Presentation of Ligands on Hydroxylapatite

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Bioconjugate Chemistry

Reprinted from
Volume 8, Number 2, Pages 103–105
Presentation of Ligands on Hydroxylapatite

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Received December 17, 1996®

Conjugates of biotin with the decamer of glutamic acid (glu10) and the trimer of D,L-2-amino-5-phosphonovaleric acid (I) have been synthesized, and it has been shown that they mediate the binding of avidin to hydroxylapatite. In a similar way a conjugate of methotrexate with glu10 mediates the binding of dihydrofolate reductase to the mineral. The presentation of ligands on the hydroxylapatite component of bone may find applications in clinical medicine.

Peptides containing several aspartic and glutamic acid residues, oligonucleotides, and other polyanions bind strongly but reversibly to hydroxylapatite; this is the basis of hydroxylapatite chromatography (1, 2). We have recently found that oligomers of glutamic acid as short as the hexamer bind quantitatively to hydroxylapatite and are not removed by washing with water or a 0.1 M NaCl solution (3). This suggests that negatively charged polypeptides might be used as linkers to bind ligands to the mineral component of bone with controllable affinity and retention time. In this paper we show that conjugates of biotin and methotrexate with negatively charged peptides may be used to mediate the binding of avidin and dihydrofolate reductase, respectively, to hydroxylapatite.

Glu10 was synthesized by the Peptide Biology Laboratory at The Salk Institute. D,L-2-Amino-5-phosphonovaleric acid (I), methotrexate (MTX), chicken liver dihydrofolate reductase (DHFR), and N-hydroxysuccinimide (N-OH-succ) were obtained from Sigma; 1,1-carbonyldiimidazole (CDI) and dicyclohexylcarbodiimide (DCC) were from Aldrich. Succinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin II) was obtained from Pierce, streptavidin from Boehringer, 125I-labeled streptavidin from Amersham, and hydroxylapatite (HA) from Bio-Rad.

The biotin derivative of glu10 was synthesized by reacting 25 nmol of the oligomer with 190 nmol of NHS-LC-Biotin II in 20 μL of 0.1 M NaHCO3 buffer at pH 8.4 for 45 min. The product was purified on a C18 column using a 0.1% TFA/acetonitrile gradient and its identity confirmed by LDMS (calculated for C96H97N13S4O34S + Na+ 1670.6; found 1670.7).

The MTX derivative of glu10 was synthesized via an N-hydroxysuccinimide intermediate (4). A solution (40 μL) containing 0.05 M MTX, 0.05 M N-OH-succ, and 0.05 M DCC in DMF was allowed to stand at room temperature for 1 h and then at 2–4 °C overnight. Ten microliters of the resulting solution was added to 5–25 nmol of glu10 in 30 μL of 0.02 M NaHCO3 at pH 8.2. The reaction mixture was shaken in the dark for 4 h and then diluted with 70 μL of water. Unreacted MTX and salts were removed by shaking the reaction mixture with 10 mg of HA overnight, removing the supernatant, and washing the HA with water. Glu10 and its MTX conjugate were eluted by shaking the HA with 2 × 50 μL of 0.02 M pyrophosphate for 30 min. The conjugate was purified by HPLC on a C18 column. Its identity was confirmed by LDMS (calculated for C76H92N18O35 + H+ 1745.6; found 1745.0).

Oligomers of D,L-2-amino-5-phosphonovaleric acid (pvl) were synthesized from the monomer (I) using carbonyldiimidazole (CDI) as a condensing agent (5). A solution of the monomer at pH 8 (0.05–0.1 M) was added to a 3-fold excess of solid CDI, and the resulting solution was allowed to stand for 6 h (or overnight). Products ranging from the dimer to the pentamer were identified by paper chromatography (n-PrOH/NH3/H2O 7:1:2), and samples of the oligomers were eluted from the paper. HPLC of the reaction mixture on an RPC-5 column gave a series of peaks that were assigned to oligomers of known length by cochromatography with the material eluted from paper.

