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Protein-Mediated Particle Assembly

Stanley Brown[†]

Department of Molecular Cell Biology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353, Copenhagen K, Denmark

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ABSTRACT

This report describes a bifunctional protein able to associate dissimilar materials. One domain of the protein adheres to metallic gold. The other domain contains a substrate for enzymatic biotin transfer. The protein is able to assemble micron-diameter gold and streptavidin-coated polystyrene spheres in a programmed manner. Both protein domains function independently and therefore can be fused to proteins displaying a variety of binding and assembly traits.

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The assembly of dissimilar materials can be mediated by bifunctional reagents (reviewed in ref 1). To mediate the programmed assembly of objects of materials "A" and "B," the bifunctional reagent must adhere to at least one of the materials in a monovalent manner. That is, it must adhere to "A" with a binding domain sufficiently small that it cannot adhere to two "A" particles simultaneously. Alternatively, that same reagent must adhere to "B" in such a manner. Furthermore, the specificity of binding must be sufficient to prevent the "A"-binding domain from adhering to "B" and vice versa.

A gold-binding protein provided one of the adhesive properties. The gold-binding protein was identified from a random population of repeating polypeptides fused to the λ receptor as described previously.[2] The repeating polypeptides recovered from this procedure were initially characterized as fusion proteins with alkaline phosphatase (AP, ref 3). One repeating polypeptide containing 11 repeats of the selected peptide, 11GB-AP, bound avidly to metallic gold but very weakly to other surfaces tested. A measurement of binding behavior by 11GB-AP and by a derivative containing only 6 repeats of the selected peptide fused to AP, 6GB-AP, is illustrated in Figure 1. In this measurement, radiolabeled hybrid APs in solution were mixed with target powders. Protein adhering to the target powder was released with detergent and separated by size with gel electrophoresis. The intensity of the band reflects the amount of protein that adhered to the powder. It can be seen in Figure 1 that AP lacking a repeating polypeptide failed to bind to gold or platinum powder but bound weakly to silver and strongly to zinc sulfide. AP bearing 11 repeats of the selected peptide bound well to gold powder, slightly to silver and zinc sulfide, and not at all to platinum. In fact, 11GB-AP bound to silver and to zinc sulfide powders less than AP lacking a repeating polypeptide, especially if we compare the samples released from 1 mg of powder. Samples containing 1 mg of ZnS perturbed electrophoresis and were not examined. Thus, the repeating polypeptide portion of 11GB-AP showed clear specificity for gold, at least among the metals tested. Although the hybrid protein with fewer repeats, 6GB-AP, retained avidity for gold powder, it had less specificity than 11GB-AP. The change in specificity can be explained if the protein conformation that adheres specifically to gold was stablilzed by the greater number of repeats. Degradation products of the hybrid proteins can be seen below the major bands. The amino acid sequence of the repeating polypeptide $(QATSIGVEKLAGMAESKPTKT)_n$ was inferred from the DNA sequence.

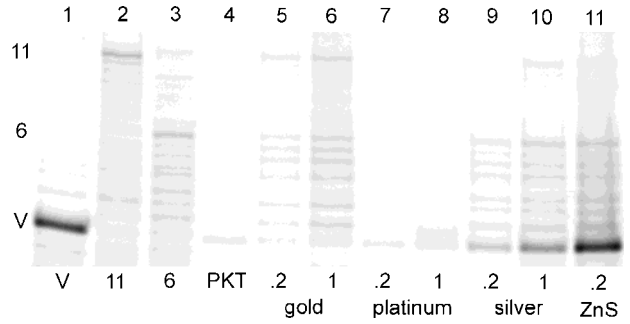

Figure 1. S2157 harboring the alkaline phosphatase vector, pSB3616 (V), or pSB3627 encoding 6GB-AP (6) or pSB3621 encoding 11GB-AP (11) was radiolabeled with ^{35}S-methionine. Osmotic shock fluid was prepared and either mixed individually with gel sample buffer (lanes 1−3) or the three extracts were pooled and allowed to adhere to various powders (lanes 4−11). Samples prepared for lanes 5, 7, 9, and 11 received 0.2 mg of powder. Samples prepared for lanes 6, 8, and 10 received 1 mg of powder. The sample prepared for lane 4 received buffer only (PKT). Samples prepared for lanes 5 and 6 received gold powder, for lanes 7 and 8 platinum powder, for lanes 9 and 10 silver powder, and for lane 11 ZnS powder. Binding to powders was conducted as described,[3] the bound proteins released and electrophoresed through an 8% acrylamide SDS gel as described.[3]

[†] E-mail: stanley@biobase.dk.

