Synthesis of a Large Water-Soluble Gold Cluster (Au_{32.36}) and its Properties as a Potential Label in Electron Microscopy

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A water-soluble gold cluster with a core of 32 to 36 gold atoms was prepared by NaBH₄ reduction of the AuCN complex of n-butyl-bis (5-morpholinocarbonyl-2-furyl)-phosphine and characterized by its mass spectrum. The chromatographic behavior, ligand exchange and binding to actin were studied.

Introduction

Large water-soluble gold clusters are used for the labelling of biological material to visualize the position of reactive sites by electron microscopy [1]. In most investigations of this type a commercial product was used, for which little chemical information has been published (for review see [9]). Large gold clusters can be prepared using phosphines as ligands [2, 3] or more easily with mercaptans in place of phosphines [4, 5, 11]. Mercaptans as ligands may interfere, however, if clusters are to be attached to the SH group of proteins, which is the most convenient way to label specific sites. For this reason, large gold clusters with phosphate ligands are still of interest. We found that a water-soluble cluster with a core of 32 to 36 gold atoms could be easily prepared using 3 as a ligand.

Results and Discussion

The reaction of tri(2-furyl)phosphine with n-butyl lithium and carbon dioxide yielded, in addition to the expected tris(5-carboxy-2-furyl)phosphine (1), n-butyl-bis (5-carboxy-2-furyl)-phosphine (2) as the main product. 2 could be easily converted to the bis-morpholide 3 using carbonyldiimidazole. Reduction of the complex formed by reaction of 3 with AuCN yielded a mixture of products, from which a dark red cluster could be isolated by ion exchange chromatography as the major product. This material on rechromatography formed a broad double peak (Fig. 1A) which could be fractionated further. It was not possible, however, to obtain pure individual species as checked by reversed phase (RP) HPLC (see below). The entire material corresponding to the peak described in Fig. 1A was used in the experiments described below and is referred to as "Au34 cluster". Electrophoresis on an IEF gel [6] showed mainly two strong red bands, accompanied by some diffuse red material, moving to the anode (Fig. 1B). RP-HPLC of the cluster using conditions for ion pair chromatography showed a broad peak with 4 to 5 spikes (Fig. 3a). In a preparative
Fig. 1. A) Rechromatography of the Au34 cluster. The cluster was applied to a DEAE Sepharose fast flow column (6 × 2.5 cm) and eluted with a gradient from 0 to 0.5 M TBK in 60 min, flow rate 5 ml/min; the material emerging after 20 - 40 min (indicated by arrows) was used for the experiments described. B) Contact print of an IEF agarose gel. The gel (pH range 3.5 - 10) was previously equilibrated for 60 min at a final voltage of 90 V/cm. The arrowhead indicates the application site of the sample; running time with the sample 10 min. In the original print, some diffuse color was seen between the bands and at the tail.

Fig. 2. Mass spectrum of the Au34 cluster.

control experiment the cluster was loaded onto a RP silica gel column. The unchanged material could be completely eluted from the gel with methanol, indicating that the peaks on the analytical column represent real species and were not the result of partial deterioration. Mass spectrometry revealed a pattern of peaks centering at a molecular weight of 11600 (Fig. 2). The mass differences between the spikes of this peak correspond very closely to AuCN (m = 222.9). There was a minor impurity having a mass of about 20000. The analytical data (C,H,N analysis) were in agreement with a formula \( \text{Au}_{34}L_{10}(\text{CN})_{8-\delta} \) (\( L = \) ligand 3). The amount of CN groups could not be calculated exactly from the elemental analysis. If one assumes that the differences between individual spikes seen in RP chromatography (Fig. 3a) represent differences in the amount of bound AuCN, the gold core may be best described as \( \text{Au}_{34}\pm\delta \). On the basis of these results the material consists of a mixture of very similar clusters, probably contaminated with a cluster of greater size. This fact is not a limitation in view of the aim of labelling biological substrates.

