Basic Studies on Heterobifunctional Biotin−PEG Conjugates with a 3-(4-Pyridyldithio)propionyl Marker on the Second Terminus

Karl Kaiser, Markus Marek, Thomas Haselgrübler, Hansgeorg Schindler, and Hermann J. Gruber*

Institute of Biophysics, J. Kepler University, Altenberger Strasse 69, A-4040 Linz, Austria. Received February 14, 1997[⊗]

Heterobifunctional poly(ethylene glycol) (PEG) derivatives with a biotin terminus have been synthesized and characterized with respect to avidin binding. Unambiguous measurement of biotinyl and pyridyldithiopropionyl end groups was established by selecting suitable assays and introducing necessary modifications. Functional studies on the binding of biotin−PEG conjugates to avidin tetramers revealed much similarity to known biotin−spacer−peptide conjugates with 7−27 atom spacers: dissociation kinetics of the initially formed 4:1 complexes were multiexponential, the complex with 2 ligands per avidin dissociating rather slowly with half-times of ∼2 days at 25 °C. The observed stability of 3:1 and 2:1 complexes with avidin is particularly significant since it allows exploitation of the additional advantages of PEG spacers, i.e. reduced steric strain in biotin−avidin−biotin bridges, reduced nonspecific adsorption of biotinylated probes and markers, and, especially, uncomparable fluorescence intensities of biotin−PEG−fluorophore conjugates as is demonstrated in the accompanying study (second of three papers in this issue).

INTRODUCTION

Avidin−biotin technology is an indispensable tool in modern bioscience (Wilchek and Bayer, 1990a). In most bioanalytical applications a probe molecule (antibody, hormone, etc.) is connected to a marker molecule (fluorophore, marker enzyme, nanogold, etc.) by a biotin−avidin−biotin (B−A−B)[1] bridge or a B−A bridge, and analogous configurations occur in biochemical and biotechnological applications (Wilchek and Bayer, 1990b). In any case, biotin residues must be covalently linked to markers and/or probes. Such biotin derivatives, however, regularly showed greatly reduced affinities for avidin/streptavidin, due to steric hindrance (Finn et al., 1980; Lavielle et al., 1983; Romovacek et al., 1983), yet high affinity and metastability of avidin−biotin interaction could be restored when 7−27 atom spacers were introduced between biotin and peptid hormones (Finn et al., 1984; Finn and Hofmann, 1985) or fluorophores (Chu et al., 1994; Schray et al., 1988).

Besides local strain of the avidin−biotin interaction, two other types of steric hindrance are to be expected in B−A−B and in B−A bridges between probes and markers: First, the four biotin binding sites on avidin/streptavidin are organized in two pairs on opposite sides of the receptor protein (Green et al., 1971), and steric repulsion between adjacent biotinylated probes/markers allows the exploitation of only one site per pair in practice (Green, 1990). Second, the recognition site of a biotinylated probe molecule (e.g. an antibody) will frequently be impaired upon B−A−B or B−A bridge formation, especially when using large markers. Such long-range steric hindrances will not be relieved by 7−27 atom spacers, and longer spacer chains are desirable.

PEG spacers appear as ideal candidates to further optimize avidin−biotin technology: (i) Any chain length can be chosen for the long-range separation of probe, avidin/streptavidin, and marker. (ii) The well-known flexibility of the PEG chains will further reduce steric interaction of all interconnected elements. (iii) As an additional benefit, PEG chains will not increase but decrease nonspecific binding of attached markers (e.g. fluorophores), due to their antiadsorptive behavior (Lim and Herron, 1992).

The crucial criterion for the usefulness of biotin−PEG conjugates was the unknown magnitude of PEG−PEG repulsion in complexes of avidin with four, three, or two biotin−PEG conjugates. To solve this question, we have synthesized heterobifunctional biotin−PEG conjugates with a reliable chromogenic marker and tested for stoichiometry and stability of avidin binding. The results demonstrated the potential of heterobifunctional biotin−PEG conjugates.

EXPERIMENTAL PROCEDURES[2]

Materials. P.a. grade materials were used as far as commercially available. Affinity-purified avidin was prepared as described (Fudem-Goldin and Orr, 1990) or obtained from Sigma (A-9271). Biotin, Boc_2O, DACA, 2,2′-dithiodipyridine, 3-mercaptopropionic acid, NHS, and 2-thiopyridone were obtained from Sigma. Biotin−NHS was prepared as described (Wilchek and Bayer, 1990c). 4,4′-Dithiodipyridine and 4-thiopyridone were obtained

* Author to whom correspondence should be addressed [telephone +43 (732) 2468-9271; fax +43 (732) 2468-822; e-mail hermann.gruber@jk.uni-linz.ac.at].

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[1] Abbreviations: ANS, 2-anilinonaphthalene-6-sulfonic acid; B−A bridge, biotin−avidin bridge; B−A−B bridge, biotin−avidin−biotin bridge; biotin, biotinoyl group; biotin-NHS, succinimidyl ester of biotin; Boc_2O, di-*tert*-butyl pyrocarbonate; Boc-, *tert*-butyloxycarbonyl group; DACA, *p*-(dimethylamino)-cinnamaldehyde; DMF, *N*,*N*-dimethylformamide; DTSP, 3,3′-dithio(succinimidylpropionate); DTT,1,4-dithiothreitol; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; Et_3N, *N*,*N*,*N*-triethylamine; 2-ME, 2-mercaptoethanol; NH_2-PEG_{800}-NH_2, *O*,*O*′-bis(2-aminopropyl)poly(ethylene glycol) 800; NH_2-PEG_{1900}-NH_2, *O*,*O*′-bis(2-aminopropyl)poly(ethylene glycol) 1900; NHS, *N*-hydroxysuccinimide; PDP, 3-(4-pyridyldithio)propionyl group; PDP-OH, 3-(4-pyridyldithio)propionic acid; PEG, poly(ethylene glycol); RT, room temperature; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; THF, tetrahydrofuran.

