Biospecific Labeling with Undecagold: Visualization of the Biotin-Binding Site on Avidin

Abstract. *The biotin-binding site on avidin has been labeled with biotin conjugated to undecagold, an organometallic cluster compound containing 11 gold atoms in a core 10 angstroms in diameter. Examination of unstained specimens by scanning transmission electron microscopy reveals the labeled sites directly, without computational averaging or filtering of the images. This approach should be widely applicable for determining the locations of subunits and functional sites in biological macromolecules at a resolution in the range of 15 angstroms.*

Numerous attempts have been made to label specific sites on biological macromolecules with heavy metal compounds for detection by electron microscopy (*1–7*). In highly periodic, multilayered specimens in which hundreds or thousands of labeled sites reinforce each other, it has been possible to locate labeling groups of one to four mercury atoms by conventional transmission electron microscopy (TEM). Detecting individual labeled sites has been possible only by scanning transmission electron microscopy (STEM) (*3, 5*); the labeling groups, however, have proved to be highly mobile under irradiation, and the electron doses required for detection are so high as to destroy most structural detail in the labeled biological molecules.

Recently, Bartlett *et al.* (*8*) reported the synthesis and characterization of a water-soluble organometallic cluster compound containing 11 gold atoms, tricyanoheptakis [4,4′,4″-phosphinidynetri (benzenemethanamine)] undecagold, and proposed that it would be useful as a high-resolution, electron-dense label. We report using the undecagold cluster for site-specific labeling on a biological molecule. We prepared a biotinyl derivative of the undecagold cluster and used it to locate the biotin-binding site on avidin.

Avidin is a 63,000-dalton protein consisting of four identical subunits arranged with twofold symmetry; each subunit binds one biotin molecule with high affinity (Michaelis constant, $10^{-15}M^{-1}$) (*9*). When avidin is complexed with bifunctional derivatives of biotin, linear polymers are formed (*9*). We used undecagold coupled with two or more biotin residues per cluster to cross-link avidin molecules into linear polymers and display the positions of the biotin-binding sites.

The biotin-coupled undecagold cluster was mixed with an avidin solution and applied to a gel filtration column. When the earliest eluting fractions were examined by conventional TEM (Fig. 1), linear chains of avidin molecules were observed along with single molecules. Similar results were reported by Green *et al.* (*9*). Examination of unstained, freeze-dried specimens by STEM showed that the avidin tetramers were frequently linked by pairs of gold clusters, visualized as electron-dense particles about 10 Å in diameter and spaced about 20 Å apart (Fig. 2). Adjacent pairs of clusters were separated by about 45 Å. The observed positions of the clusters confirm that the four biotin-binding sites are arranged in two pairs on opposite sides of each avidin molecule, as deduced by Green *et al.* (*9*). Biotin-binding sites on adjacent avidin molecules are cross-linked by two biotin residues coupled to a single undecagold cluster. The mass per repeat period determined by STEM was about 80,000, consistent with the mass of the avidin molecule (*9*) plus that of two or more gold clusters of about 5000 daltons each (*8*). Undecagold shows little tendency to move under irradiation and fades slowly after multiple scans.

Since the avidin molecule has four biotin-binding sites, a polymer of n avidin units should bind $2n + 2$ gold clusters, corresponding to 100 percent occupancy. Actual counts by visual inspection give an average value for n of 2.0 and an occupancy of about 30 percent. The short average polymer length and low occupancy may reflect a reduced binding affinity for the biotin–gold cluster conjugate, the persistence of some free biotin in the preparation, or partial degradation of the undecagold cluster.

The specificity of labeling is emphasized by the absence of unbound undecagold from the background of the specimens. Visual inspection of 47 micrographs showed only 13 undecagold clusters that were not bound to identifiable avidin molecules, corresponding to 1.3 percent of the clusters counted.