Stability

A major problem with large gold clusters is their lability in solution [2], which may be defined as the stability of the cluster itself in view of its decomposing to metallic gold and free phosphine ligands (intrinsic stability). Distinct from this may be the relative stability of a particular cluster species, which may transform to other species. On the basis of the appearance of free phosphine ligands, the Au34 cluster is relatively stable in aqueous solu-
tion for several days at a pH of seven or higher. A ca. 0.05 mM solution in 0.1 M triethanolamine/HCl buffer (pH 7.3), kept for one month at room temperature displayed a release of 3 to 4% of the phosphine ligands as revealed by HPLC (Fig. 3b). There appeared, however, a change in the pattern of the Au34 cluster peak, and small amounts of other cluster species, present in the starting material only in traces, became more visible (see Fig. 3b). Small changes in the HPLC pattern of the spikes of the cluster peak usually appeared already within a few hours in solution (not shown), indicating an easy transformation of individual cluster species which may explain the difficulty of isolating pure species by ion exchange chromatography (see above). At low pH the cluster was much less stable and deteriorated within days. Nevertheless, it was possible, for example, to perform a preparative gel chromatography in the presence of dilute acetic acid. Like other gold clusters, the material was not stable against mercaptans. A rapid decomposition occurred in the presence of cyanide. In 0.1 M KCN the color of the cluster disappeared within seconds. HPLC of the resulting solution displayed the free ligand 3, usually contaminated with a trace of the corresponding phosphine oxide. In the solid state the cluster was highly stable at least for months at room temperature.

Ligand exchange

Gold clusters undergo an intermolecular exchange of ligands and an exchange between bound and free ligands (phosphines) as well. The rate of this exchange is particularly high in large gold clusters [7]. The Au34 cluster was treated with the monoaminophosphine 4 in a molar ratio of free to bound ligands of about 1.4 to 1. Exchanged ligands were measured in the supernatant liquid after precipitation of the cluster. Under the conditions described in the caption to Fig. 4, the exchange led to an equilibrium within 40 to 50 min with a half reaction time of 1 to 2 min. The chromatogram of the resulting material on a cation exchange column displayed, in addition to a small amount of unbound ligand (which still contained 4 as ligand), a very broad peak of species with various amounts of ligands exchanged (not shown). Since a pure monoamino cluster cannot be prepared because of intermolecular ligand exchange (see below), the whole cluster material of this exchange reaction was used in the experiments described below and was referred as “amino cluster”. The intermolecular ligand exchange was demonstrated qualitatively by mixing a 2 mM solution of the Au34 cluster with the amino cluster in a ratio of 3 to 1. In ion exchange chromatography (DEAE Sepharose) after about 30 min the main peak of the Au34 cluster had almost disappeared and the major part of the material did not bind to the anion exchanger, indicating a spread of the amino ligand 4 over most of the cluster molecules (not shown).

Attachment to proteins

An important application of clusters in electron microscopy is the labelling of specific sites in a biological substrate. As a model we used G-actin, which displays one reactive SH group at Cys374. The amino cluster described above was “activated” by introducing maleimido groups, using 3-maleimidopropionic acid N-succinimidyl ester (see Exp. Part). G-actin was incubated with the activated cluster. The mixture was analyzed by gel-HPLC. Under the conditions described in Fig. 5 the labelling oc-
curred over ca. 24 h with a half reaction time of 3 - 4 h. If the molar ratio of activated cluster to protein in the reaction mixture was close to 1, one main labelled product appeared (Fig. 5d), despite the presence of statistically more than one reactive maleicimido group per cluster molecule. Clearly there is a higher probability for the free activated cluster to react with the protein compared to the reaction of a polyvalent cluster already attached to the protein [9]. In contrast, in the presence of an excess of protein over activated cluster, the main product had a lower retention time (corresponding to a higher molecular weight, Fig. 5e), indicating partial cross linking due to the polyvalent activated cluster. In the latter experiment the activated cluster reacted almost quantitatively with G-actin (Fig. 5e, dotted line), while in the experiments with equimolar or excess activated cluster the final labelling was not higher than 50% (calculated from the area of the chromatogram). This needs further investigation.