[2] Detailed procedures, NMR data, and a systematic comparison of different biotin end group assays can be found in the Supporting Information.

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from Aldrich. 2,2′-Dithiodipyridine, DMF, DTSP, DTT, Et_3N, 2-ME, NH_2-PEG_{800}-NH_2, and NH_2-PEG_{1900}-NH_2 were purchased from Fluka. NaCl, methanol, and 2-propanol were obtained from Riedel de Haen. Acetic acid and chloroform were purchased from Baker. ANS was obtained from Molecular Probes. Sephadex-based gels were obtained from Pharmacia. All other materials were purchased from Merck. Absolute DMF was prepared according to a standard procedure.

Buffers. Buffer A contained 100 mM NaCl, 50 mM NaH_2PO_4, and 1 mM EDTA, adjusted to pH 7.5 with NaOH. Buffer B contained 100 mM NaCl and 20 mM sodium acetate, adjusted to pH 4.5 with HCl. Buffer C contained 0.4 M Na_2HPO_4, adjusted to pH 11 with NaOH. Buffer D contained 0.2 M boric acid, adjusted to pH 9.0 with NaOH. Buffer E contained 0.6 M NaH_2PO_4, adjusted to pH 6.0 with NaOH. Polybuffer contained 20 mM citric acid and 20 mM NaH_2PO_4, adjusted to the desired pH by addition of HCl or NaOH, as required.

Synthesis of $CH_3COOH{\cdot}NH_2$-PEG_{800}-NH-Boc. Boc_2O (55 mmol in 50 mL of methanol) was slowly added to NH_2-PEG_{800}-NH_2 (55 mmol in 150 mL of methanol) and stirred overnight. After addition of acetic acid (3 mL) and toluene, the solvent was removed (60 g of crude product). Three grams of crude product was purified by silica chromatography in chloroform/methanol/acetic acid (90:10:0.1 and 70:30:5), yielding 1.25 g (1.17 mmol) of product, pure by TLC.

Synthesis of $HCl{\cdot}NH_2$-PEG_{1900}-NH-Boc. The procedure was the same as for the PEG_{800} homologue except that ion exchange chromatography was used because of too much peak tailing on the silica column. Fifty grams (25 mmol) of NH_2-PEG_{1900}-NH_2 was reacted with 5.5 g (25 mmol) of Boc_2O, resulting in 53 g of crude product. Three grams of the crude product was purified by ion exchange chromatography on SP Sephadex C-25 (sodium form, using distilled water for loading and washing and eluting with 20 mM NaCl). After volume reduction and saturation with NaCl, the product was extracted into chloroform, evaporated, redissolved with a minimal amount of methylene chloride, and precipitated with peroxide-free diethyl ether. Yield = 0.82 g (0.38 mmol), pure by TLC.

Synthesis of Biotin−NH-PEG_{800}-NH-Boc. Boc-NH-PEG_{800}-$NH_2{\cdot}CH_3COOH$ (0.90 g, 0.83 mmol) was reacted with 0.39 g of biotin−NHS (0.39 g, 1.14 mmol) in absolute DMF/Et_3N for 24 h at RT. Unreacted biotin−NHS was hydrolyzed with water. After evaporation, the residue was dissolved in 10 mL of 200 mM Na_2CO_3 and filtered through paper. The filtrate was saturated with NaCl, extracted with methylene chloride, and dried at 1−10 Pa for 1 h. Yield = 637 mg (0.51 mmol), pure by TLC.

Synthesis of Biotin−NH-PEG_{1900}-NH-Boc. The procedure was the same as for the PEG_{800} homologue: 1.9 g of Boc-NH-PEG_{1900}-$NH_2{\cdot}$HCl (0.90 mmol) was reacted with 0.46 g of biotin−NHS (1.41 mmol) and 0.22 mL of Et_3N. Yield = 1.92 g (0.82 mmol), pure by TLC.

Synthesis of Biotin−NH-PEG_{800}-$NH_2{\cdot}$HCl. Biotin−NH-PEG_{800}-NH-Boc (528 mg, 0.42 mmol) was deprotected in 98% formic acid (4 h at RT). After solvent removal, the crude product was purified on SP Sephadex C-25 and isolated in a similar way as $HCl{\cdot}NH_2$-PEG_{1900}-NH-Boc (see above). Yield = 389 mg (330 μmol), pure by TLC.

Synthesis of Biotin−NH-PEG_{1900}-$NH_2{\cdot}$HCl. The procedure was the same as for the PEG_{800} homologue: 1.5 g of biotin−NH-PEG_{1900}-NH-Boc (0.64 mmol) was deprotected, yielding 1.18 g of crude product. Five hundred milligrams of crude product was purified on SP Sephadex C-25, yielding 407 mg (178 μmol) of product, pure by TLC.

