Tetrairidium, a Four-Atom Cluster, Is Readily Visible as a Density Label in Three-Dimensional Cryo-EM Maps of Proteins at 10–25 Å Resolution

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Heavy metal clusters derivatized to bind to designated chemical groups on proteins have great potential as density labels for cryo-electron microscopy. Smaller clusters offer higher resolution and penetrate more easily into sterically restricted sites, but are more difficult to detect. In this context, we have explored the potential of tetrairidium (Ir4) as a density label by attaching it via maleimide linkage to the C-terminus of the hepatitis B virus (HBV) capsid protein. Although the clusters are not visible in unprocessed cryo-electron micrographs, they are distinctly visible in three-dimensional density maps calculated from them, even at only partial occupancy. The Ir4 label was clearly visualized in our maps at 11–14 Å resolution of both size variants of the HBV capsid, thus confirming our previous localization of this site with undecagold (Zlotnick, A., Cheng, N., Stahl, S. J., Conway, J. F., Steven, A. C., and Wingfield, P. T., Proc. Natl. Acad. Sci. USA 94, 9556–9561, 1997). Ir4 penetrated to the interior of intact capsids to label this site on their inner surface, unlike undecagold for which labelling was achieved only with dissociated dimers that were then reassembled into capsids. The Ir4 cluster remained visible as the resolution of the maps was lowered progressively to 25 Å.

Key Words: metal cluster; cryo-electron microscopy; molecular marker; three-dimensional image reconstruction; hepatitis B virus

INTRODUCTION

Heavy metal particles, whose high electron density renders them visible in electron micrographs, have been extensively used to label molecules of interest. In addition to considerations of specificity and efficiency of labelling, the resolution achieved depends primarily on the size of the metal particle and the distance between its center-of-mass (the feature detected) and its point of attachment (the targeted site). For instance, in colloidal gold-based immunolabelling (reviews, Griffiths, 1994; Ben-dayan, 1995), which is widely used to map the distributions of molecules in cells, the gold particles are typically 50–200 Å in diameter, and there may be up to 200–400 Å between their projected centers and the epitope marked, depending on whether intermediaries such as protein A or secondary antibodies are used.

For structural analysis of isolated macromolecules, smaller labels and more precise localizations are needed. In this context, metal clusters derivatized to bind directly to groups such as sulphhydrlys or amines are finding a growing number of applications (reviews, Hainfeld, 1996; Hainfeld and Powell, 1997; see other papers in this issue). Nanogold (Hainfeld and Furuya, 1992) has a core of 55–75 or so gold atoms, ~14 Å in diameter; in maleimide linkage to cysteine, there is a spacing of ~20 Å between its center and the sulfur of the labelled amino acid. Undecagold (Bartlett et al., 1978; Hainfeld, 1989) has 11 atoms in a cluster ~8 Å across, and ~17 Å separate its center and the targeted residue. Nanogold has sufficient scattering power to be directly visible in micrographs even against a background of negative stain (Wenzel and Baumeister, 1995; Grigori et al., 1997). Undecagold is smaller and only marginally visible in unprocessed micrographs of ice-embedded specimens. However, it has been detected in averaged two-dimensional (Crum et al., 1994) and three-dimensional (Milligan et al., 1990; Steinmetz et al., 1998) density maps at 15–30 Å resolution. Typically, the metal cluster of a labelling compound is surrounded by an organic shell that contains the

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functional group required for specific attachment to proteins and also governs its solubility. Nanogold and undecagold have triphenylphosphine shells that give them full diameters of ~26 and ~20 Å, respectively. However, because this shell poses a steric hindrance on a cluster’s ability to reach potentially labellable sites, it is of interest to have cluster labels that are smaller than undecagold.

