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Preparation and crystallization of a human immunodeficiency virus p24–Fab complex

(AIDS/capsid protein/crystallography/monoclonal antibodies)

ANDREW J. PRONGAY*, THOMAS J. SMITH*, MICHAEL G. ROSSMANN*†, LORNA S. EHRLICH‡, CAROL A. CARTER‡, AND JAN MCCLURE§

*Department of Biological Sciences, Purdue University, West Lafayette, IN 47907; ‡Department of Microbiology, State University of New York, Stony Brook, NY 11794-8621; and §Oncogen, 3005 First Avenue, Seattle, WA 98121

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ABSTRACT **A recombinant form of human immunodeficiency virus capsid protein, p24, expressed in *Escherichia coli* has been purified to homogeneity and separated into distinct isoelectric forms. A monoclonal antibody, mAb25.4, which recognizes an epitope in the amino-terminal region of p24, has been purified to homogeneity from ascites fluid and digested with papain to produce the respective antigen-binding fragment (Fab). The Fab25.4 was purified from the digestion mixture and separated into two distinct isoelectric forms. The two Fab species were each complexed with one isoelectric form of the recombinant p24 by incubating equimolar quantities of the two proteins. Two different crystal morphologies of the p24–Fab25.4 complex were obtained by the vapor-diffusion method with 12–24% PEG 3350 as the precipitant. One of these crystal forms has unit-cell parameters of a = 92.1 Å, b = 85.4 Å, c = 54.0 Å, $\alpha = \gamma = 90.0°$ and $\beta = 90.4°$ and belongs to the monoclinic space group $P2_1$, with one molecule of the complex per asymmetric unit. These crystals strongly diffracted x-rays to at least 2.7-Å resolution.**

A possible technique for inhibiting human immunodeficiency virus (HIV) infectivity is to block the assembly or disassembly of the core particle. This method has been shown to be effective against human rhinoviruses (1, 2). The capsid proteins forming the icosahedral shell of picornaviruses are eight-stranded antiparallel β-barrels (3–7), forming a hydrophobic pocket in the interior of the protein. This pocket permits some flexibility of the surrounding polypeptides, which may be of functional importance in the assembly and disassembly of the capsid structure. A series of hydrophobic organic compounds (designated by the abbreviation WIN) have been shown to bind in this pocket in human rhinoviruses and to prevent the uncoating of the virus by imparting rigidity to the capsid (2, 8). Such a pocket may also exist in other, probably homologous, virus capsids (9, 10) and would, therefore, be useful as a target for similar antiviral agents. A few secondary structure predictions have been published of HIV p24, which suggest that it folds either into an all-α structure (11) or as a β-barrel (9, 10). Regardless of the nature of the fold, it is possible that a hydrophobic pocket may be present as a functional requirement and, therefore, could be exploited for binding antiviral agents, although there is no experimental evidence for the existence of such a pocket in HIV p24. An aromatic polycyclic dione, hypericin, has been shown to be an antiretroviral agent (12) that affects either the assembly or processing of intact virions of retroviruses, possibly by inhibiting assembly and/or disassembly of the capsid (13), but there may be alternative mechanisms (14).

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The HIV capsid protein, which is encoded by the *gag* gene as part of a polyprotein, has been isolated from virally infected cells as a mixture of four isoelectric species, two of which contain phosphorylated residues (15). The primary structure of HIV p24 shows that the protein has a high degree of hydrophobic character. Not surprisingly, the protein has been shown to undergo self-aggregation (16), a property expected of a capsid protein. We have, therefore, crystallized the far more soluble complexes of p24 with antigen-binding fragments (Fabs). The purification and crystallization of one such complex is described here.