Synthesis of Biotin−PEG_{800}−PDP. Biotin−NH-PEG_{800}-$NH_2{\cdot}$HCl (93 mg, 78 μmol) was reacted with 80 mg of DTSP (200 μmol) in 10 mL of peroxide-free THF plus 50 μL of Et_3N under Ar. After overnight stirring, the solvent was removed. The residue was dissolved in buffer A (5 mL) and reduced with excess DTT (62 mg, 400 μmol) under Ar. After 15 min, the pH was lowered to 4.5 with 0.5 M acetic acid and pure biotin−NH-PEG_{800}-$NHCOCH_2CH_2SH$ was isolated by gel filtration on Sephadex G-25 in buffer B under Ar.

For synthesis of the final product, 4,4′-dithiodipyridine (35 mg, 160 μmol) was dissolved in 3.4 mL of 120 mM HCl and the column fractions containing biotin−NH-PEG_{800}-$NHCOCH_2CH_2SH$ were added under Ar. Then the pH was raised to 4.4 by addition of buffer C (~0.4 mL). After 15 min of Ar bubbling at RT, the solution was lyophilized, redissolved in water (8 mL), and filtered through paper, and pure biotin−PEG_{800}−PDP was isolated by gel filtration on Sephadex in distilled water. Yield = 19 μmol according to biotin end group titration, 99% pure by TLC.

Synthesis of Biotin−PEG_{1900}−PDP. Synthetic steps, procedures, and precautions were the same as for the corresponding PEG_{800} derivative (see above): 224 mg of biotin−NH-PEG_{1900}-$NH_2{\cdot}$HCl (98 μmol), 112 mg of DTSP (280 μmol), and 125 μL of Et_3N were reacted in 8 mL of peroxide-free THF. The resulting disulfide product was reduced with 430 mg of DTT (2.8 mmol). After acidification with acetic acid and chromatography on Sephadex G-25 M, the void peak (20 mL) containing biotin−NH-PEG_{1900}-$NHCOCH_2CH_2SH$ was added to a solution of 220 mg of 4,4′-dithiodipyridine in 2 mL of distilled water plus 220 μL of HCl concentrate. The pH was raised to 4.5 by addition of buffer C under constant bubbling with Ar. After 15 min, the mixture was lyophilized, redissolved in water, and filtered, and the filtrate was subjected to gel filtration in water as above, yielding 39 μmol of biotin−PEG_{1900}−PDP in the void peak, 99% pure by TLC.

Synthesis of 3-(4-Pyridyldithio)propionic Acid. 3-Mercaptopropionic acid (4.25 mmol; dissolved in 20 mL of methanol plus 1 mL of acetic acid) was slowly added to a solution of 4.36 mmol of 4,4′-dithiodipyridine in methanol/water/acetic acid (10 mL:10 mL:2 mL) at −10 °C, whereupon highly pure product precipitated. The crystals were successively washed with methanol/water/acetic acid, with THF, and with ethyl acetate. Drying gave 2.72 mmol of PDP-OH, pure by TLC (in chloroform/methanol/acetic acid, 70:30:0.1). The original motivation had been the synthesis of succinimidyl 3-(4-pyridyldithio)propionate, in analogy to the commercially available cross-linker SPDP. Unfortunately, the formation of succinimidyl ester was always accompanied by rapid decomposition of the 4-pyridyldithio group, the main reason being the poor solubility of the free carboxylic acid under the conditions of any known method of succinimidyl ester formation. In the present study the pure crystalline PDP-OH served as an ideal standard for the determination of ϵ_{324} of 4-thiopyridone, which is released upon addition of a slight excess of a mercaptan such as 2-ME ($4.5 < pH < 6.5$).

Quantitative Assay for 3-(4-Pyridyldithio)propionyl Groups. PDP groups were determined by quantitative release of 4-thiopyridone upon reduction with 2-ME. Typically, 300 μL of sample (containing <40 μM PDP groups in water or buffer A) was mixed with 1.2 mL of buffer E, and 25 μL of 2-ME reagent was added from an Eppendorf Multipette. Absorbance was read at 324 nm against a reagent blank. The 2-ME reagent contained 0.5 M 2-ME (3.50 mL per 50 mL) and 5 mM acetic acid

(14.3 μL per 50 mL) in distilled water. It could be stored at 4 °C at ambient atmosphere for several weeks without losing activity or producing increasing background absorbance at 324 nm, in contrast to DTT. Moreover, reaction kinetics with DTT are not as instantaneous as with 2-ME *and a gas mask is required when using DTT (but not 2-ME) for this type of assay to prevent severe irritation (noticed by all personnel after several hours of exposure).* PDP terminus concentrations were calculated from A_{324} by assuming a molar extinction coefficient of 24 000 M^{-1} cm^{-1}, as argued under Results.

Quantitative Assay for Biotin End Groups. A published fluorescence assay for avidin−biotin interaction (Mock and Horowitz, 1990) was modified. Typically, 2 mL of buffer A containing 2 μM affinity-purified avidin was mixed with 25 μL of 5 mM ANS in DMSO, and fluorescence was monitored at 328 nm excitation (slit = 10 nm) and 408 nm emission (slit = 10 nm). Refined protocols can be found in the Supporting Information.

Quantitative Assay for Empty Biotin Binding Sites in Avidin Partially Occupied with Biotin−PEG−PDP. Solutions with partially saturated avidin were obtained from gel filtrations in the course of binding studies (see below) and usually contained <1 μM unoccupied receptor sites. Two milliliters of such a solution was mixed with 25 μL of 5 mM ANS, and ANS fluorescence was monitored during titration with 5−10 μL aliquots of exactly 40 μM *d*-biotin standard as described above.