To determine the shortest oligomer that binds to HA, 2–3 μg of the dimer, trimer, tetramer, or pentamer was separately shaken with 10 mg of HA, and any oligomer retained by the HA was eluted with K2P2O7 as described above. HPLC analysis of the supernatant and K2P2O7 eluate showed that trimers and longer oligomers of pvl were found only in the eluate and therefore had been bound by the HA. Dimers were not bound to HA and were found in the supernatant fraction.

To obtain the biotin derivative of (pvl)3, 6 μg of the tripeptide isolated from RPC-5 was first adsorbed to 10 mg of HA. The solid was separated by centrifugation and washed with H2O to remove Tris and other components of the HPLC buffer. (Pvl)3 was then eluted with pyrophosphate as described above. (Pvl)3 (5–10 μg) in 20 μL of buffer containing 0.2 M pyrophosphate and 0.2 M NaHCO3 (pH 8.4) was added to 0.1 mg of solid NHS-LC Biotin II. The reaction mixture was then allowed to stand for 1 h at room temperature. The biotinyl derivative of the tripeptide was purified and isolated using an
RPC-5 column. Its identity was confirmed by ESMS (calculated for $C_{31}H_{57}N_{6}O_{16}P_{3}$ - H 893.2; found 893).

To recruit streptavidin to HA, 1 nmol of biotin-glu$_{10}$ or biotin-(pvl)$_3$ was first shaken with 1 mg of HA in 20 $\mu$L of 0.01 M Tris-CI$\text{O}_{4}$ for 6 h (or overnight) at room temperature. The supernatant was removed by centrifugation, and the HA was washed with 100 $\mu$L of water. A solution of 0.1 nmol of $^{125}$I-labeled streptavidin (25 000–50 000 cpm) in 100 $\mu$L of buffer containing 1 M KCl and 0.01 M phosphate at pH 6.5 was added to the HA and shaken for 45 min. The supernatant was removed by centrifugation and the HA washed several times with 200 $\mu$L of H$_2$O. The amounts of radioactivity found in the supernatant, wash, and HA fractions were then measured (see Table 1). In control experiments, biotin-glu$_{10}$ was replaced by glu$_{10}$ and biotin-(pvl)$_3$ was replaced by (pvl)$_3$. When a peptide bound to the HA was ligated to biotin about 70% of the streptavidin was recruited to the HA and 25–30% remained in the supernatant (Table 1). In the control experiments no more than 5% of the streptavidin was bound to the HA. Clearly the preadsorption of biotin conjugates of negatively charged polypeptides greatly enhances the adsorption of avidin to HA.

To recruit dihydrofolate reductase to HA, 1 nmol of MTX-glu$_{10}$ was adsorbed to HA as described above for biotin-glu$_{10}$. DHFR (0.52 nmol) in 200 $\mu$L of buffer containing 0.1 M ammonium sulfate, 0.01 M potassium phosphate, at pH 6.4, and 5% glycerol was added to the HA and shaken for 45 min. The HA was separated from the supernatant, washed with 100 $\mu$L of water, and then eluted twice with 20 $\mu$L of 0.02 M $K_4P_2O_7$. In control experiments the MTX-glu$_{10}$ was replaced by glu$_{10}$. The supernatant, the washes, and the pyrophosphate eluate were analyzed on a 6% acrylamide SDS gel using Coomassie Blue to visualize DHFR. Figure 1 shows that in the control experiments with glu$_{10}$ more than 75% of the DHFR was found in the supernatant (Figure 1, lane 1) and only a small amount in the pyrophosphate eluate (Figure 1, lane 3). In experiments involving MTX-glu$_{10}$ more than 75% of the DHFR was found in the pyrophosphate eluate (Figure 1, lane 6) and very little in the supernatant (Figure 1, lane 4). Preadsorption of MTX-glu$_{10}$, therefore, greatly increases the amount of DHFR that binds to HA.

