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Rat urate oxidase produced by recombinant baculovirus expression: Formation of peroxisome crystalloid core-like structures

(recombinant DNA/peroxisome nucleoid/nuclear localization/immunocytochemistry)

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ABSTRACT **Urate oxidase (EC 1.7.3.3), which catalyzes the oxidation of uric acid to allantoin, is present in most mammals but absent in humans and hominoid primates. In rats and most other mammals that catabolize uric acid to allantoin, this enzyme is localized within the crystalloid cores of peroxisomes present in liver parenchymal cells. To determine whether urate oxidase forms these crystalloid cores or whether core-forming protein(s) exist in association with urate oxidase, a baculovirus expression vector system was used to overproduce the full-length rat urate oxidase in *Spodoptera frugiperda* cells. Urate oxidase was expressed to a level of ≈30% of the total protein in this system. Immunoblot analysis demonstrated that the baculovirus-generated protein had electrophoretic and immunologic properties similar to those of urate oxidase expressed in rat liver. Immunofluorescence and electron microscopic examination revealed that the overexpressed recombinant urate oxidase is present in both the cytoplasm and the nucleus of infected insect cells as numerous 1- to 3-μm discrete particles. These insoluble protein aggregates, which were positively stained for urate oxidase by protein A-gold immunocytochemical approach, did not appear to be delimited by a single membrane. They revealed a crystalloid structure reminiscent of rat peroxisomal core consisting of bundles of tubules with an inner diameter of ≈50 Å. The recombinant urate oxidase particles, isolated by a single-step procedure, were composed entirely of 35-kDa urate oxidase subunit. These studies indicate that rat urate oxidase is capable of forming insoluble crystalloid core-like structures.**

Urate oxidase or uricase (urate:oxygen oxidoreductase, EC 1.7.3.3), an enzyme that catalyzes the oxidation of uric acid to allantoin, occupies a pivotal position in the chain of enzymes responsible for the metabolism of purines (1). The end product of purine metabolism varies from species to species; in bacteria and some marine invertebrates, purines are degraded to uric acid, allantoin, allantoic acid, urea, and then to ammonia and carbon dioxide (1). Most mammals, with the exception of humans and hominoid primates, contain urate oxidase in their livers (2, 3) and excrete allantoin as the end product of purine metabolism, as these mammals do not contain allantoinase and allantoicase. The loss of urate oxidase activity in humans and hominoid primates (such as chimpanzee, gorilla, and orangutan) results in the excretion of uric acid (1). The absence of urate oxidase in humans and hominoid primates has been shown recently to be due to nonsense mutations in the urate oxidase gene (4–6). The presence of normal urate oxidase cDNA sequences in the Old and New World monkeys [such as baboon, rhesus monkey, and squirrel monkey (4, 6)] and the indication that the structure of the urate oxidase gene is highly conserved during evolution (4–7) suggest that a mutational event leading to the silencing of this gene in humans and hominoid primates is a relatively recent evolutionary event (2, 6).

The importance of urate oxidase as an analytical tool for the determination of serum uric acid has long been recognized (8). Recently, the molecular cloning of urate oxidase cDNA from some species (4, 9) and the determination of the structure of rat urate oxidase gene (7) have generated considerable interest in the evolutionary aspects of this gene (4–6). Furthermore, since this gene is expressed exclusively in the liver of most mammals, it provides a valuable system for studying tissue-specific regulation of gene expression (9). Additional attraction for urate oxidase lies in the speculation that inactivation of this gene may be required for the development of neurological manifestations of Lesch–Nyhan syndrome in the mouse model with complete hypoxanthine-guanine phosphoribosyltransferase deficiency (10). Also of interest is the observation that this enzyme is localized within or associated with a crystalloid core or nucleoid structure present in the peroxisome of hepatic parenchymal cells (11, 12). Since such core-like structures are insoluble and appear to exhibit species-specific substructural configuration (11), this enzyme becomes potentially an attractive system for studying the structural determinants and possible urate oxidase-associated proteins, if any, in the formation of the crystalloid core (12).