Gel Filtration Assay for Binding of Biotin−PEG−PDP to Avidin. To test for metastable binding of biotin−PEG−PDP to avidin, 0.5 mL samples of buffer A containing 50 μM "functional" avidin (as determined by titration with *d*-biotin while monitoring ANS fluorescence, see above) and various amounts of biotin−PEG−PDP were incubated for 1 h at 25 °C and subjected to gel filtration on a 1 × 48 cm column of Sephadex G-100 at RT. Samples were loaded rather quantitatively by rinsing the loading reservoir with another 200 μL of buffer A. Elution was at 0.25 mL/min with buffer A; fractions were collected at 5 min intervals. All fractions were assayed for PDP group concentrations as described above. From the resulting PDP profile the "avidin peak" and the "free PEG peak" could be identified to pool corresponding fractions (see Figure 3).

Total avidin in the peak was determined from A_{282} using the known absorbance of 1.54 at 1 mg/mL of avidin and the molecular mass 67 000 g/mol (Green, 1990) and correcting for the functional purity of 98−99% as determined by the ANS fluorescence method (which always confirmed batch specifications by Sigma). It was also demonstrated that gel filtration did not change the functional purity of avidin in control runs without ligand. Empty receptor sites on partially occupied avidin were assayed as described above.

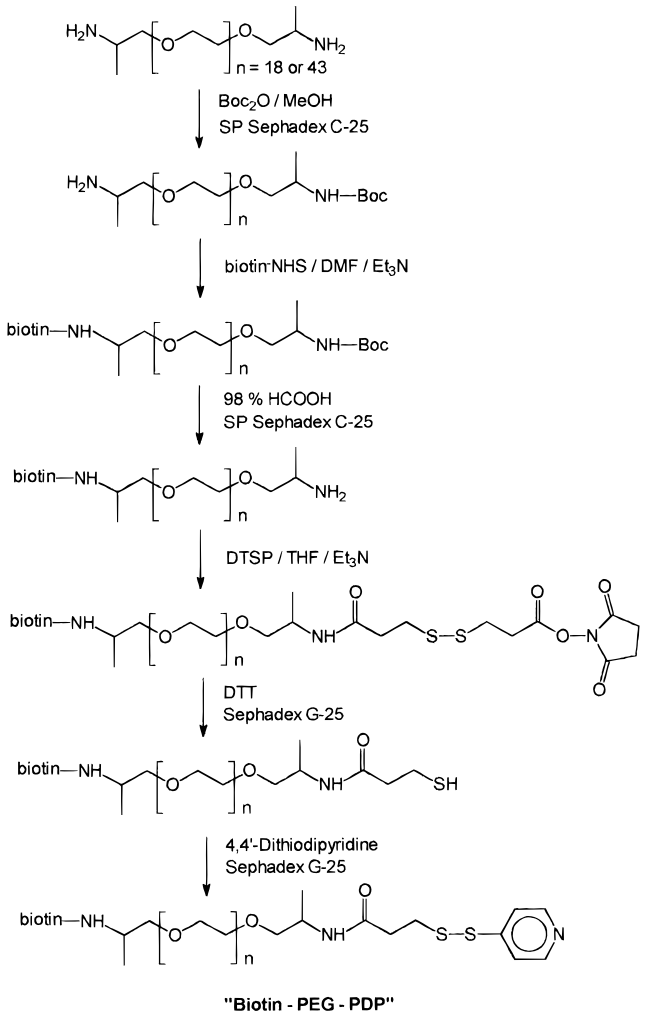
PDP group concentrations in the "avidin peak" and in the "PEG peak" were determined as described above. Biotin terminus concentrations in the PEG peak were determined according to the "mixed ANS titration method" described above.

All assays on pooled peaks were done in triplicate. Free *d*-biotin, free PDP-OH, or free 4-thiopyridone was never observed at the elution volume typical for small volumes, in spite of routine checks.

RESULTS[2] AND DISCUSSION

Synthetic Route to Biotin−PEG−PDP. The starting materials NH_2-PEG_{800}-NH_2 and NH_2-PEG_{1900}-NH_2 contained sizable fractions with one or no primary amino group, as judged from TLC in chloroform/methanol/acetic acid and from chromatrography on SP Sephadex C-25 with salt gradient elution. The sequence of steps in Scheme 1 was devised to get rid of these impurities and to arrive at maximum purity of biotin−PEG−PDP. The mono-Boc derivative collected from the first SP Sephadex C-25 column was still contaminated with monoamine impurities from the starting materials, and these amines were also biotinylated in the following step. However, after deprotection of biotin−NH-PEG-NH-Boc only the correct product biotin−NH-PEG-NH_2·HCl was retained on SP Sephadex C-25.

Scheme 1. Synthesis of Biotin−PEG_{800}−PDP and Biotin−PEG_{1900}−PDP



Having arrived at pure heterobifunctional biotin−NH-PEG-NH_2·HCl, it was essential to prevent deterioration of end group purity because further product isolations on Sephadex G-25 only selected for PEG derivatives as opposed to small molecules but not for PEGs with different end groups. It was, therefore, necessary (i) to provide for quantitative end group conversion in every subsequent step and (ii) to prevent NH_2 or SH oxidation by rigorous application of Ar atmosphere, especially for the switch to basic reaction conditions. Consequently, it was possible to synthesize biotin−PEG_{800}−PDP and biotin−PEG_{1900}−PDP with exactly 1 biotin residue on each chain and with 96% or 89%, respectively, of the expected PDP group contents. Such heterobifunctional purity favorably compares with the 15% PDP group content of CH_3-O-PEG−NH-PDP recently synthesized according to a different procedure (Woghiren et al., 1993).