In a preparative experiment the partially labelled actin was separated from unbound cluster by gel chromatography on a Sephadex G100 column in a 2 mM Tris/0.1 mM ATP buffer (not shown). The purified labelled G-actin could not be polymerized to filaments of F-actin by addition of KCl but formed unordered aggregates instead, as was revealed by electron microscopy (not shown). These aggregates formed in the presence of MPAA as well, excluding the possibility of crosslinking due to remaining maleicimido groups. It was not possible to block maleicimido groups remaining after the reaction with mercaptoethanol, due to the sensitivity of the cluster to mercaptans. Maleicimido groups, however, are not very stable in water and are expected to deactivate spontaneously during preparation. The aggregation may be a particular feature of actin; other biological substrates can perhaps be labelled without aggregation or denaturation.

In a control experiment, the SH group of G-actin was blocked by treatment with an excess of MPAA. The following incubation with activated cluster (with MPAA still present) led to the binding of only a very small amount of cluster (0.04 to
0.05 mole per mole actin) as revealed by gel-HPLC (Fig. 5f), indicating that the attachment of the activated cluster to the protein was really by reaction with the free Cys374. The binding of a small amount of cluster after blocking of the SH group was not surprising in principle, because maleimido groups may also interact with amino groups. There may be some other mechanism effective as well.

A further control experiment was performed by incubating G-actin with a large excess (2.6 mole cluster per mole actin) of non-activated Au34 cluster. Within 4 days, about 0.2 mole cluster per mole actin became bound, as revealed by gel-HPLC (not shown). This reaction was almost completely suppressed if the actin was first treated with MPAA. The binding of a non-activated gold cluster to a protein with a free SH group is to be expected as a consequence of the reactivity of the cluster against mercaptans [9]. It is not clear, however, why this reaction does not go to completion. A small amount of cluster-containing material appeared in the last control experiment within the exclusion volume of the gel-chromatography column independent of the blocking of SH groups (not shown) indicating a side reaction which cannot be explained by the information available at present.

Summary

Through the use of phosphine 3 as ligand, a large gold cluster with a core of 32 to 36 gold atoms could be easily prepared. This cluster was relatively stable in aqueous solution at room temperature and physiological pH. The cluster undergoes fast ligand exchange, allowing the introduction of the monoaminophosphine 4. After attachment of maleimido groups to this "amino cluster", the cluster was shown to bind covalently to G-actin. Control experiments have indicated that at least small amounts of the "non activated" cluster also bind to G-actin, probably by direct interaction with the SH group of the protein.

Experimental

Abbreviations used: HPLC = high pressure liquid chromatography; IEF = isoelectric focussing; TLC = thin layer chromatography; Rf relative position of spots in TLC; RT retention time in analytical HPLC; L = phosphate ligand 3; MPAA = 3-maleimidopropionic acid amide; TBK = triethylammonium bicarbonate; TEA = triethylamine. Mass spectra of the clusters were obtained by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) using a Bruker BOFLEX instrument in the linear mode with pulsed ion extraction switched on. Sinapinic acid matrix was used in the positive-ion mode. For FAB mass spectra a Joel JMS-SX 102A instrument was used. 1H-NMR spectra were measured on a Bruker AM-500 spectrometer operating at 500 MHz with dimethyl sulfoxide-d6 as solvent and tetramethylsilane as reference. HPLC was carried out using the "Gold®" system (Beckman) with a dual wavelength detector. A Super-ODS column 4.6 x 50 mm (Toso Haas) was used for reversed phase chromatography with a gradient formed of solvent A (10 mM of tetra-butylammonium bromide in 100 mM of Na-phosphate buffer, pH ≈ 6.8) and acetonitrile (0 to 100% within 10 min, flow rate 1.2 ml/min); the detector wavelength was 260 nm and/or 400 nm. Retention times given are merely a rough guide, and they varied due to minor changes in the solvent concentration and other factors. A TSKgel G2000SWXL, 7.8 x 300 mm column (Toso Haas) was used for gel-HPLC with solvent A at a flow rate of 1 ml/min. For preparative reversed phase chromatography a Lobar LiChropep® RP18 (310-25) column (Merck) was used. Tri(2-furyl)phosphine and AuCN were obtained from Strem; 1,2-bis(2-aminoethoxy)ethane was purchased from Aldrich; MPAA was prepared from 3-maleicimidopropionic acid N-succinimidyld ester [8] by brief treatment with NH3; TBK buffer was removed from collected fractions by evaporation i. vac., followed by addition of methanol and repeated evaporation, if necessary several times. Reactions of phosphines were done under argon. A rigorous exclusion of air was not necessary; chromatography was performed without protection from air which led to a small contamination of phosphines with the corresponding phosphine oxides (identified as reaction products of treatment of phosphines with KMnO4 at high pH). G-actin was prepared according to [10]; IEF gels were prepared according to the prescription of the supplier using 1% agarose and Ampholine™ 3.5-10 (Pharmacia). Aluminium sheets Silica gel 60 F254 (Merck) were used for TLC, solvent n-butanolic/acetic acid/water (4:1:1 v/v).