In the rat liver peroxisome, the peroxisomal core or nucleoid has been frequently described as crystalline, crystalloid, or multilamellated on the basis of ultrastructural appearance (11, 13). There is considerable controversy as to whether the enzyme urate oxidase forms a crystalloid core by itself (14) or whether such a core is formed by other protein(s) and urate oxidase is simply compartmentalized in such nucleoid cores (12, 15–18). To examine this issue, we have chosen to express a full-length rat liver urate oxidase cDNA (9, 19) in a baculovirus/insect cell expression system because of the high levels of foreign gene expression obtained with the viral polyhedrin promoter (for review, see ref. 20). This system offers the additional advantage that the infected eukaryotic insect cells carry out many of the post-translational modifications found in mammalian proteins (21, 22). In this paper we report that *Spodoptera frugiperda* fall worm cells infected with recombinant baculovirus encoding the entire rat urate oxidase produce large quantities (2–3 mg of urate oxidase per 2×10^7 infected cells) of enzymatically active urate oxidase protein. The observations that urate oxidase forms insoluble crystalloid core-like aggregates within the infected *S. frugiperda* cells and that such structures can be easily purified by a single-step procedure are of considerable interest.

MATERIALS AND METHODS

Baculovirus Vectors and Insect Cells. The baculovirus transfer vector pVL1392, a wild-type baculovirus *Au-*

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tographa californica multiple nuclear polyhedrosis virus (AcMNPV strain) and the insect host cultures of *S. frugiperda* cells were obtained from Invitrogen. Cell cultures were grown in a modified Grace's insect cell culture medium (GIBCO).

Construction of pUOX-3. We have isolated (9, 19) two cDNA clones, pUOX-1 and pUOX-2, coding for rat urate oxidase. Clone pUOX-1 lacked the nucleotide region encoding the first 10 amino acids in the amino-terminal region of the enzyme. pUOX-2 contained the full-length cDNA but had a single nucleotide change (T → C) at position 372. This resulted in a Tyr → His change (19). pUOX-3 containing the full-length correct amino acid sequence, based on the genomic sequence (7), was constructed by joining the 5′ *Eco*RI–*Nco* I fragment of pUOX-2 to the 3′ *Eco*RI–*Nco* I fragment of pUOX-1 (Fig. 1).

Construction of PVL1392-UOX3. The full-length urate oxidase cDNA, containing the entire coding and most of the 3′ untranslated region without the polyadenylylation signal, was released by digesting pUOX-3 with *Eco*RI and *Bam*HI (Fig. 1). This 1.2-kilobase fragment was then cloned into *Eco*RI–*Bam*HI site in the expression vector pVL1392, and the resulting plasmid was designated pVL1392-UOX3.

Generation and Purification of Recombinant Virus pUOX3-AcMNPV. Transfer of urate oxidase cDNA into the AcMNPV genome was achieved by cotransfection of PVL1392-UOX3 (2 μg) and AcMNPV (1 μg) DNA into exponentially growing insect cells by the $CaCl_2$ precipitation method modified for insect cells (23). After 6 days, the supernatant from this transfection culture was serially diluted and used to infect fresh insect cells growing in a 96-well microtiter plate. Recombinant virus was preselected by a limiting dilution dot-blot hybridization and plaque purification method (24).

Purification of Urate Oxidase. Exponentially growing *S. frugiperda* cells in a 750-ml tissue culture flask were infected with the recombinant virus encoding urate oxidase. Approximately 48 hr after infection, the cells were dislodged, transferred to sterile centrifuge tubes, and sedimented at 1000 × *g* for 5 min. The cell pellet was then lysed with 10 mM Tris·HCl (pH 7.5) containing 2% (wt/vol) sodium deoxycholate and stirred at 4°C for 1 hr. This suspension was then centrifuged at 12,000 × *g* for 30 min. The pellet, which contained essentially all urate oxidase, was solubilized by stirring with 0.1 M Na_2CO_3 (pH 11) for 1 h (16).

Immunomorphological Procedures. Electron microscopy and protein A-gold staining were performed essentially as described (3). Immunofluorescence localization of urate oxidase used polyclonal antibodies against rat urate oxidase and cells that were transferred onto glass slides by a cytocentrifuge at 1000 rpm for 5 min. Fluorescein-conjugated anti-rabbit antibody (Sigma) was used to visualize antigen–antibody complexes. Controls were fixed cells incubated with conjugated secondary antibodies alone and with preimmune rabbit serum.