The overall yields of biotin−PEG−PDP could certainly be optimized if larger amounts of such products are

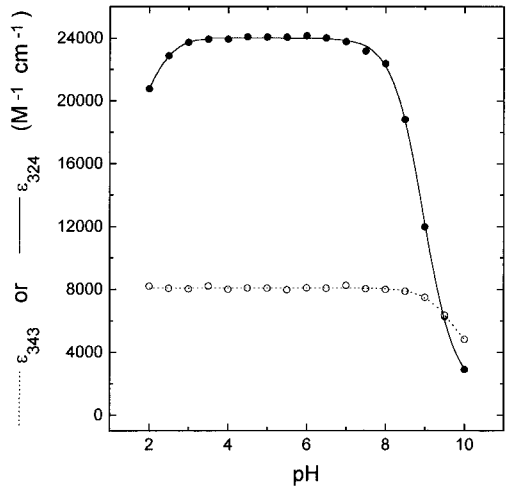


Figure 1. pH dependence of molar extinction coefficients of 4- and 2-thiopyridone. To 2 mL of polybuffer with the indicated pH value was added 50 μL of 2 mM 4-thiopyridone or 2-thiopyridone (in water), and A_{324} or A_{343}, respectively, were read against a water reference. Background absorbance of relevant reagent blanks was subtracted.

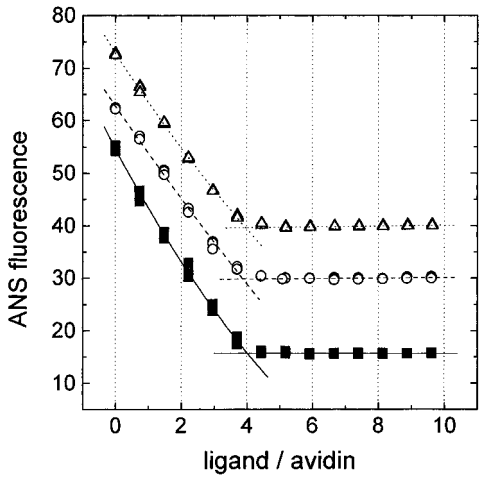


Figure 2. Demonstration of 4:1 stoichiometries in saturated complexes of biotin−PEG conjugates with avidin. Avidin with 99% functional purity was titrated with known solutions of *d*-biotin (■), biotin−NH-PEG$_{1900}$−NH$_2$·HCl (○), or biotin-PEG$_{1900}$-PDP (△). The latter two curves were displaced by +10 and +20 fluorescence units, respectively, to improve legibility of the plot.

desired. The main losses occurred during the two filtration steps (after reaction with DTSP and after lyophilization/redissolution in the final step) in which product was probably occluded in the precipitates formed by excess of reagents and/or byproducts. However, the primary goal of the present study was successfully reached, i.e. to arrive at biotin−PEG−PDP with sufficient purity for the functional experiments reported below.

Use of 3-(4-Pyridyldithio)propionyl as a Marker for Biotin−PEG Elements. PDP groups are well-known and widely used for specific coupling to free SH groups (Carlsson et al., 1978; Woghiren et al., 1993). Of course, it is implied that biotin−PEG−PDP should be useful to attach biotin−PEG elements to biomolecules with SH groups. For the purpose of this study, however, the PDP group was strictly viewed as a stoichiometric marker for biotin−PEG conjugates. The special advantage of the PDP group lies in the fact that the chromophoric marker is not the PDP group itself but free 4-thiopyridone, which is released from the PDP group after thiol addition (Grassetti and Murray, 1967), thereby eliminating any unpredictable influence of the PEG chain upon the UV−vis absorbance of a covalently attached chromophore. Such an influence has indeed been observed in subsequent studies with some biotin−PEG−dye conjugates (see the second of three papers in this issue). While the need for PDP cleavage required much pipetting (e.g. to measure the PDP profiles in Figure 3), the payoff was the unambiguous quantitation of biotin−PEG conjugates, whether bound to avidin or not.

The 4-pyridyldithio group was far more attractive than the 2-pyridyldithio function because of its much higher UV absorbance after cleavage (Grassetti and Murray, 1967). Unfortunately, conflicting values have been given in the literature for the molar extinction coefficients of 4- and 2-thiopyridone (Carlsson et al., 1978; Grassetti and Murray, 1967; Jou et al., 1983; Leserman et al., 1984). Figure 1 shows a re-examination, as well as the pH dependence of, the UV absorbance of 4- and 2-thiopyridone.

For 4-thiopyridone, ϵ_{324} = 24 000 M^{-1} cm^{-1} was obtained in the plateau region (3.5 ≤ pH ≤ 6.5). In parallel, 25 300 and 22 500 M^{-1} cm^{-1} were measured for 4-thiopyridone released from known concentrations of 4,4′-dithiodipyridine and from newly synthesized PDP-OH, respectively. Thus, the values from all available standards closely agreed, and the average value ϵ_{324} = 24 000 M^{-1} cm^{-1} was chosen for determination of PDP concentrations.

The UV absorbance of 2-thiopyridone was re-examined by DTT-induced cleavage of 2,2′-dithiodipyridine preparations from Fluka and Sigma and of crystalline 3-(2-pyridyldithio)propionic acid synthesized in the course of a preceding study (Haselgrübler et al., 1995). Obtained values for ϵ_{343} were 8040, 8050, and 8150 M^{-1} cm^{-1}, respectively. The pH profile was measured with 2-thiopyridone from Sigma, which initially gave 8600 M^{-1} cm^{-1} in the plateau region (pH ≤7.5) when 100% purity was assumed. The plateau in Figure 1 was set to 8080 M^{-1} cm^{-1} to compensate for impurities and to comply with the best possible estimate.