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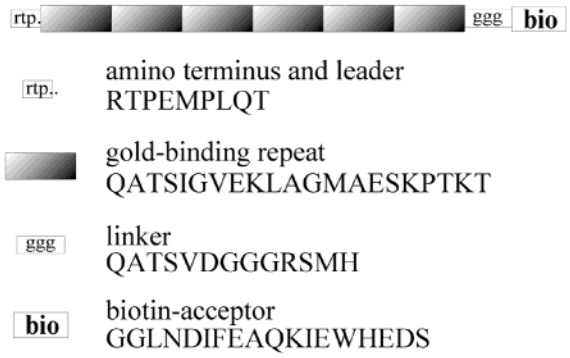

Figure 2. Schematic diagram of 6GB-bio. The lysine (K) in the biotin-acceptor portion is the site of biotin transfer.

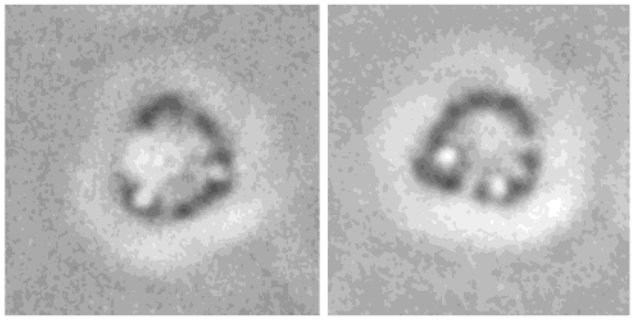

Figure 3. Optical micrograph of polystyrene spheres coating larger gold sphere. Assembled spheres in wet mounts were observed with phase-contrast microscopy using a 100× oil-immersion objective.

The second adhesive property relied on the biotin−streptavidin complex. The vitamin, biotin, is tightly bound by avidin and streptavidin. As a cofactor, biotin is covalently bound to carboxytransferases (reviewed in ref 4). In *Escherichia coli*, biotin is transferred to the apoenzyme by biotin holoenzyme synthetase (BHS), the product of the *birA* gene (reviewed in ref 5). A minimal biotin acceptor peptide was found by Beckett, Kovaleva, and Schatz,[6] and this sequence was used to construct the gene for a hybrid protein. The DNA encoding the gold-binding repeating polypeptide was transferred from pSB3627 to the DNA encoding the biotin-acceptor peptide by polymerase chain reaction (PCR) using a vector primer upstream[2] and 5′ TTCCAGAACGTC-GACGCTGGTC 3′. The resulting plasmid, pSB3647, contained 6 repeating oligonucleotides and encoded 6GB-bio (Figure 2). The protein depicted in Figure 2 contains only one biotin-acceptor site. Thus, the bio domain provides a monovalent binding property. The linker region connecting the two domains includes the gly-gly-gly-arg-ser sequence, similar to that used in the construction of single-chain antibodies.[7] Two similar plasmids were also prepared. pSB3652 contained 4 repeating oligonucleotides and encoded 4GB-bio. pSB3657 contained a short oligonucleotide encoding the tripeptide gly-thr-ala instead of a repeating polypeptide and encoded GTA-bio. GTA-bio provided a control lacking a gold-binding domain.