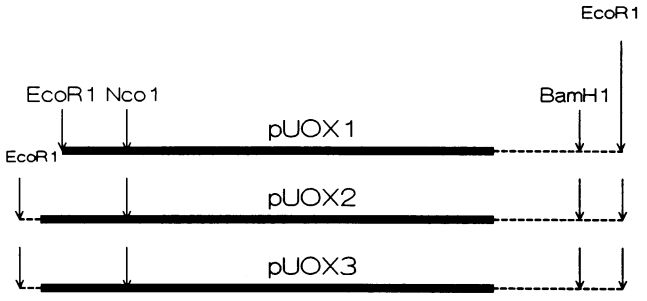

FIG. 1. Construction of pUOX-3. pUOX-1 and pUOX-2 cDNAs were double-digested with *Eco*RI and *Nco* I, and the fragments were separated on an agarose gel. The 5′ *Eco*RI–*Nco* I fragment of pUOX-2 and the 3′ *Eco*RI–*Nco* I fragment of pUOX-1 were electroeluted and ligated. The resulting full-length pUOX-3 cDNA was sequenced to confirm the correct predicted amino acid sequence. The dashed line denotes noncoding region.

Other Methods. The urate oxidase activity was determined as described by Kalckar (25). One unit of enzyme activity is defined as the activity that converts uric acid to allantoin at the rate of 1 μmol/min. Protein was estimated by the Bradford procedure (26). $NaDodSO_4$/PAGE and immunoblot analysis were performed as described (27, 28). Plasmid isolation, endonuclease digestion, ligation, and hybridization were performed according to standard protocols (29).

RESULTS AND DISCUSSION

Expression of Full-Length Rat Urate Oxidase in Insect Cells. Recombinant baculoviruses have become very attractive for foreign gene expression because of their ability to express large quantities of functional eukaryotic recombinant proteins (20–22). The pVL1392 baculovirus transfer vector, used in the current study, was designed specifically with a mutation in the polyhedrin ATG translation initiation codon to allow expression of native eukaryotic proteins lacking additional fused leader sequence encoded by the vector (22). In the present study, under optimal conditions of infection of *S. frugiperda* cells with the recombinant virus pUOX3-AcMNPV containing the cDNA of rat liver urate oxidase, the maximum quantity of recombinant protein was produced 60 h after infection. The specific activity of urate oxidase in *S. frugiperda* cells, 48 h after infection, was ≈100 times greater than that found in rat liver (0.54–0.65 units/mg of protein in *S. frugiperda* cells versus 0.005 unit/mg of protein in rat liver). A time course of expression of urate oxidase activity in insect cells infected with the recombinant virus is illustrated in Fig. 2. A marked elevation of urate oxidase activity occurred 36 h after infection and the levels continued to increase 60 h after infection, the last time point tested.

$NaDodSO_4$/PAGE analysis of whole cell lysates of *S. frugiperda* cells infected with recombinant baculovirus containing rat urate oxidase cDNA for 60 or 70 h revealed a major 35-kDa band corresponding to the expected molecular weight of the urate oxidase subunit (3, 19). Densitometric scanning of Coomassie blue-stained gels showed that this 35-kDa band accounted for 30–40% of the protein in the total homogenate 60 h after infection in three experiments (data not shown). This 35-kDa protein in infected cells increased in amount in a time-dependent manner beginning 24 h after infection and

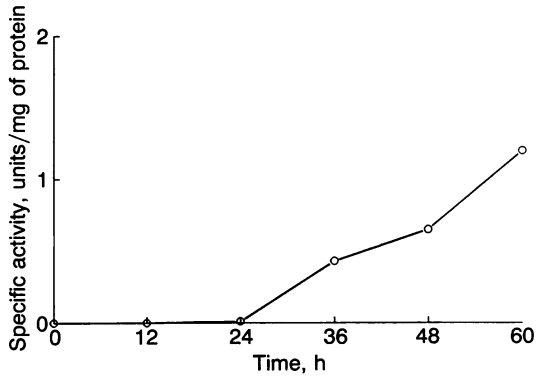

FIG. 2. Time course of expression of rat urate oxidase in *S. frugiperda* cells infected with the recombinant virus pUOX3-AcMNPV. Insect cells were grown in a monolayer in a 75-cm tissue culture flask. When the growth reached 75% confluency, they were infected with the recombinant virus at time 0. Cells were then harvested 12, 24, 36, 48, and 60 h after infection and assayed for urate oxidase activity. The results are expressed as the average of two infections.