Figure 1 shows that the less common 4-isomer of the PDP group is a 3-fold better chromogenic marker than the 2-isomer, but knowledge of pH dependence is even more critical because absorbance becomes pH-dependent at pH ≥6.5, rather than at pH ≥7.5, respectively. Fortunately, cleavage of the 4-isomer by DTT or 2-ME under typical assay conditions is rapid or instantaneous, respectively, even at pH 6.0 (data not shown).

Quantitative Determination of Biotin End Group Content. As shown below, the functional characterization of biotin−PEG−PDP required accurate determination of the PDP marker functions, as well as of the biotin termini. In a systematic investigation[2] the ANS assay (Mock and Horowitz, 1990) was found to yield unambiguous results if complete titrations were performed, as shown in Figure 2. In the absence of a biotin derivative (= ligand) the pseudoligand ANS weakly binds to all four functional biotin binding sites on avidin, resulting in high ANS fluorescence. Successive addition of *d*-biotin (solid squares, calibration), biotin−PEG$_{1900}$−PDP (open circles), or biotin−NH-PEG$_{1900}$-NH$_2$·HCl (open triangles) results in displacement of weakly bound ANS, with concomitant loss of ANS fluorescence. The breakpoint unequivocally corresponds to saturation of avidin with exactly 4 biotin derivatives per functional avidin tetramer. Such titration method is very robust, i.e. insensitive to the second functional group on the PEG chain, because only the breakpoint in the titration profile and not the magnitude of the fluorescence signal is used for quantitative evaluation. Thus, even intensely colored biotin−PEG−fluorophore conjugates could be characterized according to

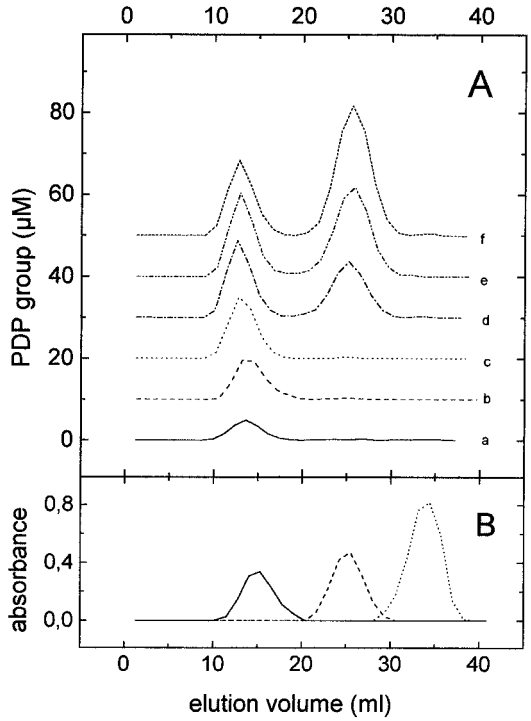


Figure 3. Chromatography of avidin complexes with biotin−PEG_{1900}−PDP on Sephadex G-100: (A) loaded samples contained 1, 2, 3, 6, 8, or 10 ligands per avidin tetramer, yielding PDP profiles a, b, c, d, e, or f, respectively; (B) control of nonspecific binding and of column performance. Avidin (—, A_{282}) had been blocked with *d*-biotin (177 mol/mol of avidin) before the usual incubation with biotin−PEG_{1900}−PDP (- - -, PDP profile, A_{324} after thiopyridone release) and chromatography. In a parallel run, 0.5 mL of 4 mM NaN_3 was eluted (• • •) to mark the peak of small molecules. Virtually identical results (as shown in panels A and B) were seen in fully equivalent series with biotin−PEG_{800}−PDP except that the free PEG_{800} derivative eluted slightly later than the free PEG_{1900} derivative, yet the PEG_{800} peak was still well resolved from the salt peak marked by NaN_3.

this method (see the second of three papers in this issue). The same biotin end group contents as with the ANS assay were also found with other methods,[2] albeit less clear-cut and with much less convenience.

Assessment of Purity of Heterobifunctional Biotin−PEG−PDP Conjugates. For a final assessment the following findings had to be taken into account: (i) The biotin/PDP ratios in biotin−PEG_{800}−PDP and in biotin−PEG_{1900}−PDP were 100:96 and 100:89, respectively, when assayed as described above. (ii) All biotin and PDP termini were covalently linked to PEG molecules since they coeluted in the void volume of Sephadex G-25 in the final synthetic step (see Experimental Procedures). (iii) According to TLC, both the immediate precursors and the two products were homogeneous, by all specific staining criteria.[2] (iv) Most importantly, the "bindability" of biotin−PEG_{800}−PDP and biotin−PEG_{1900}−PDP toward slight excess of avidin was ≥99%, while nothing was bound to avidin preblocked with *d*-biotin (see Figure 3). Taken together, the data consistently indicate that every PEG chain had exactly one biotin terminus, while the slight imperfection on the PDP side may in part be due to residual uncertainty about ϵ_{324} of 4-thiopyridone and/or a small percentage of symmetric disulfides (biotin−NH-PEG-$NHCOCH_2CH_2S]_2$).