Since undecagold clusters do not move between successive scans, distances between clusters can be determined to within the limit of resolution of the scanning transmission electron microscope, or about 2.5 Å under the conditions of this study. Biologically meaningful resolution is limited by the distance between the center of the gold

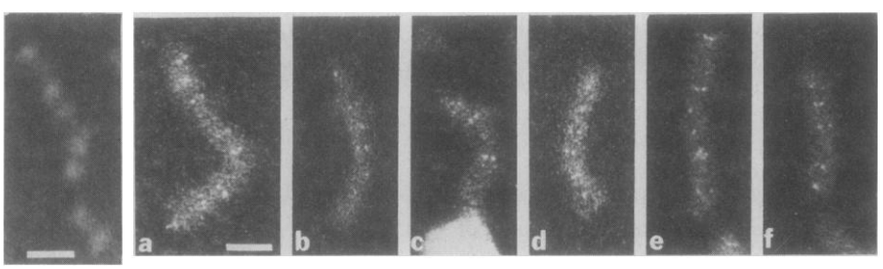

Fig. 1 (left). Conventional transmission electron micrograph of avidin-biotinyl undecagold complex negatively stained with sodium phosphotungstate (*p*H 7.4). Undecagold, synthesized by a modification of the procedure of Bartlett *et al.* (*8*), was reacted in a solution containing 1*M* triethanolamine and 0.167*M* HCl with a tenfold excess of *N*-hydroxysuccinimidobiotin (*10*). After 4 to 16 hours at room temperature, a 200-fold excess of acetic anhydride was added, and after an additional hour the mixture was centrifuged at 30,000*g* for 10 minutes and applied to a 1 by 20 cm column of Sephadex G10 in 20 m*M* triethanolamine HCl (*p*H 8) to remove excess reactants. The orange biotinyl undecagold was collected and its concentration was estimated from its absorbance at 305 nm ($E_{305} = 10^5 M^{-1}$ cm^{-1} for the unmodified cluster). Small portions of biotinyl undecagold were then added to a 0.1 m*M* solution of avidin in 5 m*M* imidazole HCl (*p*H 8) to give an approximate final stoichiometry of one gold cluster per avidin subunit. The mixture was then applied to a 1 by 50 cm column of Sephadex G100 in 0.2*M* triethanolamine HCl (*p*H 8.0) (*9*). Grids were prepared from the earliest eluting fractions by a modified double-film procedure (*11, 12*) and were examined in a Philips 201 electron microscope operated at 80 kV. Micrographs were recorded at an approximate magnification of 80,000. Scale bar, 100 Å. Fig. 2 (right). Scanning transmission electron micrographs of unstained avidin–biotinyl undecagold complex. Preparation of freeze-dried specimens and their examination by STEM were performed essentially as described by Mosesson *et al.* (*13*). The micrograph in (a) was analyzed by integrating the mass in a set of 24 circular spots 5 Å in radius. The spots were arranged in pairs separated by 20 Å, with approximately 45 Å between pairs, and were aligned with the visible undecagold clusters. Of the 24 spots, 13 were occupied by undecagold clusters and had an average molecular weight of 3729 ± 531. The remaining unoccupied areas had an average molecular weight of 2094 ± 369. The molecular weight difference between occupied and unoccupied spots, 1635 daltons, equals the molecular weight of isolated undecagold clusters as measured by STEM under identical conditions (*14*). Scale bar, 100 Å.

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cluster and the bound biotin residue, or about 12 Å (*8*). Because the avidin molecule itself is small and lacks identifiable structural features, we did not attempt to relate the position of the gold cluster to any specific feature of the protein structure.

Further applications of gold cluster labeling are likely to involve larger molecules in which the positions of labeled sites must be determined relative to features of the biological structure itself; effective resolution will be limited by radiation damage to the biological structure. With the use of negatively stained specimens and reduced electron doses, resolution in the range of 15 to 20 Å should be obtainable. The combination of specific labeling with heavy-metal cluster compounds and detection by STEM offers a powerful new method for studying the locations and interactions of subunits and functional sites in biological macromolecules.

DANIEL SAFER
Johnson Research Foundation/G4, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia 19104
JAMES HAINFELD
JOSEPH S. WALL
Department of Biology, Brookhaven National Laboratory, Upton, New York 11973
JOHN E. REARDON
Institute for Enzyme Research, University of Wisconsin, Madison

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Hemoglobin Parchman: Double Crossover Within a Single Human Gene

Abstract. *Structural analysis of a new variant hemoglobin revealed tryptic peptides with the amino acid composition of normal δ-globin, except for two internal peptides, which had the compositions of normal β-globin. The most likely explanation for these findings is that a double, nonhomologous crossover between the δ- and β-globin genes had occurred.*