Two such candidates are the four-atom clusters, tetratetramethylmercury (Hg₄) and tetrariumid (Ir₄). The mercury compound has been used to label the filamentous bacteriophage fd (Lipka et al., 1979), but it was concluded that the Hg was too volatile under the electron beam to be generally suitable as an EM label, and little subsequent use has been made of this reagent. Ir₄ was originally developed for X-ray crystallography of large proteins whose native reflections are insufficiently perturbed by single heavy atom derivatives to provide adequate phases (Jahn, 1989; Weinstein et al., 1989). In EM, Ir₄ has been visualized in dark-field STEM micrographs (Furuya et al., 1989), but its scope as a protein label was limited by the state of preservation of freeze-dried specimens. Here, we report that Ir₄ is a highly effective label for ice-embedded specimens, which retain their native conformations (Adrian et al., 1984).

Recently we localized the C-terminus of the hepatitis B virus (HBV) capsid protein by appending a cysteine residue at this site and labelling it with undecagold (Zlotnick et al., 1997). HBV capsids are icosahedral shells of two sizes—280 Å in diameter (90 dimers of 17-kDa subunits) and 320 Å (120 dimers), respectively. Unusually for viral capsids, these shells are perforated with sizable holes whose diameters, at ~20 Å, are similar to that of undecagold. Our attempts to label intact capsids were unsuccessful, but dissociated dimers were readily labelled and could be reassembled into capsids. In the density map, undecagold was conspicuously present at a specific site on the inner surface of the capsid; on this basis, we attributed the nonlabelling of this smaller cluster.

Materials and Methods

Preparation of the tetrariumid cluster. To prepare Ir₄(OC)₁₂[Pt-p-C₆H₄C(O)NHMe]₂[Pt-p-C₆H₄C(O)NHMe]₂[Pt-p-C₆H₄C(O)NH(CH₂)₃NH₂]₂ and its maleimido derivative, one-hundred milligrams of Ir₄(OC)₁₂, 69 mg of Tris p-N-methylcarboxamidophenylphosphine, P-p-C₆H₄C(O)NHMe₂, and 62 mg of the amino-substituted phosphine, P-p-C₆H₄C(O)NHMe₂(p-p-C₆H₄C(O)NH(CH₂)₃NH₂), were refluxed for 4 h in 50 mL of anhydrous toluene under N₂, cooled to room temperature, and then separated by gel filtration on a 500-mL column (LH-20, Pharmacia-Biotech, Piscataway, NJ) eluted with methanol. Fractions with a yellow–orange color were pooled and evaporated to dryness. Fractions with similar UV-Vis and IR spectra were combined. Pure product was characterized by UV-Vis and IR spectroscopy. Preparative-scale resolution of the product was performed by HPLC on a C₆H₄C(O) NHMe₂ column (5 μm; Phenomenex). The protein concentration was determined using [1H], [13C], and [31P]NMR spectral data. A total of 1.4 mg of this cluster and 11.4 mg (150-fold excess) of N-methoxycarbonyl-maleimide were each dissolved in 0.5 mL of methanol, and the two solutions were combined and left at 5°C for 30 min. The maleimido cluster was isolated by gel filtration (LH-20 column, 80 mL, eluted with methanol at 2-mL/min flow rate). Fractions 8–11 (15-mL fractions) were pooled, and the solvent was quickly removed under reduced pressure.