Synthesis of 1 and 2

928 mg (4 mmol) of tri(2-furyl)phosphine was dissolved in diethyl ether (~100 ml). To the cooled solution (~10 to ~30 °C) was added an excess of 1.6 M n-butyl lithium (10 ml, 16 mmol). The solution was allowed to warm up and kept at room temperature for about 60 min. HPLC of a hydrolyzed (water) sample revealed after that time the presence of about 40% starting material (RT = 7.86 min) and 60% of the assumed butyl-bis(2-furyl)phosphine (RT = 9.04 min), based on the absorption at 260 nm.
The solution was put on solid dry ice. After evaporation of the carbon dioxide, water (100 ml) was added. The solution was made strongly alkaline with 2 M sodium hydroxide and extracted once with diethylether (150 ml). The ether solution was discarded. The water phase was made acidic with concentrated HCl and the reaction product was extracted with diethylether. The residue after evaporation of the solvent was dissolved in methanol/water (~12 ml) and transferred to a preparative RP18 column, previously equilibrated with methanol/water/acetic acid (25:100:1 v/v). The column was eluted using a linear gradient from 25% up to 100% methanol within 80 min at a flow rate of 5 ml/min. 1 (RT = 5.54 min; yield: 310 mg) appeared after 36 - 48 min, 2 (RT = 5.96 min; yield: 685 mg) emerged after 58 - 70 min. Compound 2 can be recrystallized from toluene. 1: 1H NMR: δ = 7.062 and 7.284 (d, furyl-3 and -4-H). - C_{15}H_{20}O_{5}P_{2}H_{2}O (382.2) calcd. C 47.12, H 2.36; found C 47.20, H 2.76. - MS (FAB+): m/z = 449. - C_{47}H_{42}O_{7}P_{2} (540.1) calcd. C 58.33, H 6.76 N 6.25; found C 58.33, H 6.47 N 6.25; Rf = 0.6. 2: 1H NMR: δ = 0.85 (t, CH₃), 1.38 (2m, -CH₂-), 2.20 (t, P-CH₂), 7.22 and 6.98 (d, furyl-3 and -4-H). 13.3 (s, COOH). - MS (FAB+): m/z = 311. - C_{12}H_{15}O_{5}P (310.2) calcd. C 54.19, H: 4.84; found C 54.01 H: 4.78. HPLC revealed the presence of 2 to 4% of the corresponding phosphine oxide (RT = 4.98 min).

**Synthesis of 3**

340 mg of 2 (1.1 mmol) was dissolved in 2 ml of DMF, 670 mg (4.1 mmol) of carbonyldimidazole was added and the mixture was stirred for 20 min at room temperature followed by addition of morpholine (0.5 ml). After further 10 min the reaction was stopped by addition of water (20 ml). The solution was extracted twice with diethylether (2×50 ml), the residue from the ether phase was dissolved in a minimum amount of 25% methanol and put on a RP column, equilibrated with 25% methanol and 1% acetic acid in water. Elution was done with a gradient from 25 to 100% methanol in 90 min at a flow rate of 5 ml/min. Compound 3 (RT = 6.8 min) emerged after 74 - 84 min, preceded by a small amount of the corresponding oxide (RT = 4.7 min) after 50 - 60 min, yield: 290 mg (non-crystalline material, 59%). - MS (FAB+): m/z = 449. - C_{22}H_{29}O_{5}N_{2}P (448.5) calcd. C 58.94 H 6.47 N 6.25; found C 58.33 H 6.76 N 6.10.