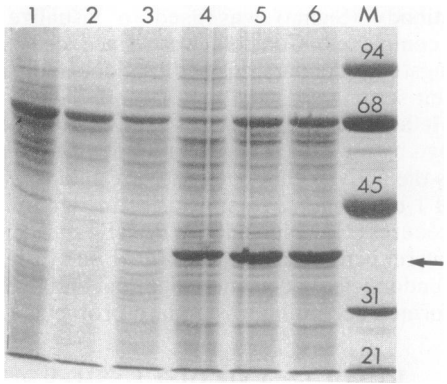

FIG. 3. $NaDodSO_4$/PAGE analysis of whole cell lysates of insect cells infected with the recombinant virus containing urate oxidase cDNA. Cells were grown and infected as described in Fig. 2 and harvested at various times after infection. They were then suspended in 0.1 M Na_2CO_3, lysed by brief sonication, and analyzed by $NaDodSO_4$/PAGE. Approximately 75 μg of protein was loaded in each lane. Lanes: 1, lysate of uninfected *S. frugiperda* cells; 2–6, cell lysate of infected cells harvested 12, 24, 36, 48, and 60 h after infection, respectively. Molecular mass markers (in kDa) are in lane M.

became prominent 36 h after infection (Fig. 3). This agrees well with the increases in urate oxidase enzyme activity (Fig. 2). Immunoblot studies demonstrated that this protein was specifically recognized by polyclonal antibodies to rat urate oxidase (data not shown). Uninfected insect cells or cells infected with the wild-type virus did not show any immunoreactive protein. This may be due to the possibility that the anti-rat urate oxidase antibody did not recognize the insect urate oxidase or because it was present in quantities below the limit of immunodetection. These results clearly suggest that insect cells infected with recombinant baculovirus express huge quantities of enzymatically active urate oxidase.

Subcellular Localization of Baculovirus-Expressed Urate Oxidase. In rat and in other mammalian livers, urate oxidase is localized exclusively within the peroxisomes of hepatic parenchymal cells (3, 11, 12). Recent studies have identified a tripeptide peroxisomal targeting signal that targets proteins to peroxisomes (30, 31) (Ser-Lys-Leu or a conservative variant such as Ser-Arg-Leu), located at the carboxyl terminus of the majority of peroxisomal proteins, including urate oxidase. It has been suggested that the tripeptide peroxisomal targeting signal can also function in diverse mammalian species and in yeast, plants, and insects (31). This implies the presence of a receptor on the peroxisome membrane that is capable of recognizing proteins with the peroxisomal targeting signal for internalization. Because proteins expressed in baculovirus/insect cell system appear for the most part to undergo necessary post-translational modifications and are targeted to the correct organelle (20, 21), we decided to investigate the cellular and subcellular distribution of recombinant urate oxidase in insect cells by immunofluorescence and protein A–gold immunocytochemical methods. Further, the ultrastructural examination of *S. frugiperda* cells infected with a baculovirus harboring the urate oxidase cDNA would reveal whether the recombinant protein forms crystalloid core-like structures in these insect cells. *S. frugiperda* cells infected with recombinant virus when examined by immunofluorescence microscopy with polyclonal antibody against rat urate oxidase revealed the presence of numerous intensely fluorescent discrete granular structures, measuring 1–3 μm in diameter (Fig. 4 *A* and *B*). These round to oval immunofluorescence-positive bodies were found in the cytoplasm and the nucleus of infected cells. In some cells that ruptured during cytospin preparation, these granules were found to be scattered but to retain their configuration. Uninfected insect cells and cells infected with wild-type virus showed no discernible fluorescence for urate oxidase (data not illustrated). It is also pertinent to note that uninfected insect cells also showed no catalase-positive cytoplasmic organelles on immunofluorescence analysis with polyclonal antibodies against rat catalase (Fig. 4*C*). The failure to detect peroxisomes in the uninfected insect cells by these immunofluorescence methods may be due to the inability of the antibodies against rat catalase and urate oxidase to recognize these proteins in insect cells or that these insect cells contain few or no intact peroxisomes. It may be worthwhile to microinject purified peroxisomal proteins into these insect cells to determine whether such proteins can be sorted to peroxisomes.

