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Dissociative binding of α- and β-sulphoxides of biotinylamidoethyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionamide to avidin

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Previously we reported the dissociative binding of biotinylamidoethyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionamide to avidin [Garlick & Giese (1988) J. Biol. Chem. **263**, 210–215]. In the present paper we report the corresponding binding of the α- and β-sulphoxides of this parent compound to avidin. The 1:1 complex (obtained with avidin in excess) of the α-sulphoxide derivative with avidin has a dissociation half-life ($t_{\frac{1}{2}}$) of 25 days, only 1.6 times as fast as the parent compound ($t_{\frac{1}{2}}$ 41 days). However, the corresponding β-sulphoxide dissociates 446 times faster ($t_{\frac{1}{2}}$ 0.092 day) than the parent compound, this apparently being due to a steric effect. The α-sulphoxide is attractive as a tracer reagent to facilitate studies and applications of the avidin–biotin system.

INTRODUCTION

In a previous study we investigated the dissociative binding properties of three radiolabelled biotin derivatives to avidin [1]. Each of the compounds was an *N*-acyl derivative of biotinylethylenediamine. The three *N*-acyl groups were acetyl, 3-(4-hydroxy-3-iodophenyl)propionyl and 3-(4-hydroxy-3,5-diiodophenyl)propionyl. A variation in dissociation rates was observed, with the monoiodo compound having an intermediate value (dissociative $t_{\frac{1}{2}}$ 41 days), starting as a 1:1 molar complex with avidin (obtained with avidin in excess). The purpose of the work was to establish a radiolabelled biotin derivative to facilitate studies and applications in general of the avidin–biotin system. In the present investigation we extend this previous study by preparing and examining the dissociative binding of the α- and β-sulphoxide derivatives of the monoiodo compound to avidin.

MATERIALS AND METHODS

Materials

Affinity-purified avidin (14.6 units/mg) and (+)-biotin were from Sigma Chemical Co. ^{125}I-labelled Bolton–Hunter reagent (NEX-120) was from duPont, NEN Research Products.

Biotinylamidoethyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionamide (compound **I**), ^{125}I-labelled and non-radioactive, was prepared as described previously [1]. Dimethylformamide and ethylenediamine were redistilled before use. All other chemicals were of the highest purity available.

Chromatography

H.p.l.c. was done on a gradient system equipped with a variable-wavelength detector. γ-Radiation from ^{125}I was monitored externally through Tygon tubing by an NaI scintillation crystal and a ratemeter from Ludlum Instruments.

T.l.c. was done on silica-gel plates from Analtech. For detection of radioactivity after cutting, plastic-backed silica from Eastman–Kodak was used. Solvent *A* was butan-1-ol/acetic acid/water (70:7:10, by vol.) and solvent *B* was butan-1-ol/acetic acid/water/2-mercaptoethanol (70:7:10:2.8, by vol.). Biotin and its derivatives were specifically stained with *p*-(dimethylamino)cinnamaldehyde as described by McCormick & Roth [2]. Separation of free from bound radioactive biotin derivative in the avidin-binding assays (see below) was accomplished by spotting 3–5 μl of sample on an instant-t.l.c. strip (1.2 cm × 9 cm, from Gelman), allowing the strip to dry for 3–4 min and then developing it in 0.15 M-NaCl/ethanol (19:1, v/v).

Biotin α- and β-sulphoxides (authentic)

Biotin sulphoxides were prepared by oxidation of biotin with H_2O_2 in acetic acid followed by fractional crystallization [3]. Biotin β-sulphoxide was also prepared by an improved method using *N*-bromosuccinimide [4].