MATERIALS AND METHODS

Protein purifications were performed either on a fast protein liquid chromatography system employing Mono Q, Mono S, and Superose 12 columns or by conventional chromatography using protein A-Sepharose and protein G-Sepharose resins, all purchased from Pharmacia. p24–antibody interactions were measured by an ELISA using ScreenType purchased from Boehringer Mannheim. Absorbance measurements were recorded with a Perkin–Elmer model λ 3B spectrophotometer. SDS/PAGE was performed as described by Laemmli (17). PEG 3350 was purchased from Fisher Scientific. Ultrapure ammonium sulfate was purchased from ICN. Papain was purchased from Sigma. ^{125}I-labeled protein A was purchased from ICN. All other reagents were of the highest quality available.

Recombinant HIV p24 was expressed in *Escherichia coli* BL21(DE3) using a plasmid, pHIV-FSII, encoding truncated HIV-1 *gag/pol* genes (18). The protein was purified to ≈90% purity as described (16) and subsequently purified to homogeneity on a Pharmacia fast protein liquid chromatography system by binding to a Mono Q column equilibrated with 20 mM Tris Cl, pH 8.0, containing 0.1% (wt/vol) 1-octyl-β-glucopyranoside and eluting with a linear gradient from 0 to 500 mM NaCl in 20 mM Tris Cl, pH 8.0. The protein was fractionated into distinct isoelectric forms by chromatography on a Mono S column equilibrated with 10 mM citric acid/10 mM sodium phosphate, pH 4.5, and eluted with a linear pH gradient from 4.5 to 7.5. Between 80 and 90% recovery was generally achieved.

The anti-p24 monoclonal antibody (mAb) mAb25.4 was purified by passing ascites fluid over a protein A-Sepharose column equilibrated with 3.0 M NaCl/1.5 M glycine, pH 8.9. After washing with this same buffer, the mAb was eluted

Abbreviations: Bistris Cl, [bis(2-hydroxyethyl)amine]tris(hydroxymethyl)methane chloride; CHESS, Cornell high-energy synchrotron source; HIV, human immunodeficiency virus; mAb, monoclonal antibody; mAb25.4, a monoclonal antibody directed against HIV p24; Fab25.4, the antigen-binding fragment resulting from papain digestion of mAb25.4; p24, the major capsid protein of HIV; Tris Cl, tris(hydroxymethyl)aminomethane chloride.

†To whom reprint requests should be addressed.

from the column with 0.1 M citric acid/NaOH, pH 3.9. The fractions containing antibody were immediately neutralized with 1.5 M Tris Cl, pH 8.8, and the purity of the antibody was shown by 0.08% SDS/6% PAGE. The ability of mAb25.4 to form a complex with the recombinant p24 was demonstrated by an ELISA with hydrogen peroxide and *o*-phenylenediamine as substrates. The purified mAb was digested with papain (1 mg of papain/200 mg of mAb) in 0.1 M sodium phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol at 37°C for 3 hr. The digestion was stopped by the addition of either iodoacetamide or 5,5′-dithio-bis(2-nitrobenzoic acid) to give a final concentration of 30 mM. The digestion mixture was extensively dialyzed at 4°C against several 1-liter volumes of 20 mM Tris Cl, pH 8.0, and concentrated by size-exclusion centrifugation through an Amicon Centriprep membrane filter with a 30,000 M_r cutoff. The digestion mixture was fractionated into Fab, Fc, and partially digested mAb components on a Mono Q column equilibrated in 20 mM Tris Cl, pH 8.0, using a linear gradient from 0 to 500 mM NaCl. Usually 30% by weight of the starting material was recovered, corresponding to ≈50% of the Fab.