The absence or paucity of peroxisomes in insect cells maintained as a cell line may be relevant in view of the cytoplasmic and nuclear localization of aggregates of recombinant urate oxidase expressed in these cells (Fig. 5 *A* and *B*). The urate oxidase is present in infected *S. frugiperda* cells as electron-dense bodies measuring up to 3 μm in size that do

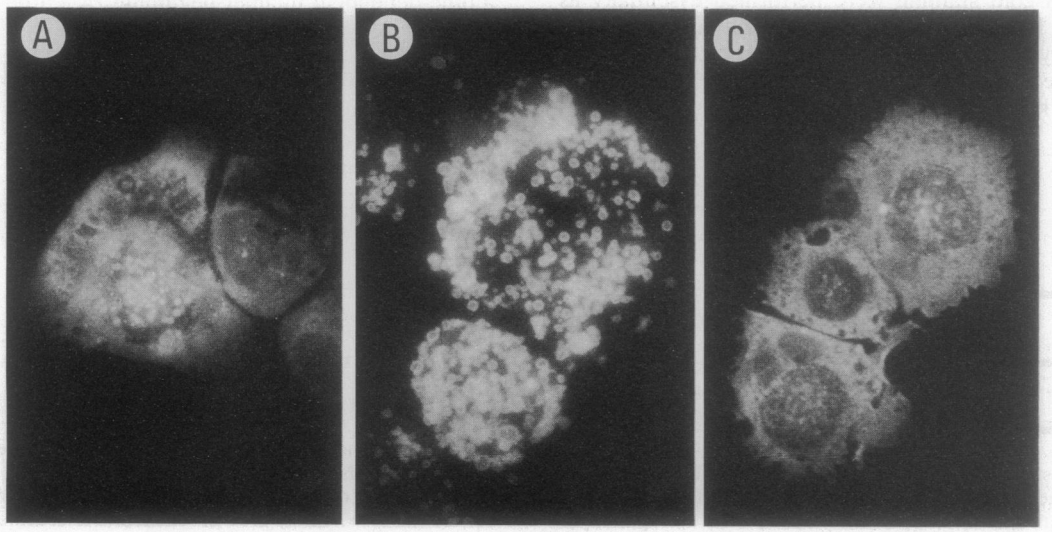

FIG. 4. Immunofluorescence analysis of *S. frugiperda* cells. (*A* and *B*) Representative cells infected with recombinant baculovirus containing rat urate oxidase cDNA immunostained with antibodies against rat urate oxidase. Numerous intensely fluorescent round to oval structures are seen in the nucleus and cytoplasm. (*C*) Uninfected insect cells immunostained with antibodies against rat catalase.