α- and β-sulphoxides of biotinylamidoethylamine

Biotin sulphoxide (α or β) (20 μmol) in 0.3 ml of dry dimethyl sulphoxide was allowed to react with 30 μmol of *NN'*-carbonyldiimidazole in 5 μl of dimethyl sulphoxide at ambient temperature for 1 h with magnetic stirring.. Ethylenediamine (200 μmol) was added, followed, after 30 min, by 1 ml of 0.53 M-acetic acid. A sample containing 125 μg of sulphoxide was diluted with 0.5 ml of aq. 0.1% (v/v) acetic acid containing 5 mM-sodium heptanesulphonate, and purified by h.p.l.c. on a Waters C_{18} column with a linear gradient of 0–10% (v/v) acetonitrile in aq. 0.1% acetic acid containing 5 mM-sodium heptanesulphonate in 10 min at 2 ml/min. The absorbance at 214 nm was monitored. Each biotinylamidoethylamine sulphoxide as a heptanesulphonate ion-pair was eluted at 16 min and was collected and immediately freeze-dried. T.l.c. of each compound in solvent *A* gave a single spot (R_F 0.05–0.1), which was positive with *p*-(dimethylamino)cinnamaldehyde and ninhydrin. It was apparent, because of other unsuccessful attempts to isolate these biotin derivatives, that they were unstable except as the heptanesulphonate salts.

Abbreviations used: compound **I**, biotinylamidoethyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionamide; **Iα(O)**, α-sulphoxide of compound **I**; **Iβ(O)**, β-sulphoxide of compound **I**.

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α- and β-Sulphoxides of compound I [Iα(O) and Iβ(O)]

Method 1. Compound I was prepared as described previously [1] except that the 2-mercaptoethanol was omitted from the 50% (v/v) ethanol used for the final dilution. Compound I (0.8 mCi in 3 ml) was oxidized to the corresponding α- and β-sulphoxides by adding 50 μl of 1 M-H_2O_2 in water at ambient temperature. After 30 min of reaction the solvent was vacuum-evaporated to about 0.3 ml, and the sulphoxide isomers were separated by preparative t.l.c. (1 mm silica thickness) in solvent *A*. The plate was scanned for radioactivity and the β-sulphoxide (R_F 0.31) and α-sulphoxide (R_F 0.44) were eluted from the scraped silica gel with 2 × 2 ml of ethanol/acetic acid/water (1:1:1, by vol.). Essentially only two radioactive products were seen. Each isomer was evaporated to dryness, redissolved in 0.5 ml of aq. 0.1% acetic acid and repurified by h.p.l.c. on a Waters C_{18} column eluted with 20% (v/v) acetonitrile in aq. 0.1% acetic acid. This additional purification was necessary to remove suspected silica contaminants from the extraction. Failure to remove these contaminants resulted in the precipitation of the lysozyme during the avidin-binding assay (see below). Each sample was evaporated to near-dryness by vacuum, and then dissolved in aq. 50% (v/v) ethanol to 50 μCi/ml and stored at −20 °C.

Method 2. H.p.l.c.-purified biotinylamidoethylamine α- or β-sulphoxide (35 μl of 1–4 μg/μl solution) in 0.5 M-potassium phosphate buffer, pH 8.0, was separately added to dried ^{125}I-labelled Bolton–Hunter reagent (10 μCi) and the reaction was allowed to proceed for 1 h at 4 °C. Each conjugate was stabilized by the addition of 200 μl of aq. 50% ethanol and stored at −20 °C. T.l.c. was done on plastic-backed strips (100 μm silica thickness) in solvent *B*. Radioactivity bands were detected by cutting 0.5 cm fractions and counting the radioactivity (γ-radiation counter efficiency was 63.5% for ^{125}I).

Reduction of Iα(O) and Iβ(O)

A modification of the procedure for the reduction of methionine sulphoxide was used [5]. Iα(O) and Iβ(O) (2 μCi each) were separately dried by vacuum, redissolved in 100 μl of mercaptoacetic acid/water (1:1, v/v) and incubated for 13 h at 63–64 °C. T.l.c. (250 μm silica thickness) was done in solvent *B*.

Dissociation kinetics for I-avidin, Iα(O)-avidin and Iβ(O)-avidin complexes

Into duplicate 12 mm × 75 mm polystyrene test tubes were added 0.25 ml of 50 mM-potassium phosphate buffer, pH 7.4, containing 0.15 M-NaCl, 0.05% lysozyme, radioiodinated tracer (0.5 μCi; 0.23 pmol) and avidin (2.5 μg, 37 pmol). After incubation for 20 min, biotin (21 μg, 85 nmol) was added and the temperature was maintained at either 4 °C or 20 °C. A temperature-controlled recirculating water bath (Haake) was used to maintain 20 °C. The percentage of tracer bound was determined at subsequent time intervals by spotting 4 μl from each sample on an instant-t.l.c. strip (1 cm × 6 cm) and developing in 0.15 M-NaCl/ethanol (19:1, v/v). The avidin and any bound compound I, Iα(O) or Iβ(O) stayed at the origin whereas any free I, Iα(O) or Iβ(O) migrated to the solvent front. The strip was cut in half and the radioactivity of each section was counted in a γ-radiation counter. Percentage bound was calculated as [(origin c.p.m.)/(origin + solvent-front c.p.m.)] × 100.