The ability of the bifunctional reagent to mediate programmed assembly was tested. In this test, smaller polystyrene spheres were assembled around larger gold spheres by biotinylated 6GB-bio. A periplasmic extract containing 6GB-bio was prepared as described[3] from strain S2576[8] harboring pSB3647. The extract was diluted into biotinylation buffer[9] containing BHS[10] to quantitatively transfer biotin to the hybrid protein. The unincorporated biotin was removed by extensive dialysis against 10 mM potassium phosphate, pH 7, 0.1 M KCl using 6000−8000 MW cutoff dialysis membranes (Spectra/Por 1, Spectrum Labs). The dialysate was clarified at 15 000 g, 10 min, 4° and adsorbed to 0.53 μm streptavidin-coated polystyrene spheres (Bangs Labs) in 10 mM potassium phosphate, pH 7, 0.1 M KCl, 1% Triton X-100 (PKT, ref 3). The polystyrene spheres were recovered by centrifugation, washed once with PKT, and resuspended with PKT. A 100 μg sample of 6GB-biocoated polystyrene spheres was incubated with 0.2 μg of gold powder as 1.5−3 μm spheres[11] in 1 mL of PKT at room temperature with constant mixing overnight. The gold with any adhering polystyrene spheres was allowed to settle for 2 h and examined by optical microscopy using a phase-contrast objective. Examples of the resulting complexes are shown in Figure 3. The polystyrene spheres in the focal plane appeared black in the images. Polystyrene spheres slightly above or below the focal plane appeared white in the images. The gold spheres appeared gray in the images. Although polystyrene spheres outside the equatorial plane of the gold sphere were not visualized here, the tight packing observed around the equatorial plane suggests similar tight packing around the rest of the gold sphere. The self-assembly of similarly sized spheres has been described using negatively charged smaller spheres with positively charged larger spheres.[12] Although contact areas of the surfaces and efficiency of coating will influence assembly, at least in this study, gold-binding by 6GB-bio was sufficient.

Are both constituents of the bifunctional protein required to associate two dissimilar micron-diameter spheres? The requirement for the gold-binding constituent was tested by preparing periplasmic extracts containing either 6GB-bio, which contains the gold-binding domain, or GTA-bio, which lacks the gold-binding domain, as above. The requirement for the streptavidin-binding property was tested by enzymatically adding biotin to the hybrid proteins. Proteins related to AP, like 6GB-bio, are secreted from the cytoplasm to the periplasm, where an N-terminal signal sequence is removed. BHS is a cytoplasmic protein and biotin transfer to secreted proteins is inefficient.[13] However, secreted proteins can be efficiently biotinylated by BHS in vitro.[9] Biotin was added by diluting the extracts into biotinylation buffer[9] either containing or lacking BHS.[10] Following the biotin-transfer reaction, the constituents were diluted with PKT and allowed to adhere to gold powder as 0.8−1.5 μm spheres.[11] Unincorporated biotin was removed by allowing the gold spheres to settle and discarding the supernatant. A 0.5 mg sample of "coated" gold spheres was allowed to adhere to 25 μg of streptavidin−polystyrene paramagnetic beads (Dynal). The mixed beads were incubated in 1 mL of PKT for 30 min with constant mixing. The beads were recovered by magnetic separation, and the buffer with the sedimented, unattached gold spheres was removed by aspiration. The beads with any adhering gold spheres were gently resus-

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Table 1. Requirements for Reagent Activity[a]

extract	BHS	beads		fraction
		total	adjacent	adjacent
GTA-bio	−	37	1	0.03
GTA-bio	+	91	1	0.01
6GB-bio	−	83	11	0.13
6GB-bio	+	109	48	0.44

[a] Periplasmic extracts were osmotic shock fluids from cultures expressing GTA-bio or 6GB-bio. The second column indicates whether BHS was added in vitro to conjugate biotin to the biotin acceptor site of GTA-bio or 6GB-bio. The total number of polystyrene beads counted and those with adjacent gold spheres are listed in the third and fourth columns, respectively.

pended with PKT buffer and examined by optical microscopy. The polystyrene spheres were counted and classified as to whether they had adjoining smaller gold spheres. Although gold spheres bound to polystyrene spheres will appear adjacent, the observation of adjacency does not demonstrate binding. The results of this analysis are reported in Table 1. There was a marked difference due to the presence of a gold-binding domain (GTA-bio vs 6GB-bio). When examined with Fisher's exact test, the probability the results with GTA-bio were the same as with 6GB-bio was less than 10^{-8}.