Preparation and labelling of capsids. Capsid protein constructs were expressed in Escherichia coli and isolated in a procedure that involves dissociation to dimers, further purification, and reassembly (Wingfield et al., 1995; Zlotnick et al., 1997). All labelling experiments were performed on preformed particles prepared as follows: 2 mL of dimeric protein at 1 mg/mL in 100 mM NaHCO₃, 20 mM DTT; pH 9.6, was mixed with 0.4 mL of 500 mM Hepes, 2 M NaCl, pH 5.0, and incubated for 1 h at room temperature. For labelling, the buffer was changed to 20 mM Hepes, 2 mM EDTA, pH 6.9, with a PD10 gel filtration column (Pharmacia-Biotech). The protein concentration was determined by absorbance, using ε₂₈₀ = 2.95 × 10⁴ M⁻¹ cm⁻¹ (Zlotnick et al., 1997). Fifty nanomoles of tetrariumid monomaleimide dissolved in 200 μL of DMSO was immediately added to 50 nmol of protein in a final volume of 1200 μL. After an overnight incubation at room temperature, MgCl₂ was added to a final concentration of 100 mM to alleviate capsid aggregation. Unbound label and unassembled protein were then removed by gel filtration on a 12-mL, 15-cm-long column of CL-6B (Pharmacia-Biotech) equilibrated with 20 mM Hepes, 50 mM MgCl₂, pH 8.0. Particles were concentrated to ~2 mg/mL by ultrafiltration (Ultrafree-15, Millipore, Bedford, MA). Labelling was quantitated as follows: the concentration of Ir₄ was calculated from the absorbance at 320 nm (corrected for the contribution by light scattering alone, estimated from the UV spectrum of the reduced unlabelled particles) using ε₃₂₀ = 2.55 × 10⁴ M⁻¹ cm⁻¹. Protein concentrations were calculated from the absorbance at 280 nm, corrected for the contribution by Ir₄ at this wavelength, using A₃₂₀/A₂₈₀ = 2.08.

Cryo-electron microscopy. Capsid samples were frozen in a thin film of vitrified ice suspended over holey carbon films, as described by Zlotnick et al. (1996). Micrographs were recorded at a magnification of ×23 000 on a CM200-PEG microscope (Philips, Eindhoven, Netherlands), fitted with a model 626 cryoholder (Gatan, Pleasanton, CA) and operating at 120 keV. Pairs of micrographs at different defocus values were recorded using low-dose techniques. In the focal pairs analyzed in calculating the reconstructions, the first exposure had a defocus value in the range 0.8–1.1 μm, so that the first zeros of the contrast transfer function (CTF) were at spatial frequencies of (20 Å⁻¹)⁻¹ – (20 Å⁻¹)⁻¹, which was increased by 0.4 Å for the second exposure.

Calculation of density maps. Micrographs were digitized on an SCAl scanner (Zeiss, Englewood, CO) at 7 μm per pixel, corresponding to 1.8 Å per pixel at the specimen. Particle images were extracted and processed using a semiautomated procedure (Conway et al., 1993). Their orientations were determined by means of the PFT algorithm (Baker and Cheng, 1996), using as starting models preexisting maps of Cp147 capsids: T₅ = 4 (Conway et al., 1997); T = 3 (Conway et al., 1998). The CTF was corrected and the focal pairs were combined as described by Conway et al. (1997). The maps were calculated using methodology reviewed by Fuller et al. (1996), including all particles with correlation coefficients of 0.5 or higher for each of the three statistics calculated by the PFT program (Baker & Cheng, 1996). The number of particles included in each reconstruction and the resulting resolution are listed in Table I.
RESULTS

Labelling HBV Capsids with Ir₄. The capsids studied represent variants of the HBV assembly domain expressed in E. coli. The complete assembly domain is 149 residues long. In the native capsid protein, it is coupled to a 34-residue, C-terminal domain that is highly basic and binds RNA in nucleocapsid assembly. The wildtype assembly domain has cysteines at positions 48, 61, and 107 that are replaced by alanines in the 149-residue construct, Cp*149. Cp*150 has an additional residue, a cysteine, appended at the C-terminus.

Labelling was performed by adding Ir₄ (Fig. 1) dissolved in DMSO to preassembled, freshly reduced capsids at a 1:1 molar ratio of Ir₄ clusters:protein monomers and incubating for 12 h at room temperature. At lower temperatures or at Ir₄ concentrations much above 50 nmol/mL, the reagent began to precipitate. In early experiments, spectroscopy gave erratic estimates of labelling levels, which were explained by the presence of microprecipitates of Ir₄ seen by cryo-EM (e.g., Fig. 2d). Subsequently, precipitation of the reagent was largely alleviated by including up to 20% DMSO or 2-propanol, additives that are well tolerated by most (but not all) proteins. No adverse effects of the DMSO on HBV capsid structure were observed, at least to 11 Å resolution (see below).