Proof of 4:1 Stoichiometry and Measurement of Metastability in the Complex of Avidin with Biotin−PEG−PDP. The main idea of this study was to test whether four biotin−PEG conjugates can bind to avidin tetramers in a stoichiometric way, in spite of

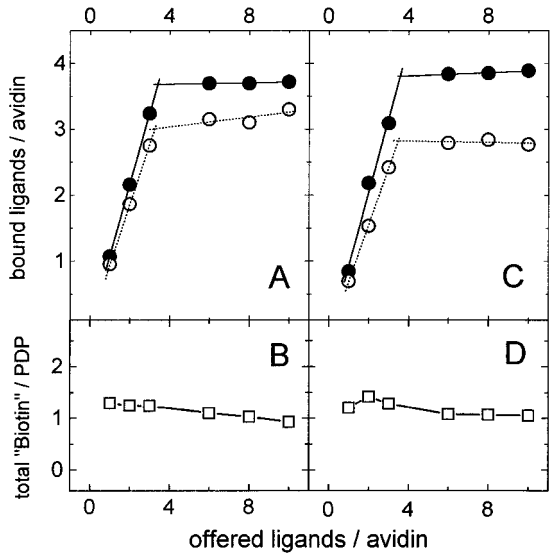


Figure 4. Stoichiometric ratios of biotin−PEG−PDP/avidin after chromatography on Sephadex G-100: (A) binding of biotin−PEG_{1900}−PDP to avidin [eluted avidin peaks from Figure 3 were analyzed for occupied sites/avidin (●) or for PDP markers/avidin (○; before plotting, the numbers were multiplied by 100/89 to account for those 11% of PEG_{1900} molecules which had a biotin end group while lacking the PDP terminus)]; (B) "biotin" groups in the total chromatograms of Figure 3 were calculated by adding the number of occupied sites on avidin to the number of biotin groups seen in the PEG_{1900} peak (PDP groups in the total chromatograms of Figure 3 were calculated by adding the number of PDP markers in the avidin peak to the PDP groups in the PEG_{1900} peak; the ratio of total "biotin"/total PDP is plotted); (C) binding of biotin−PEG_{800}−PDP to avidin, analogous to panel A (the number of PDP markers/avidin were corrected for those 4% of biotin−PEG conjugates which lacked the PDP terminus); (D) ratio of "biotin"/PDP in experiments with biotin−PEG_{800}−PDP, analogous to panel B.

possible PEG−PEG repulsion, and how stable such complexes would be. This implied the use of 98−99% functionally pure avidin tetramers for all relevant studies. The ANS assay (Figure 2) clearly demonstrated that biotin−NH-PEG_{1900}−NH_3^+ Cl^- (open triangles) and biotin−PEG_{1900}−PDP (open circles) bind to intact avidin tetramers in the same 4:1 ratio as does *d*-biotin (solid squares) under equilibrium conditions.

Metastability and specificity of binding was tested by gel filtration on Sephadex G-100 (Figure 3), by which three well-resolved peaks were obtained for avidin, for free PEG molecules, and for small molecules (see Figure 3B and legend to Figure 3). Speaking in the terminology of receptor pharmacologists, we tested for "bindability", "saturation", and "displacement".

Bindability was 1 because all PDP marker groups comigrated with avidin at excess of receptor sites/ligands (traces a−c in Figure 3A).

Saturation was tested at excess of ligand/receptor sites (traces d−f in Figure 3A). As expected, a constant amount of PDP marker groups comigrated with avidin, while the variable excess of PDP markers was eluted at the position of free PEG molecules.

Quantitative evaluation of ligand/avidin stoichiometry after gel filtration is shown in parts A and C of Figure 4 for biotin−PEG_{1900}−PDP and biotin−PEG_{800}−PDP, respectively. As expected, avidin appeared to be occupied by almost 4 ligands/tetramer when judged by measurement of occupied and empty binding sites (solid circles). In contrast, maximum ligand binding appeared to be only ~3 (open circles) when PDP markers per avidin were counted. Obviously, PDP markers had been lost during

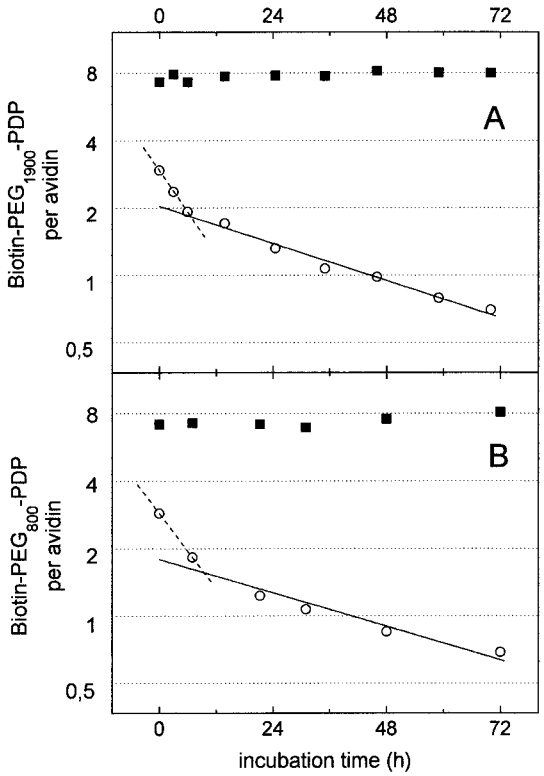


Figure 5. Displacement of biotin−PEG−PDP from complexes with avidin. See text for details.

gel filtration, while biotin endings had been retained in the receptor sites of avidin.