The above results show that conjugates of various ligands with anionic polypeptides adsorbed noncovalently on hydroxyapatite could be used as supports for affinity chromatography. More importantly, HA presents special opportunities in a related context, because it is the main mineral component of bone. The surface of bone is freely accessible to molecules in the extracellular fluid even if they are as large as proteins (6). The bisphosphonates, small molecules carrying four negative charges, have been used extensively to attach technetium to hydroxyapatite for bone scintigraphy (7). One example of the recruitment of an anticancer drug, methotrexate, to bone using a bisphosphonate has been reported (8). We believe that anionic polypeptides may prove particularly convenient as carriers of ligands to bone and may sometimes have advantages over the bisphosphonates.

### Table 1. Biotin-Mediated Binding of Streptavidin to Hydroxyapatite

<table>
<thead>
<tr>
<th></th>
<th>$^{125}$Istreptavidin in supernatant</th>
<th>$^{125}$Istreptavidin on hydroxyapatite</th>
</tr>
</thead>
<tbody>
<tr>
<td>glu$_{10}$</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>pvl$_3$</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>biotin-glu$_{10}$</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>biotin-pvl$_3$</td>
<td>32</td>
<td>68</td>
</tr>
</tbody>
</table>

Figure 1. Coomassie Blue stained SDS gel showing DHFR in the supernatant (lanes 1 and 4), the wash (lanes 2 and 5), or the pyrophosphate eluate (lanes 3 and 6) after shaking a solution of the enzyme with glu$_{10}$-bound hydroxyapatite (HA) (lanes 1–3) or with MTX-glu$_{10}$-bound HA (lanes 4–6). One nanomole of glu$_{10}$ or MTX-glu$_{10}$ was shaken with 1 mg of HA for 6 h. Excess peptide was removed by washing. The glu$_{10}$ or MTX-glu$_{10}$-bound HA was then shaken with 0.52 nmol of DHFR for 45 min. The supernatant was removed by centrifugation and the HA washed with 100 $\mu$L of water. The glu$_{10}$ and MTX-glu$_{10}$ together with any bound DHFR were eluted from the HA by shaking with 2 x 50 $\mu$L of pyrophosphate solution for 30 min.

The mechanism of action of bisphosphonates on bone resorption is not fully understood, but it seems clear that it is not entirely a matter of adsorption to hydroxyapatite. The properties of osteoblasts are profoundly affected by micromolar concentrations of bisphosphonates, suggesting that they attach to receptors, possibly pyrophosphate receptors, on the cell surface (9). The structures of polypeptides are completely unrelated to that of inorganic pyrophosphate, so by using them as carriers it should be possible to dissociate the direct effects of adsorption to HA from the indirect effects due to interaction with extracellular receptors on osteoblasts (or osteoclasts).

Polypeptides are uniquely convenient as carriers, because effective automated methods are already available for their synthesis, and the use of combinatorial peptide libraries is well-established. The strength of adsorption of the carriers could easily be controlled via their length, while more or less degradable carriers could be obtained by varying the ratio of D- to L-residues. In the special case of a peptide ligand, the ligand and the anionic carrier could be assembled in a single single-phase peptide synthesis. Presentation of ligands on HA that interact directly with receptors on osteoblasts or osteoclasts, or which recruit proteins to bone, may find applications in medicine.

### ACKNOWLEDGMENT

This work was supported by Grant GM33023 from the National Institute for Allergy and Infectious Diseases and Grant NAWG-1860 from the National Aeronautics and Space Administration. We are grateful to Prof. A. Michael Parfitt (University of Arkansas for Medical Sciences) for much helpful advice. We thank Aubrey R. Hill, Jr., for technical assistance and Sylvia Bailey for manuscript preparation.

### LITERATURE CITED