RESULTS AND DISCUSSION

We previously prepared compound I (see Fig. 1), a mono[^{125}I]iodo Bolton–Hunter derivative of biotinylamidoethylamine, as a radioactive biotin derivative for analytical studies with the avidin–biotin system [1]. The compound had

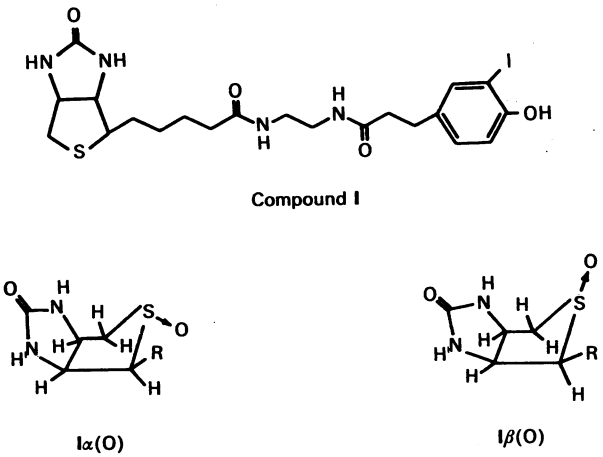

Fig. 1. Structures of the biotin derivatives

R is the valerylethylenediamine Bolton–Hunter moiety shown in compound I.

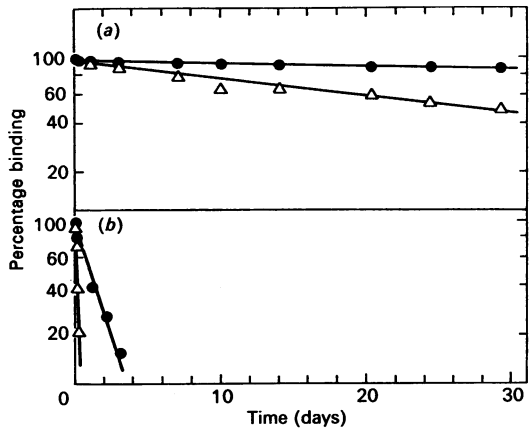

Fig. 2. Dissociation of avidin complexes of (*a*) Iα(O) and (*b*) Iβ(O)

●, 1:1 complex at 4 °C; △, 1:1 complex at 20 °C. The concentration of unlabelled biotin was 0.10 mM.

favourable binding properties to avidin, but required the presence of a thiol stabilizer such as 2-mercaptoethanol to minimize its decomposition during storage to two more-polar products, evident by t.l.c. analysis.

Suspecting (see below) that these products were the α- and β-sulphoxides of compound I [designated as Iα(O) and Iβ(O), the structures of which are shown in Fig. 1], we proceeded to prepare authentic samples starting with authentic biotin α- and β-sulphoxides. The absolute configurations of these latter sulphoxides have been established by n.m.r. [6,7]. DeTitta *et al.* [8] confirmed the structure of the α-sulphoxide by X-ray crystallography. Paton *et al.* [9] similarly confirmed the structure of an indole derivative of biotin α-sulphoxide, consistent with a prior n.m.r. assignment of this latter compound.

We separately coupled the authentic sulphoxides that we had prepared with ethylenediamine, and in turn these products were allowed to react with ^{125}I-labelled Bolton–Hunter reagent, yielding Iα(O) and Iβ(O). Both compounds were chemically stable in the absence of 2-mercaptoethanol. The products each re-formed compound I, as shown by t.l.c., when heated with mercaptoacetic acid in a procedure that converts methionine sulphoxide into methionine [5]. In this reduction Iα(O) re-formed compound I (88% in 13 h) in a slightly higher yield than did Iβ(O) (78%).