Complex formation was achieved by incubating the p24 with the Fab at approximately equimolar concentrations in 20 mM Tris Cl, pH 8.0. The presence of a complex was demonstrated by isoelectric focusing in 1% agarose containing Pharmalytes in the pH 4.0–8.0 range, and the protein bands were made visible with Coomassie brilliant blue stain after fixing with trichloroacetic acid. The complexed protein was separated from the uncomplexed proteins by chromatography on a Mono Q column using either a linear gradient from 0–500 mM NaCl in 20 mM Tris Cl, pH 8.0, or a linear pH gradient from 8.5 to 5.8 in 20 mM Tris Cl. Crystallization of the complex was achieved at room temperature (≈25°C) by the vapor-diffusion method using both hanging drops and sitting drops with PEG 3350 as precipitant. The reservoir solution contained 1 ml of 12–24% PEG 3350/40 mM [bis(2-hydroxyethyl)amine]tris(hydroxymethyl)methane chloride (Bistris Cl), pH 6.2. Crystals with a cubic morphology grew in approximately 8 weeks in hanging drops. Subsequently, crystals with cubic-like as well as parallelopiped-like morphologies were grown from sitting drops, both *de novo* and by the microseeding technique, in 12–24% PEG 3350/40 mM Bistris Cl in the pH range of 6.0 and 7.5 and when either $CaCl_2$ or $MgSO_4$ was present at 20 mM final concentration both in the drop and in the reservoir. The diffraction patterns of the cubic-like crystals and the parallelopiped-like crystals were observed on film with an oscillation camera using x-rays from a rotating anode source and the Cornell high-energy synchrotron source (CHESS), respectively. The data were processed using the OSC123 autoindexing program (19) and the OSC-CYB oscillation film processing program (20). Reflections from different films were scaled together and postrefined (21).

The composition of the crystalline material was determined after washing a few of the parallelopiped crystals by transferring them into three successive 300-μl volumes of 30% (wt/vol) PEG 3350/50 mM Bistris Cl, pH 7.0. The crystals were dissolved in 20 μl of Nanopure grade water; 5 μl of a sample loading buffer was added, and the solution was heated to 100°C and maintained there for 2 min. The material was analyzed by 0.08% SDS/13% PAGE, and the protein bands were detected by a silver-staining technique (22). In addition, the dissolved crystalline material was analyzed by a Western immunoblot procedure (23) subsequent to electrophoresis through a 0.08% SDS/13% polyacrylamide gel from which the protein bands had been transferred with a semi-dry blotting apparatus to a nitrocellulose paper presoaked in 39 mM glycine/48 mM Tris Cl, pH 9.2/0.0375% (wt/vol) SDS/20% (vol/vol) methanol. The nitrocellulose blot was soaked overnight (≈15 hr) at ≈25°C in 3% (wt/vol) bovine serum albumin/0.2% (wt/vol) gelatin/0.9% (wt/vol) NaCl/15 mM Tris Cl, pH 7.4. After several washing steps, the nitrocellulose blot was soaked in a solution of 3% (wt/vol) bovine serum albumin/0.9% (wt/vol) NaCl/15 mM Tris Cl, pH 7.4, containing an anti-p24 mAb at 100 μg/ml for 3 hr at 25°C, subjected to several washes, and soaked in a solution of 3% (wt/vol) bovine serum albumin/0.9% (wt/vol) NaCl/15 mM Tris Cl, pH 7.4, containing 5 μCi (1 Ci = 37 GBq) of ^{125}I-labeled protein A for 2 hr at ≈25°C, after which the nitrocellulose blot was again washed several times. Between each soaking the blot was washed once with 0.9% (wt/vol) NaCl/15 mM Tris Cl, pH 7.4, then washed twice with 0.9% (wt/vol) NaCl/15 mM Tris Cl, pH 7.4/0.05% Tween 20, and finally washed twice with 0.9% (wt/vol) NaCl/15 mM Tris Cl, pH 7.4. Each wash was done in a 100-ml volume for 10 min at 25°C, except after the ^{125}I-labeled protein A soak the washes were for 8 hr each. The bands were visualized by exposing the blot for 48 hr at −70°C to Kodak DEF-5 x-ray film using a DuPont Cronex intensifying screen.