After the incubation, unbound label was removed by gel filtration and capsid labelling was assessed by measuring absorbance by Ir₄ at 320 nm and by protein at 280 nm. These data reproducibly indicated a labelling efficiency of about 11% for Cp*150 under the conditions used. Nonspecific binding, estimated by treating Cp*149 capsids—which have no cysteines—in the same way, was found to be less than 0.5%.

Cryo-EM of Ir₄-labelled capsids. Micrographs of labelled and unlabelled capsids are compared in Fig. 2a and Figs. 2b and 2c, respectively. There is no clear-cut difference between them, and we conclude that individual Ir₄ clusters are not directly visible in cryo-micrographs. The images shown were recorded quite close to focus (~0.8 μm), conditions that we

![Figure 1](image-url)

**FIG. 1.** Schematic diagram of the tetrairidium cluster compound used. The diameter of the cluster core is 5 Å and its full diameter, including the organic shell, is ~17 Å (Weinstein et al., 1989). The electron density in the metal core is 4.7 electrons/Å³. The average Ir-Ir bond distance is expected to be 2.736 Å. Although the Ir atom at the apex of the cluster is directly bonded to six atoms or ligands, it satisfies the 18-electron rule (as applied to coordinate-covalent bonds in organometallic clusters), as do the other three iridium atoms (Ros et al., 1986).

**TABLE I**

Summary of Three-Dimensional Reconstructions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total number of particles</th>
<th>Resolution (Å)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FSC</td>
<td>FRC</td>
<td></td>
</tr>
<tr>
<td>T = 3: Cp * 149</td>
<td>1041 (2)</td>
<td>13.7</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>T = 3: Cp * 150 + Ir₄ Expt 1</td>
<td>1096 (4)</td>
<td>14.8</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>T = 4: Cp149</td>
<td>1296 (2)</td>
<td>11.3</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>T = 4: Cp * 150 + Ir₄ Expt 1</td>
<td>1460 (4)</td>
<td>12.6</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>T = 4: Cp * 150 + Ir₄ Expt 2</td>
<td>600 (4)</td>
<td>14.7</td>
<td>10.5</td>
<td></td>
</tr>
</tbody>
</table>

a The total number of particles used in each reconstruction is given. The number of focal pairs combined to yield these data is given in parentheses. All particles present were analyzed; of these, all that had all three PFT correlation coefficients (PFT, PRJ, and CMP—see Baker and Cheng, 1996) of 0.5 or higher were included in the reconstruction. This fraction varied from ~30 to ~80% in different focal pairs.

b Resolution was calculated by two criteria—the Fourier shell correlation (FSC, van Heel, 1987) and the Fourier ring correlation coefficient (FRC, Saxton and Baumeister, 1982; Conway et al., 1993). In both cases, the criterion used to define the resolution limit was the frequency at which the correlation dropped below 0.5 for the last time. The relatively large discrepancy between the two measures for the last reconstruction (T = 4, Expt 2) simply reflects that the correlation hovers about the 0.5 value over a considerable range, i.e., between approx (14 Å)⁻¹ and (10 Å)⁻¹, in this analysis.
considered to be conducive to visualizing the small (5 Å) metal clusters while still conveying the capsids, albeit with relatively low contrast. The clusters were no more visible at other defocus values used, up to ~2 µm (data not shown).

Density maps of Ir₄-labelled capsids. In an experiment in which 11% labelling of Cp*150 was obtained, density maps were calculated for both the T = 4 and the T = 3 capsids to 12 and 14 Å resolution, respectively (Table I, Fig. 3). In both cases, conspicuous focal densities are present beneath the fivefold and quasi-sixfold lattice sites. These densities are absent from Cp*149 control capsids that were subjected to the same labelling reaction (cf., Fig. 3). This localization of the Ir₄ clusters and, consequently, of the C-termini of the capsid protein confirms the result previously obtained by visualizing undecagold bound to Cp*150 at ~20 Å resolution (Zlotnick et al., 1997). Consistent results were obtained for a T = 4 capsid reconstruction in an earlier labelling experiment in which the DMSO was omitted (Table I, cf., Figs. 4a and 4b). Although the labelling efficiency of the latter experiment could not be determined reliably by spectroscopy (see above), it must have been very similar to that of the experiment illustrated in Fig. 3, to judge by the intensity of the cluster-associated densities in the respective maps.