The frequency of adjacent particles was elevated by in vitro biotin transfer to 6GB-bio. This can be seen in Table 1 by comparing adjacency with and without prior incubation with BHS. When examined with Fisher's exact test, the probability the results with 6GB-bio incubated without BHS were the same as with 6GB-bio incubated with BHS was less than 10^{-5}. It can be seen in Table 1 that particle behavior with GTA-bio was unaffected by incubation with BHS. Therefore, BHS did not alter particle behavior in the absence of a gold-binding domain.

The avidity of binding by repeating polypeptides can vary with the number of repeats.[3] Initial attempts at particle assembly included 4GB-bio, but 4GB-bio gave inconsistent results. Perhaps any particle association mediated by 4GB-bio was sensitive to mechanical disruption during subsequent manipulations. 4GB-bio was not examined further.

A characteristic of binding proteins is their disassociation constant. This value is known for many proteins. The use of already characterized proteins in particle assembly would be facilitated by correlating assembly properties with dissociation constants. Thus, the binding properties of the gold-binding domain used here may assist in the selection of proteins for other experiments. Binding of 6GB-bio to metallic gold was measured with radiolabeled peptide, as described[3] using ^{35}S-potassium sulfate and gold powder (0.8−1.5 μm spheres, ref 11). 6GB-bio had an apparent disassociation constant of 34 pM for gold powder. Since some biotin transfer occurs prior to secretion, binding to streptavidin-coated polystyrene spheres was tested with the gold-binding repeats fused to AP as in Figure 1. No binding was detected. Thus, the programmed particle assembly observed in Figure 3 can be explained by a bifunctional reagent displaying surface-specificity for adhesion. That is, the gold-binding domain bound to gold and not streptavidin-coated polystyrene as the radiolabeled protein, and the bio domain as GTA-bio did not bind to gold in the microscopy experiment summarized in Table 1.

The use of bifunctional reagents to aggregate dissimilar particles has been described[14] although uniform coating of one type of particle by the other was not reported. Perhaps the ability to adjust the affinity of recombinant proteins permitted the assembly observed here, six repeats of the gold-binding sequence having been just adequate. If micron-diameter gold spheres, or gold-coated spheres[15] of sufficiently uniform size become available, an exciting potential use of this reagent would be in the assembly of crystalline arrays. If coordination number can be determined exclusively by relative sphere diameter, various types of arrays would be observed. It is likely a bifunctional reagent having a lower avidity for the spheres may prove more suitable for assembling three-dimensional arrays. Also, longer linker regions may accommodate small variations in particle diameters.

The biotin-acceptor domain retains its activity when fused to many different proteins (reviewed in ref 16). Similarly, the gold-binding domain described here retained its binding activity as part of the λ-receptor, AP and 6GB-bio. Previously described repeating polypeptides also retain their binding properties when fused to various proteins.[2,3] Therefore, the gold-binding domain may be joined to proteins having a variety of traits by recombinant DNA. Other potential protein partners include those forming 2D crystalline arrays such as S-layer proteins[17,18] and proteins adhering to other inorganic surfaces such as chromium[3] and semiconductors.[19]

Since the sensor surface of many biosensors including surface plasmon resonance sensors is gold, the gold-binding domain could be fused to a receptor protein and thus immobilize the receptor protein with controlled orientation. Receptors modified with an oligo-histidine tag have been immobilized on modified sensor surfaces and found to function well.[20] A use of recombinant receptor proteins in biosensors is particularly attractive since the receptor could be attached to the sensor in situ.

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(8) S2576: F$^-$ *lacI^Q* Δ(*proC-phoA*) Δ*lamB* Δ(*ompT-fepC*)*266 endA hsdR17 supE44 thi1 relA1 gyrA96 degP41*. *degP41* is described in: Strauch, K. L.; Beckwith, J. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1576−1580.
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(11) Gold powder as 0.8−1.5 μm spheres was purchased from Alfa, and 1.5−3 μm spheres was purchased from Aldrich. Prior to use, gold powder was washed with HF as described[3] and then, immediately before use, washed with 2-propanol followed by H_2O and PKT. Caution, hydrofluoric acid is toxic and corrosive.
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