RESULTS

When an HIV *gag/pol* precursor is expressed in *Escherichia coli*, it is efficiently processed into the individual *gag* proteins (18). Substantial purification of the mature p24 arising from this processing had been attained (16) by using a "facilitated" aggregation step. Further purification to homogeneity was achieved by anion-exchange chromatography on a Mono Q column using a linear gradient of increasing ionic strength. However, initial attempts to dissolve the precipitated protein at concentrations >5 mg/ml in 20 mM Tris Cl, pH 8.0, resulted in a solution containing multiple aggregated states, a large proportion of which did not bind to the Mono Q resin. Addition of 1-octyl-β-glucopyranoside (0.1% final concentration) to the protein solution significantly decreased the aggregation, as indicated by binding of p24 to the resin and its elution as a single peak at 50 mM NaCl (data not shown). Purification of the protein to homogeneity by this chromatographic step was confirmed by 0.1% SDS/13% PAGE. The hypothesis that the detergent altered the aggregation state of the protein was tested by removal of the detergent from the protein fraction eluting at 50 mM NaCl by using extensive dialysis at 4°C against several 1-liter volumes of 20 mM Tris Cl, pH 8.0. This procedure resulted in a solution of p24 that bound to the resin and eluted as a very broad peak at a higher ionic strength (data not shown), suggesting that a different aggregated state may be present after removal of the detergent. Crystallization depends on the ordered packing of highly purified protein; therefore heterogeneity of the protein sample can prevent the crystallization process. Thus, the apparent presence of multiple aggregated forms of this recombinant p24 in the absence of detergents might cause difficulties in crystallization. Therefore, to minimize the aggregation, 1-octyl-β-glucopyranoside was added to all p24 solutions.

The purified recombinant p24 was analyzed by isoelectric focusing and shown to be a mixture of two isoelectric forms with pI values of ≈6.55 and 6.75 (Fig. 1 *Inset*). The protein was fractionated into the two distinct isoelectric forms by chromatographic separation on a Mono S column using a linear pH gradient (Fig. 1). Treatment of the mixture with bacterial alkaline phosphatase had no effect on distribution of the protein in these two isoelectric forms (data not shown). The relative amounts of the two isoelectric forms appeared to be preparation dependent—some preparations consisted predominantly (as much as 95%) of the species having a pI value of 6.75, whereas in other preparations the distribution approached a 50/50 mixture of the two species (Fig. 1 *Inset*). Storage of the individual isoelectric forms at 4°C for longer than a week did not result in the appearance of the other

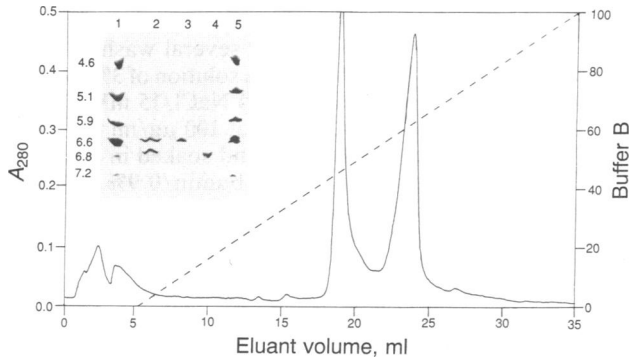

FIG. 1. Chromatographic separation of recombinant HIV p24 (*E. coli*) into distinct isoelectric forms. The protein sample in 10 mM citrate/10 mM phosphate, pH 4.5, was applied to a Mono S column equilibrated in the same buffer. p24 was eluted as two distinct isoelectric forms with a linear pH 4.5–7.5 gradient. (*Inset*) Isoelectric focusing of recombinant HIV p24. Samples of recombinant HIV p24 expressed in *E. coli* were applied in 20- to 30-μg lots to agarose gel containing Pharmalytes in the pH 4.0–8.0 range. The gel was focused with an electrophoretic current of 13 mA at 5°C. Lanes: 1 and 5, protein standards of known pI values; 2, recombinant HIV p24 before separation into distinct isoelectric forms; 3, recombinant HIV p24, peak 2 of Mono S chromatographic separation by linear pH gradient; 4, recombinant HIV p24, peak 3 of Mono S chromatographic separation by linear pH gradient.

isoelectric form, suggesting that the individual forms are not different aggregated states or conformations. The cause of this variability is not known, but it might arise as a result of a different posttranslational modification of the protein.