The closely linked human δ- and β-globin genes are located on the short arm of chromosome 11 (*1*) and are expressed in the ratio 1:40 (*2*). Nonhomologous crossing-over between these genes results in a family of fusion genes that produce the Lepore and Miyada type hemoglobins. The Lepore hemoglobins are characterized by globin chains with a δ-amino terminus, a β-carboxyl terminus, and reduced synthesis, resembling that seen in normal δ-globin synthesis (*3*). The reciprocal products of the Lepore hemoglobin genes, the Miyada type hemoglobins, have β-amino and δ-carboxyl termini and also exhibit reduced synthesis (*2*). We found, in a black patient, an abnormal hemoglobin that appeared to have arisen as a result of a double nonhomologous crossing-over between the δ- and β-globin genes. The hybrid globin chain had a δ-amino terminus, an internal β-chain fragment, and a δ-carboxyl terminus. This hemoglobin, which we call hemoglobin Parchman, is the only fusion hemoglobin gene which, on the basis of protein structure, unequivocally contains the intervening sequence 1 (IVS-1) of the β-globin gene, and thus may offer additional insight into the role of untranslated DNA in the differential expression of the δ- and β-globin genes.

The patient was not anemic, had no evidence of hemolytic disease, and had very mild microcytosis (mean corpuscular volume, 79 fl). There was no evidence of hemoglobin instability. There was an imbalance in globin chain synthesis with a deficit of α chain relative to β chain ($\alpha/\beta = 0.75$), consistent with the presence of α-thalassemia. Gene mapping studies indicated that the patient had heterozygous α-thalassemia 2, since digestion of cellular DNA with the restriction endonuclease Eco RI yielded two DNA fragments, 19 and 23 kilobase pairs (kbp) long, containing α-globin genes after hybridization with a complementary DNA probe for α-globin (*4*). Hemoglobin studies showed that hemoglobin A_2 was lower than normal (1.2 percent) and hemoglobin F was elevated (4.5 percent). The reason for the increase in hemoglobin F is not known. An additional hemoglobin fraction migrated in the same position as sickle cell hemoglobin (hemoglobin S) on alkaline electrophoresis and eluted near

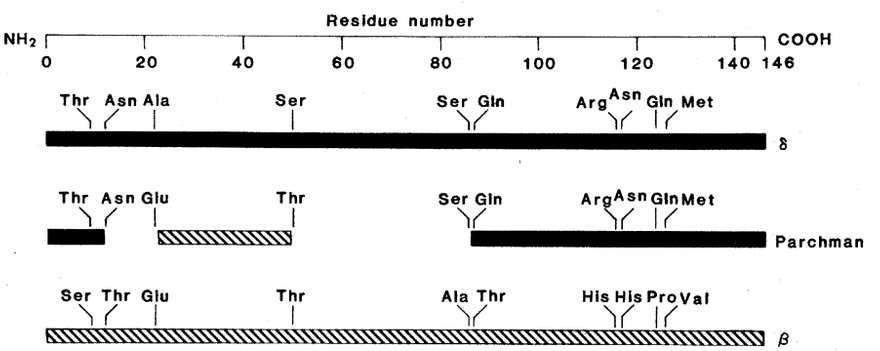

Fig. 1. The position of the pertinent amino acids of hemoglobin Parchman. The δ chain differs from the β chain by ten amino acids. The positions of these amino acids are shown for the δ chain, which is the non-α chain of hemoglobin Parchman, and for the β chain. Hemoglobin Parchman was purified by DEAE-Sephadex column chromatography (*10*), and its constituent chains were separated by chromatography on columns of *O*-(carboxymethyl)cellulose (*11*). The purified non-α chain was digested with trypsin, and the resulting peptides were separated by chromatography on Beckman PA-35 resin and by high-performance liquid chromatography (HPLC) (*12*). Peptides were repurified by column chromatography on Bio-Rad AG50W × 2 resin and by HPLC. Purified peptides were hydrolyzed in 5.7*M* HCl for 22 and 72 hours and analyzed on a Dionex amino acid analyzer with DC-4A resin and *O*-pthalaldehyde fluorescence detection. The amino acids shown are Thr, threonine; Asn, asparagine; Ala, alanine; Ser, serine; Arg, arginine; Gln, glutamine; Met, methionine; Glu, glutamic acid; His, histidine; Pro, proline; and Val, valine. Automatic sequence analysis of T-3 revealed the sequence of Val-Asn-Val-Asp-Glu (Asp, aspartic acid), demonstrating glutamic acid at position 22.

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