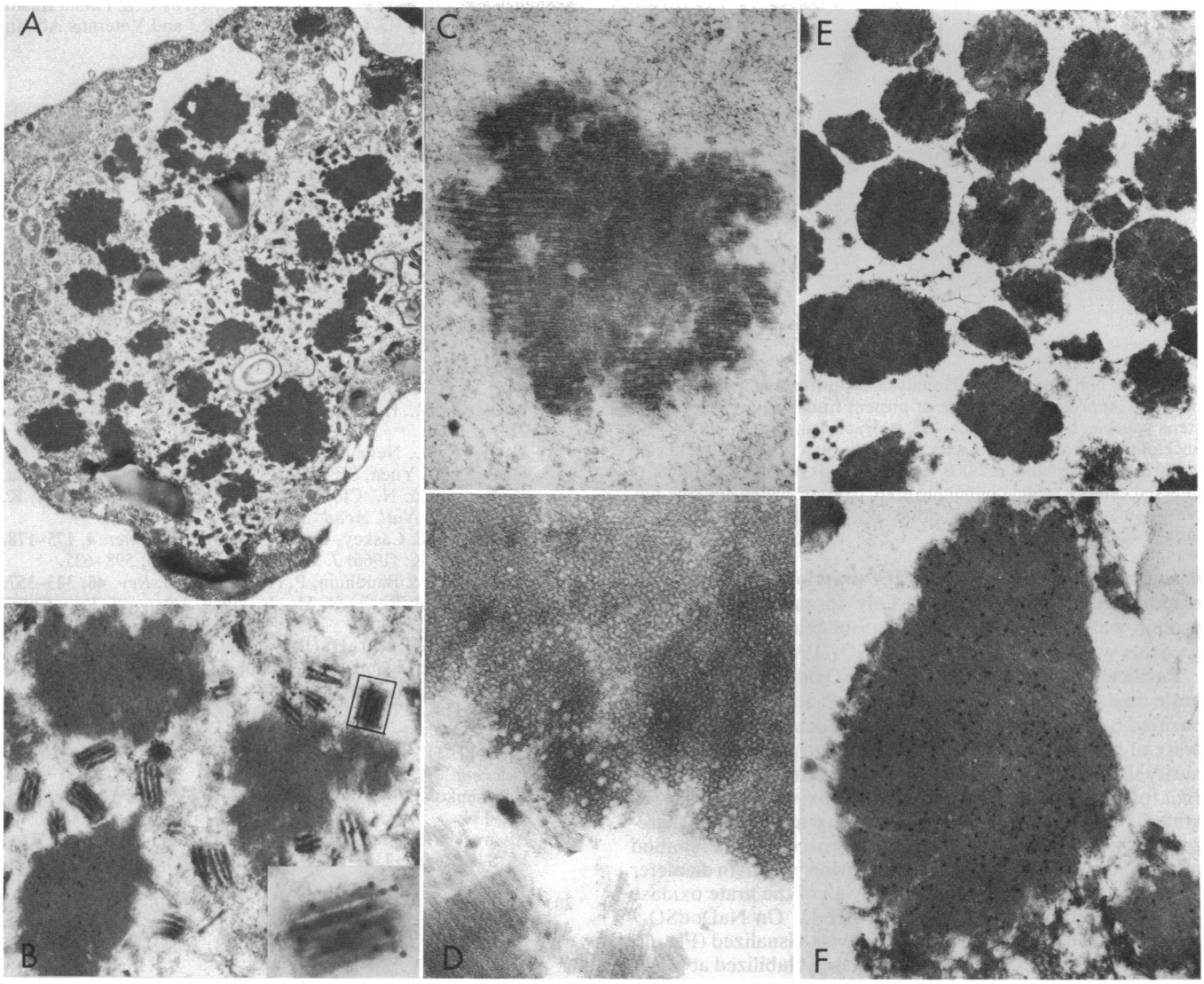

FIG. 5. Electron microscopic features of *S. frugiperda* cells infected with recombinant baculovirus containing rat urate oxidase cDNA and purified fractions of insoluble recombinant protein aggregates. (*A*, *C*, and *D*) Regular transmission electron micrographs of an intact cell (*A*) and longitudinal and cross sections of isolated recombinant urated oxidase aggregates (*C* and *D*). Immunogold localization of urate oxidase in intact cells (*B*) and purified aggregates (*E* and *F*). Note that the recombinant protein aggregates are present in the nucleus and cytoplasm of insect cells (*A*). (*B Inset*) Enlargement of the boxed area showing virus particles heavily and specifically labeled with the protein A–gold particles suggests that urate oxidase protein translocates to the nucleus and sticks to the virus.