Table 1. Dissociation of biotin derivatives complexed to avidin

The radioiodinated biotin derivatives were incubated at room temperature with excess avidin for 0.3–2 h to form 1:1 complexes before the incubation temperature was set and excess biotin was added at time zero. All of the biotin derivatives gave an initial $(B/B_0) \times 100$ value of 99–100 % (obtained immediately after addition of excess non-radioactive biotin).

Compound	Dissociation half-life (days)	Temperature (°C)
I	41	20
	380	4
Iα(O)	25	20
	140	4
Iβ(O)	0.092	20
	1.0	4

Likewise, Melville [3] observed that biotin (+)-sulphoxide (later determined to be biotin α-sulphoxide) underwent more rapid reduction to biotin than did biotin (−)-sulphoxide (later determined to be biotin β-sulphoxide).

The chromatographic behaviour of Iα(O) and Iβ(O) matches that of the parent α- and β-sulphoxides of biotin. The α-sulphoxide of biotin migrates faster than the corresponding β-sulphoxide on paper chromatography [10], which is consistent with our observation that Iα(O) migrates faster than Iβ(O) on silica t.l.c.

The two decomposition products from compound I co-migrate with Iα(O) and Iβ(O) on silica t.l.c. The formation of the two products from compound I is accelerated by the addition of H_2O_2. When free biotin is oxidized with H_2O_2, the α-isomer is formed in a 4:1 ratio over the β-isomer as a consequence of steric hindrance [4]. Similar oxidation here of compound I gives a 7:1 ratio of Iα(O) to Iβ(O). The storage products from compound like Iα(O) and Iβ(O) synthesized from corresponding biotin sulphoxides, also re-form compound I when heated with mercaptoacetic acid.

We determined the dissociation half-life for a 1:1 complex (avidin binding sites in excess) of Iα(O) with avidin at both 20 and 4 °C, as shown in Fig. 2(*a*). The corresponding data for Iβ(O) are shown in Fig. 2(*b*), and the data for both are included in Table 1. Whereas Iα(O) at 20 °C dissociates from avidin only 1.6 times as fast as does compound I, Iβ(O) dissociates 446 times faster. Lowering the temperature from 20 to 4 °C slows down the dissociation of Iα(O) by 5.6-fold (from 140 to 25 days), whereas this same change in temperature slows down the dissociation of Iβ(O) by 9.3–11.6 fold (results from two experiments).

The structural details of the complex of streptavidin with biotin have been elucidated by X-ray crystallography [11]. From several considerations that have been summarized [11], one can expect that the binding of biotin by avidin will be similar. It is therefore reasonable to speculate about the faster dissociation of Iβ(O) than Iα(O) from a complex with avidin on the basis of the details of the streptavidin–biotin complex.

There may be a hydrogen bond between the α side of the biotin sulphur atom and the hydroxy group of Thr-90 in the complex of biotin and streptavidin [11]. From visual examination of the X-ray-crystallographic model of this complex (F. R. Salemme & P. C. Weber, personal communication) it is difficult to predict the net change in binding of biotin to streptavidin by converting the biotin sulphur atom into an α-sulphoxide. Nevertheless, converting the sulphur atom into a β-sulphoxide would appear to position an oxygen atom unfavourably in a region occupied by Trp-79, Trp-92 and Tyr-43 (F. R. Salemme & P. C. Weber, personal communication). Perhaps this latter type of interaction (corresponding residues in avidin are Trp-70, Phe-79 and Tyr-33, respectively [12]) is what basically gives rise to the faster dissociation of Iβ(O) than of Iα(O) from its complex with avidin.

Probably biotin sulphone, because it incorporates the unfavourable structural feature of biotin β-sulphoxide, will be found to dissociate more rapidly than biotin when complexed to avidin. Currently it is only known that the dissociation equilibrium constant for avidin binding of biotin sulphone is less than 10^{-8} M, the limit of measurement for the analytical technique that was used [2].

CONCLUSION

The dissociative binding properties of a 1:1 complex of avidin with Iα(O) and Iβ(O) have been defined. The former compound, because of its ease of preparation, chemical stability, sensitive detection, small size and slow dissociation as a complex with avidin, is attractive as a tracer reagent for use in binding studies with the avidin–biotin system.

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