* [24,25] from the *CCP4* package. The final model was validated by *PROCHECK* [26] and the statistics parameters of processing and refinement are shown in Table 1.

2.5. Isothermal Titration Calorimetry

The microcalorimetric isothermal titration experiment was performed on a VP-ITC Micro Calorimeter (Micro Cal Inc, Northampton, MA). All experiments were carried out using a 287 μl Hamilton syringe. The reagents in the cell were mixed with a stirring speed of 307 rpm. Samples were degassed prior the usage. The measurements were preceded by two initial injections of 2 μl biotinylruthenocene solution into the perfusion vessel. Next, the injections were repeated 53 times at an interval of 210 s with 5 μl of ligand solution. Data were corrected by subtracting the heat of dilution. All measurements were conducted in 20 mM phosphate buffer, pH 7.4 and 5% methanol at 25 °C. Concentration of avidin

Table 1
Data collection statistics and refinement results for avidin–biotinylruthenocene complex.

PDB ID	4I60
Space group	$P4_22_12$
Unit cell (Å)	$a = 60.22$, $b = 60.22$, $c = 62.47$
Molecules in asymmetric unit	1
Solvent content (%)	37.80
X-ray data collection	
Radiation source	DESY, X-13 beam-line, Hamburg
Wavelength (Å)	0.8015
Temperature (K)	100
Rotation range (°)	1
Resolution range (Å)	43.3–2.5 (2.6–2.5)[a]
Completeness (%)	99.8 (99.6)[a]
Redundancy	9.07 (8.59)[a]
I/σ(I)	19.49 (4.75)[a]
Reflections collected	39000
Unique reflections	4301
R_{marge}[b] (%)	13.2 (50.5)
Refinement	
R_{work}[c]/R_{free}[c] (%)	16.47/21.10
RMSD bond lengths (Å)	0.018
RMSD bond angles (°)	2.264
Average B factor (Å[b])	34.05
Ramachandran plot:	
Most favoured regions (%)	88.5
Allowed regions (%)	11.5
Generously allowed regions (%)	0.0
Disallowed regions (%)	0.0

[a] Values in parentheses correspond to the last resolution shell.

[b] $R_{merge} = \sum_h\sum_j|I_{hj} - \langle I_h\rangle|/\sum_h\sum_j I_{hj}$, where I_{hj} is the intensity of observation j of reflection h.

[c] $R = \sum_h||F_o|-|F_c||/\sum_h|F_o|$ for all reflections, where F_o and F_c are observed and calculated structure factors, respectively. R_{free} is calculated analogously for the test reflections, randomly selected and excluded from the refinement.