Dependence of cluster visibility on resolution. To estimate the minimum resolution required to visualize Ir₄ labels by cryo-EM, we progressively lowered the resolution of these density maps. As shown in Fig. 4, the cluster-associated density becomes progressively more diffuse as the resolution falls, but it remains significantly above the level of residual background noise to at least 25 Å resolution.

DISCUSSION

Visibility of tetrairidium as a density label. Hitherto, undecagold is the smallest metal cluster to have been used as a density label in cryo-EM. Ir₄ is significantly smaller (2 vs 6.5 kDa, 4 vs 11 heavy atoms). Although it was essentially invisible in micrographs covering a considerable range of defocus values (Fig. 2), Ir₄ was clearly visualized in three-dimensional density maps, even at incomplete occupancy and moderate resolution (Figs. 3 and 4), reflecting the large improvement in signal-to-noise ratio in such a density map compared to the original projection images.

We reproducibly obtained a labelling stoichiometry of ~11% for Ir₄ relative to the capsid protein under our conditions of incubation, corresponding to ~26 clusters per T = 4 capsid and ~20 clusters per T = 3 capsid. However—as with undecagold (Zlot-
nick et al., 1997)—the bound clusters are located on the fivefold and quasi-sixfold axes, where occupancy by one cluster blocks the binding of clusters to the other four (or five) subunits surrounding the lattice point. Thus, the maximum binding to be expected is 42 \( (T_5^4) \) and 32 \( (T_5^3) \) clusters per capsid, so that the binding obtained represents about 60% of maximum.

As visualized (Figs. 3, 4a, and 4b), the 5 Å-diameter clusters appear considerably larger than expected; in fact, their full width at half-height is \( \sim 20 \) Å. Only part of this broadening may be attributed to the limited resolution (11 Å), because a step function 5 Å across has a full width at a half-height of \( \sim 9 \) Å when band-limited to this resolution. The remainder of the broadening must arise from other effects, such as local disorder of the C-termini, or slight displacement of each cluster from the adjacent symmetry axis in a direction that depends on which of the surrounding subunits it is bound to. The latter effect would give rise to averaging-related smearing of the cluster density as depicted in the reconstruction.

Initially, we were surprised that a label as small as Ir4 should be so conspicuous under these conditions of observation (Fig. 3). However, the difference in specific density between the cluster and the adjacent protein \( (\Delta \rho \sim 21.5 = 22.8 \text{ vs } 1.3) \) is so much higher than that between protein and vitreous ice \( (\Delta \rho \sim 0.35 = 1.3 \text{ vs } 0.95) \) that it remained distinctly visible in the map, despite attenuation by partial occupancy and delocalization arising from averaging (see above). We anticipate that Hg4, which is similar to Ir4 in electron scattering power, should also be detectable under similar conditions, i.e., in 3D density maps of ice-embedded specimens imaged at low electron doses.