Loss of *small* PDP fragments could be ruled out by the absence of small PDP-containing molecules in the Sephadex G-100 chromatograms (see Figure 3A and legend to Figure 3). Loss of *full-sized* PEG−PDP elements, however, was clearly observed because the "free PEG peak" in Figure 3A always contained an excess of biotin-free PEG−PDP elements in addition to intact biotin−PEG−PDP. This excess quantitatively corresponded to the "missing" PDP markers in the avidin peak. In other words, the total "biotin" (= measured *occupied* sites in the avidin peak plus measured biotin groups in the free PEG peak) nicely fitted with the total eluted PDP markers within experimental uncertainty (see Figure 4B,D).

The observed phenomenon necessarily means that ∼1/2 or ∼1 mol of PEG−PDP element per mole of avidin had been lost from saturated complexes with biotin−PEG_{1900}−PDP (solid circles minus open circles in Figure 4A) or biotin−PEG_{800}−PDP (solid circles minus open circles in Figure 4C), respectively, and that the missing biotin termini appeared stuck in their original receptor site on avidin. Here it seems appropriate to speculate that PEG chain crowding might provide the driving force and some nucleophilic group on avidin might provide the catalysis for "de-biotinylation" of bound biotin−PEG−PDP. At least, any alternate explanation seems even less acceptable.

Dissociation kinetics were analyzed over long time periods as shown in parts A and B of Figure 5 for biotin−PEG_{1900}−PDP and biotin−PEG_{800}−PDP, respectively. Mixures (8:1) of ligand/avidin were subjected to active ligand displacement by adding *d*-biotin (340 mol/mol of avidin) at zero incubation time. After the indicated incubation times (at 25 °C), aliquots were gel filtered on Sephadex G-100 as before. Recovery of protein and of biotin−PEG−PDP was almost quantitative (the small losses being due to imperfect loading). In particular, the PDP/avidin ratios in the total elution volumes (∼8:1, solid squares in Figure 5A,B) were equivalent to those of the loaded samples; that is, no selective adsorption of any component had occurred. Bound ligands/avidin in the protein peak were determined from the ratio of PDP marker groups/avidin (open circles in Figure 5A,B).

The time course of biotin−PEG−PDP displacement by *d*-biotin was very similar for biotin−PEG_{1900}−PDP (Figure 5A) and for biotin−PEG_{800}−PDP (Figure 5B), and both were apparently biphasic.

The starting point at "zero time" (no addition of *d*-biotin) always showed ∼3 PEG−PDP markers bound per avidin tetramer, mainly due to *chemical* loss of PEG−PDP elements (as argued above) and partly due to physical dissociation of intact biotin−PEG−PDP molecules during the 1 h elution time.

Displacement by *d*-biotin of biotin−PEG−PDP from the 3:1 complex with avidin then proceeded with a half-times of ∼10 and ∼11 h for PEG_{1900} and PEG_{800}, whereas the 2:1 complexes showed much slower dissociation kinetics, the half-times being ∼44 and ∼48 h, respectively.

Thus, the measured dissociation kinetics of biotin−PEG conjugates represent no exception to the rule that biotin derivatives with bulky residues display biexponential time courses (Finn and Hofmann, 1985; Hofmann et al., 1982). The longevity of the 2:1 complexes is known to originate from the actual geometry of the avidin tetramer: The pairwise arrangement of receptor sites renders the *trans* complex with one ligand in each pair of sites most stable (Green, 1990).

Displacement of biotin−PEG−PDP by excess of *d*-biotin was inapplicable for testing the *specificity* of ligand binding because complete dissociation would have taken weeks at room temperature. Instead, avidin was irreversibly blocked by a large excess of *d*-biotin [dissociation half-time of 200 days according to Green (1990)] before addition ofbiotin−PEG−PDP and loading on Sephadex G-100. As shown by the results in Figure 3B, there was absolutely no nonspecific binding between avidin (solid lines) and biotin−PEG_{1900}−PDP (dashed lines) or biotin−PEG_{800}−PDP (equal results, not shown).

Conclusions. The goal of this study was to test the practical potential of heterobifunctional biotin−PEG conjugates. Basic steps were to synthesize biotin−PEG−PDP and to establish analytical procedures for unequivocal counting of biotin and PDP termini in aqueous samples. Consequently, it was necessary to analyze the stoichiomety and metastability of avidin binding by such novel biotin−PEG ligands.

The results showed that avidin indeed binds 4 biotinylated PEG chains per protein tetramer and that the dissociation kinetics are slow enough to utilize 3:1 complexes (∼10 h half-life) and 2:1 complexes (∼2 day half-life) in typical applications. Similar biphasic dissociation kinetis and half-lives have been reported for biotin derivatives with 7−27 atom spacers (Finn et al., 1984; Finn and Hofmann, 1990; Hofmann et al., 1982). In the accompanying study with biotin−PEG−fluorophore conjugates it is shown that even 4:1 complexes are sufficiently stable for the minute time scale of most applications (see the second of three papers in this issue).

The essential point of the present study, however, does not depend on any gradual advantage/disadvantage of PEG spacers as opposed to conventional 7−27 atom spacers! Most important is the mere fact that biotin−PEG conjugates are indeed good ligands for avidin−and thus allow exploitation of all other advantages of PEG spacers: (i) minimized steric strain on probe molecules (e.g. antibodies) in B−A−B or B−A bridges, (ii) reduced

nonspecific adsorption of markers (e.g. fluorophores) to biological specimen, and (iii) virtually unquenched fluorescence in avidin/streptavidin-bound biotin−PEG−fluorophore conjugates, as demonstrated in the accompanying study (see the second of three papers in this issue).

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Supporting Information Available: Detailed procedures, NMR data, and a systematic comparison of different biotin end group assays (20 pages). Ordering information is given on any current masthead page.

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