not appear to be delimited by a clearly identifiable single membrane (Fig. 5 *A* and *B*). These structures are present in the cytoplasm and nucleus, but in most cells they are more abundant in the nucleus (Fig. 5 *A* and *B*). They are composed of bundles of hollow tubules with an approximate internal diameter of 50 Å (Fig. 5 *B–F*). The polytubular pattern of the recombinant urate oxidase in the insect cells infected for 70 h (Fig. 5 *C* and *D*) was essentially identical to that of the crystalloid configuration of nucleoid cores of rat liver peroxisome cores (14, 15). The urate oxidase was localized in these dense polytubular cytoplasmic and nuclear aggregates by the protein A–gold immunocytochemical procedure (Fig. 5 *B, E and F*). Numerous gold particles were distributed over these structures revealing antigenic sites. It is of interest to note that the observation that recombinant viral particles present in the nucleus of infected cells were also decorated by the protein A–gold particles (Fig. 5*B Inset*) provides additional evidence for the translocation of urate oxidase to the nucleus. The reasons for the translocation of this recombinant protein destined for peroxisomes to the nucleus of these insect cells remain unclear but may reflect an overwhelmed peroxisomal import system. In uninfected insect cells or in cells infected with the wild-type virus or with the recombinant virus expressing β-galactosidase, polytubular crystalloid structures resembling those observed in cells expressing recombinant urate oxidase were not found (data not included).

The observation that recombinant rat urate oxidase forms large crystalloid core-like structures in insect cells, somewhat reminiscent of those seen in rat liver peroxisomes, is of interest. Hruban and Swift (14) pointed out that the rat liver peroxisome crystalloid core is essentially similar to the crystals of commercial preparation of urate oxidase and concluded that the core is made up of urate oxidase. DeDuve and his coworkers (12, 16) questioned this conclusion. They suggested that urate oxidase contributes no more than 25% of the protein of the cores in rat liver peroxisomes and that the cores contain other components that have about the same molecular weight as urate oxidase (16). This led to the suggestion that urate oxidase-associated proteins may be required for the crystalloid core formation. The results of the present study with the recombinant protein expressed in

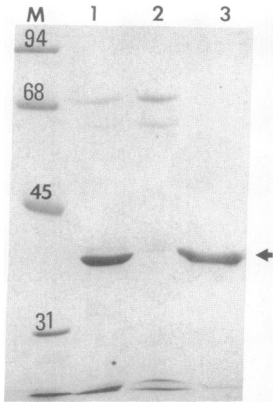

FIG. 6. $NaDodSO_4$/PAGE analysis of purified recombinant urate oxidase. Approximately 50 μg of protein from various purification steps was electrophoresed on 10% polyacrylamide gels in the presence of 0.1% $NaDodSO_4$. Lanes: 1, cell lysates from a 48-h infected culture; 2, supernatant (12,000 × *g*) after deoxycholate treatment of infected cells; 3, pellet containing urate oxidase aggregates (see Fig. 5*E*) dissolved in 0.1 M Na_2CO_3. Molecular mass markers (in kDa) are in lane M.

insect cells strongly argue that the urate oxidase by itself is responsible for the crystalloid core formation as no other protein was identified when these isolated recombinant aggregates were analyzed by $NaDodSO_4$/PAGE (see below).

Purification of Recombinant Urate Oxidase. The rat liver urate oxidase expressed in *S. frugiperda* cells could be easily purified by a single-step purification procedure. The insolubility of mammalian urate oxidase at neutral pH was used to purify the protein. When the cells were stirred in 10 mM Tris·HCl (pH 7.4) containing 2% sodium deoxycholate, >95% of urate oxidase was pelleted at 12,000 × *g* as insoluble aggregates. These pellets on electron microscopic examination revealed crystalloid aggregates measuring ≈2 μm in diameter (Fig. 5*E*). They contained essentially all of the urate oxidase and dissolved in 0.1 M Na_2CO_3 at pH 11. On $NaDodSO_4$/PAGE only one 35-kDa protein band was visualized (Fig. 6). The specific activity of the urate oxidase solubilized at pH 11 was 7.2 units/mg of protein, which is near the specific activity (8.3 units/mg) of the homogeneous porcine enzyme (32).

The present study demonstrates the utility of the baculovirus expression system for highly efficient production of urate oxidase that can form relatively large insoluble aggregates in the cytoplasm and nucleus of insect cells. Such aggregates consisting of only the enzyme protein are very easy to isolate by a one-step method. The finding that these recombinant urate oxidase protein aggregates in insect cells display a substructure similar to that seen in rat liver peroxisomal crystalloid cores—the intracellular sites of urate oxidase in mammals—will certainly assist in biochemical and biophysical analyses of wild-type urate oxidase and mutant urate oxidases. Expression of mutant and truncated urate oxidase cDNAs in the baculovirus system will be necessary for elucidating structural determinants.

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