The low solubility of p24 and its tendency to aggregate prompted attempts to crystallize p24–Fab complexes. The mAb 25.4, which recognizes an epitope at the N terminus of HIV p24 (J.McC. unpublished work), was used in the current study. The isolation of this mAb from ascites fluid by immunoaffinity chromatography yielded 95–100% pure mAb as shown by 0.08% SDS/6% PAGE under reducing and nonreducing conditions (data not shown). After digestion with papain, the Fab fragment was purified from the digestion mixture by ion-exchange chromatography on a Mono Q column using a linear gradient of increasing ionic strength, which fractionated the Fab into three peaks (Fig. 2). Isoelectric focusing analyses of the Fab fractions revealed (Fig. 3) that at least two isoelectric forms of Fab, with pI values of

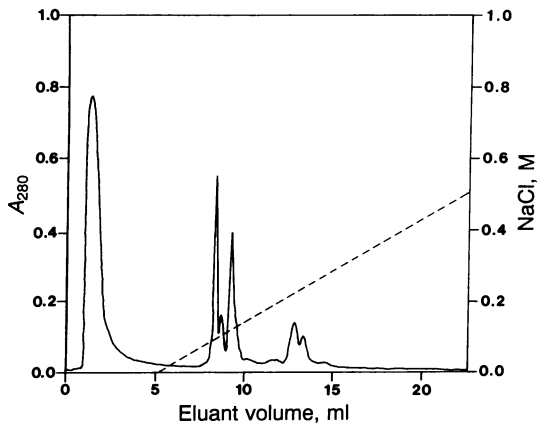

FIG. 2. Chromatographic purification of Fab25.4. After digestion and dialysis against 20 mM Tris Cl, pH 8.0, the digest of mAb25.4 was applied to a Mono Q column equilibrated with 20 mM Tris Cl, pH 8.0/1 mM azide. Fab and Fc fragments were separated with a linear gradient from 0–0.5 M NaCl in 20 mM Tris Cl, pH 8.0/1 mM azide. The pregradient peak (peak 1) is residual thiol reagent; peaks 2–4 are Fab, and peaks 5 and 6 are Fc.

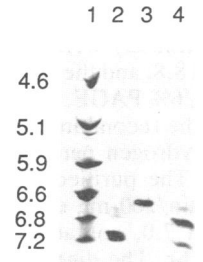

FIG. 3. Isoelectric focusing analysis of the formation of a complex between recombinant HIV p24 (expressed in *E. coli*) and Fab25.4. Samples of p24, Fab25.4, and the complex p24–Fab25.4 were applied to an agarose gel containing Pharmalytes in pH 4.0–8.0 range. The gel was focused with a current of 13 mA at 5°C. Lanes: 1, standard proteins of known pI values; 2, first peak of Fab25.4 eluted from Mono Q column; 3, p24; 4, complex of p24 and Fab25.4 (taken from peak 2 in Fig. 2).

≈6.8 and 7.1, were present in each of the three peaks and that some fractionation into distinct isoelectric forms occurred during chromatographic separation of the Fab and Fc fragments. Further separation of the two isoelectric forms is achieved by a second passage over a Mono Q column of the material collected in each peak from the first column.

Complexes were prepared between recombinant p24 expressed in *E. coli* (the more abundant species with pI 6.75) and Fab25.4. Complex formation was confirmed by isoelectric focusing analyses—the complexes each had isoelectric points with values between those of the uncomplexed p24 and Fab (Fig. 3), and these were separated from the uncomplexed proteins by ion-exchange chromatography (data not shown).