**Synthesis of 4**

432 mg (1.4 mmol) of 2 was dissolved in DMF (1 ml) and mixed with an excess of carbonyldimidazole (0.6 g, 3.7 mmol). After 30 min at room temperature morpholine was added (140 µl, 1.6 mmol) followed after additional 15 min by an excess of 1,2-bis(2-aminoethoxy)ethane (0.4 ml, 2.7 mmol); after ~1 h water (5 ml) and acetic acid (2 ml) were added and the mixture was applied to a RP18 column, previously equilibrated with water / 2% acetic acid. The column was washed with the same solvent, followed by a gradient from 0 to 100% methanol / 1% acetic acid in 90 min at a flow rate of 5 ml/min. Fractions were checked by TLC. There appeared two peaks which strongly reacted with ninhydrin. The first peak after 40 - 52 min (Rf = 0, RT = 3.6) was assumed to be the bis-amino derivates; 4 (~130 mg) emerged after 58 - 62 min (Rf = 0.38, RT = 5.3). By acetylation with a stoichiometric amount of acetic anhydride the appearance of just one reaction product was confirmed by TLC, indicating the presence of only one reactive NH₂ group.

**Synthesis of the Au34 cluster**

100 mg of AuCN was added to a solution of 200 mg of 3 in methanol (5 ml) and stirred under argon for 60 min. Undissolved AuCN was removed by centrifugation and the supernatant liquid was treated with an excess of NaBH₄ (11 mg, dissolved in 0.5 ml ethanol with a trace of triethylamine). The black-brown solution was kept under argon for 60 min. Water (20 ml) was added and the mixture was extracted once with diethylether (100 ml). The ether phase contained 115 mg of 3 which could be re-used without further purification. The water phase was put on a DEAE fast flow Sepharose column (Pharmacia; 6×2.5 cm, previously equilibrated with 1M TBK and washed with water). The column was washed with water, followed by a gradient of TBK up to 500 mM within 60 min at a flow rate of 5 ml/min. There appeared first a small amount of green-brown material (after 10 - 12 min), followed by a green cluster (14 - 20 min) and some brown material (22 - 24 min). The dark red cluster was collected after 32 - 46 min (about 233 OD₄₀₀, 33 mg). The material was purified by rechromatography under the same conditions (see Fig. 1); yield: 150 OD₄₀₀ (22 mg, about 10%). An analytical sample was prepared by analogous chromatography, using NaCl as eluent. This sample was desalted by adsorption of the cluster to a small amount of RP18 silica gel (Merck; 10 ml gel for 20 mg), washing with water and elution with methanol. Au₃₄L₁₀(CN)₇Na₂ = Au₃₄C₂₆H₉₂O₅N₉P₆Na₁₂ (11430) (the amount of Na was an estimate): calcd. C 23.93, H 2.54, N 3.43; found (mean of 3 samples) C 24.02, H 2.65, N 3.34. The extinction of a 1% solution in water at 400 nm (d = 1 cm) was 67.3, corresponding to a molar extinction coefficient of 78 × 10³.

**Activation of the amino cluster**

50 µl of a ca. 2 mM (E₄₀₀ = 156) methanolic solution of the amino cluster (product from the reaction shown in Fig. 4, purified by precipitation from methanol with diethylether three times) was mixed with 50 µl of 0.5 M triethanolamine/HCl buffer (pH 7.8). After addition of
6μl of 200 mM 3-maleicimidopropionic acid N-succinimidyl ester [8] in acetonitrile the solution was kept for 15 min at room temperature, followed immediately by purification on a Sephadex G25 column (50×0.8 cm) with water as solvent; most of the colored peak, except the very last part, was collected and kept at −80 °C until use.

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