**Resolution of metal cluster labels.** The resolution attainable with these markers depends more on the spacing between the center of the cluster-associated density and the labelled residue than it does on the...
FIG. 3. Density maps of Ir₄-labelled and unlabelled HBV capsids. (Top) T = 4 capsids of the labelled Cp*150 construct and the control Cp*149 construct. The small black arrowhead (middle panel, top row) points to the organic linker connecting the Ir₄ cluster to the capsid protein. (Bottom) A similar comparison for the corresponding T = 3 capsids. For each density map, isodensity renderings of the outer and inner surfaces are shown, together with a central section depicting local variations in density. The capsids are viewed along a twofold symmetry axis and are contoured to enclose 100% of expected mass. The specifics of the reconstructions are given in Table I. For both capsids, labels are visible at corresponding sites on their inner surfaces. Bar = 50 Å.
size of the cluster. With undecagold, this spacing is \( \sim 17 \, \text{Å} \), and with \( \text{Ir}_4 \), it is slightly less, at \( \sim 15 \, \text{Å} \). However, it is possible to achieve more precise localizations than these figures imply because, under favorable circumstances, the organic linker connecting the cluster to the labelled protein may be visualized (arrowhead in Fig. 3, middle panel, top row; see also Fig. 4 of Zlotnick et al., 1997). Extrapolating an appropriate distance along this vector from the cluster center should give a precise localization of the residue of interest, i.e., to within \( \pm 5 \, \text{Å} \) or so. While clusters as small as \( \text{Ir}_4 \) are readily visible in density maps at 25–30 Å resolution (cf., Fig. 4), one advantage of extending their resolution to 10–15 Å is enhanced prospects of visualizing this linker and, consequently, of effecting a more precise localization of the targeted residue.

Although shifting to progressively smaller clusters does not bring a major gain in resolution, these reagents have the advantage of being less susceptible to steric constraints that may limit their reactivity. The relatively bulky organic shell surrounding the cluster prevents it from accessing confined spaces in such a way that an atomic force microscope is limited in the vertical dimension by the size of its tip. Thus, smaller clusters have superior penetrating power, as illustrated by the observed positive \( \text{Ir}_4 \) labelling of a residue inside HBV capsids that is inaccessible to undecagold. An alternative approach to this problem of steric impedance may be to engineer variants of undecagold or \( \text{Ir}_4 \) with longer linkers between the relatively bulky label and the functional group that may be better able to reach into restricted sites, albeit at possible cost in terms of resolution (see above).

**Preferential labelling of B-subunits in capsids assembled from labelled dimers; implications for regulation of capsid assembly.** In the \( T = 4 \) capsid, pairs of three nonequivalent subunits surround the quasi-sixfold axis. They are called the B-, C-, and D-subunits, respectively (Fig. 5). For capsids assembled from dimers labelled in solution with undecagold, it was observed that the linker connecting the cluster to the B-subunit was markedly more visible than that for the C- and D-subunits, implying that the single cluster per quasi-sixfold site was preferentially associated with a B-subunit (Zlotnick et al., 1997). Although this linker is also discernible in our map of preformed capsids labelled with \( \text{Ir}_4 \) (Figs. 3 and 4), the preference for B-subunits is much less pronounced. This distinction may reflect a feature of the assembly pathway; for instance, binding of the cluster to the C-termini of unassembled dimers might switch them into a state of enhanced receptivity for binding other dimers, leading to a cooperative aggregation around a local symmetry axis, and in so doing, may mimic an effect of RNA binding to the C-terminal domain in nucleocapsid assembly. If the symmetry axis in question were fivefold, this would explain the preferential labelling of B-subunits, with additional dimers subsequently adding on to connect the resulting pentamers, thereby completing the formation of either a \( T = 4 \) or a \( T = 3 \) capsid.

**Prospects for mapping the 3D structures of protein complexes by cysteine scanning and cluster labelling.** Although ample scope remains for further development of clusters with other functionalities, the reaction of maleimide with the sulfhydryl of cysteine has been found to work well with undecagold (Hainfeld, 1988), Nanogold (Hainfeld and Furuya, 1992), and \( \text{Ir}_4 \) (Jahn, 1989). In addition to naturally occurring cysteines, current molecular biology techniques readily allow the insertion or substitution of this amino acid at virtually any site of interest. In principle, therefore, the possibility already exists to use cluster labelling to delineate the overall path of a polypeptide chain in any protein complex amenable to analysis by cryo-EM. Given enough constraints of this kind, it should then be possible to fold in information on conformational preferences of specific peptides, secondary structure predictions, and other data such as intramolecular cross-links to build detailed molecular models, starting from cryo-EM density maps in the 10 Å-resolution range.
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