The complex crystallizes in two apparently different crystal morphologies—a cubic-like morphology with a maximum dimension of 0.15 mm on a side and a parallelopiped-like morphology with a maximum dimension of 0.3 mm × 0.2 mm × 0.2 mm (Fig. 4). The presence of both protein species in the crystalline material was demonstrated by 0.08% SDS/13% PAGE analyses of the dissolved crystals (Fig. 5). The Fab was present as two species (giving rise to three bands)—a species containing an interchain disulfide that migrated at a M_r of 45,000 and a species in which this disulfide has been reduced and migrated as two bands of M_r = 25,000 and 20,000 (representing heavy and light chains, respectively). Differences in the relative affinity for the stain may explain the unequal distribution of intensity of these two bands. The p24

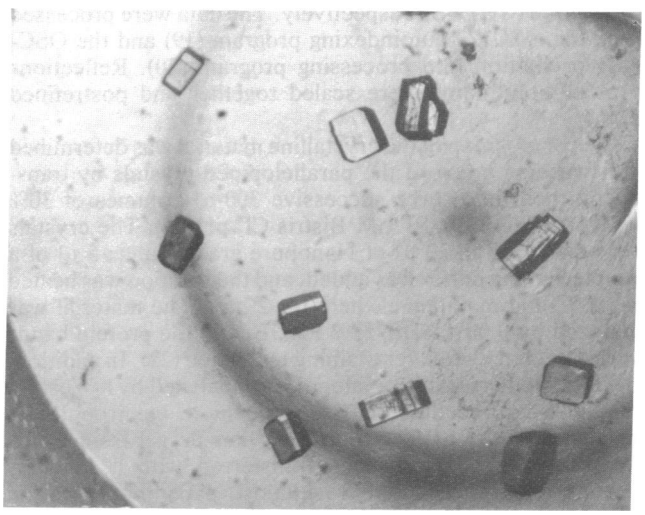

FIG. 4. Parallelopiped-like crystals of the p24–Fab25.4 complex. The largest crystals had dimensions of 0.3 mm × 0.2 mm × 0.2 mm.

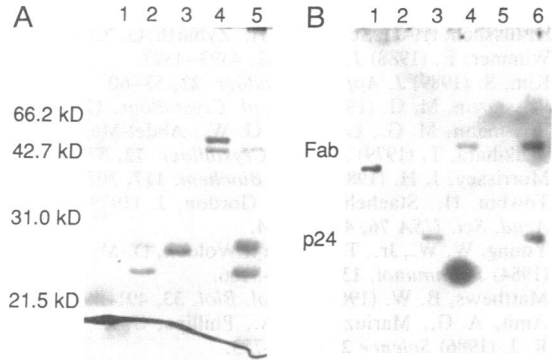

FIG. 5. SDS/PAGE analysis of the protein content of the parallelopiped-like crystals of p24–Fab25.4. A few crystals were washed three times in 30% PEG 3350/40 mM Bistris Cl, pH 7.0, and dissolved in 20 μl of Nanopure-grade water sample buffer. After dissolution 5 μl of 40% (vol/vol) glycerol/200 mM Tris Cl, pH 6.8/5% SDS was added, and the sample was heated in a boiling water bath for 3 min. The material was electrophoresed through 0.08% SDS/13% polyacrylamide gels. Note that it was difficult to assess the amount of protein present in loading the sample derived from dissolving crystals. (*A*) Bands were visualized by silver staining (22). Lanes: 1, M_r standards; 2, Fab25.4; 3, p24; 4, p24–Fab25.4 complex; 5, dissolved crystal of p24–Fab25.4 complex. In the crystal Fab appears as two species with the sulfhydryls in both oxidized and reduced states. (*B*) Bands were visualized by Western immunoblot method with ^{125}I-labeled protein A. Lanes: 1, ^{14}C-labeled M_r markers; 2, empty; 3, p24; 4, complex of p24–Fab25.4; 5, Fab25.4; 6, dissolved crystalline material of p24–Fab25.4 complex.

migrated as a single band of M_r = 24,000, close to the heavy chain of the Fab. Because p24 and Fab heavy-chain bands are not well resolved, the presence of p24 in the dissolved crystalline material was demonstrated by a Western immunoblot procedure (Fig. 5*B*, lane 6). The disulfide-crosslinked Fab species also gives a positive signal (Fig. 5*B*, lanes 4 and 6), presumably because protein A has been shown to bind to the Fabs of some IgG isotypes (24). The absence of a signal for the crosslinked Fab species in lane 5 of Fig. 5*B* possibly resulted from the transfer of a quantity of this species below a level detected by the blotting procedure.

The cubic-shaped crystals diffract x-rays to at least 3.9-Å resolution but are somewhat disordered (data not shown). The parallelopiped-shaped crystals have unit-cell dimensions of a = 92.1 Å, b = 85.4 Å, c = 54.0 Å, $\alpha = \gamma = 90°$ and β = 90.4° (after postrefinement). The Laue symmetry is 2/m, showing that the crystals are monoclinic. There are no general (*hkl*) systematic absences. Inspection of the axial reflections (0*k*0) revealed the presence of a 2_1 screw axis along b, demonstrating the space group is $P2_1$. The molecular mass of the complex is ≈75 kDa. Thus, V_M = 2.82 Å^3/Da (25), assuming one complex per crystallographic asymmetric unit, which is a most reasonable value. These crystals diffract x-rays well to at least 2.7-Å resolution (Fig. 6). Crystals grown at pH 7.5 are well ordered, but those grown at pH 6.2 show rather large mosaicity. Twenty degrees of data have been processed, and the $I/\sigma(I)$ value is 6.35 at 2.8-Å resolution. The R_{sym} value is 8.8% for 3432 independent reflections derived from 11 film packs.

CONCLUSION

The structure of a number of antigen–antibody complexes has been determined (26–28). These authors observed that the conformational changes induced on the antigen in the formation of such a complex while real was, nevertheless, small. In the present case, the objective is to use such a complex as a method of crystallization in anticipation that the structure of the p24 antigen will not be altered by formation of the antigen–antibody complex.

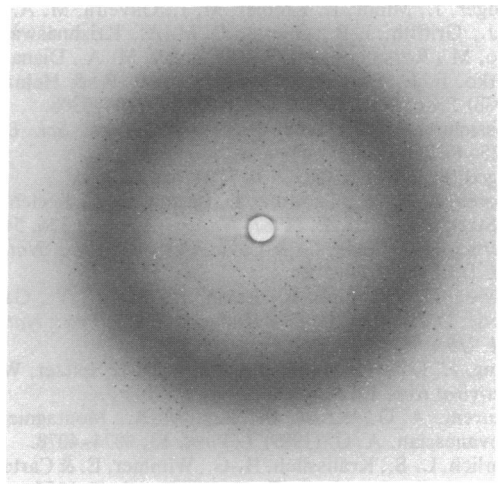

FIG. 6. Diffraction pattern of a p24–Fab25.4 crystal taken at CHESS. Crystal-to-film distance was 75 mm, and the oscillation angle was 1.5°; the x-ray wavelength was 1.54 Å.

Knowledge of the structure of p24 may provide insight into the packing arrangement of the capsid structure and show whether a hydrophobic pocket is present. Such a structure might, therefore, provide the basis for modifying antiviral agents that bind to the core protein or finding new compounds that bind into the putative functional WIN pocket. The structure of the complex will provide information about the interaction between p24 and the antigen-binding domains of antibodies. Furthermore, this structure together with known Fab structures can be used with the molecular replacement method for determining the structure of other complexes of p24 with different antibodies. At present two such complexes have been crystallized, one of which is the p24–Fab25.4 complex described above, and the other is a p24 complex with Fab25.3. The resultant information of p24–Fab interactions might prove useful for developing a vaccine against HIV.

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