Crystallization and Preliminary X-ray Studies of Human Recombinant Interleukin-2*

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Two different forms of crystals (potentially) suitable for x-ray structure analysis were obtained for recombinant human interleukin-2 (IL-2) using ammonium sulfate as a precipitant in the pH range of 6.3-7.3 (in the case of hexagonal bipyramidal crystals) and 4.5-5.5 (in the case of plate crystals). The hexagonal bipyramidal crystal belongs to a hexagonal space group P6$_2$2 or P6$_2$2 with $a = b = 105.8$ Å and $c = 122.2$ Å. The crystal diffractions up to 3.4 Å resolution and contains 2 or 3 IL-2 molecules in an asymmetric unit. The plate crystal belongs to an orthorhombic space group P2$_1$2$_1$2 with $a = 47.9$ Å, $b = 79.6$ Å, and $c = 31.9$ Å. The crystal diffractions up to 2.5 Å resolution and contains only 1 IL-2 molecule in an asymmetric unit. These facts reconfirmed crystallographically the high homogeneity of the present preparation of human recombinant IL-2.

Interleukin-2 (IL-2), also referred to as T-cell growth factor, is a lymphokine produced by activated T-cells (1, 2). The protein is reported to promote long-term in vitro proliferation of antigen-specific effector T-lymphocytes and to induce cytotoxic T-lymphocyte reactivity (3-6), indicating its potential clinical value for treatment of neoplastic diseases. Recently, mRNAs for human IL-2 from various sources, such as a leukemic T-cell line (7), splenocytes (8), tonsillar mononuclear cells (9), and peripheral blood lymphocytes (10) have been isolated, and their cDNAs have been cloned, sequenced, and expressed in Escherichia coli. All these nucleotide sequences turned out to correspond to one common amino acid sequence. The expressed protein was purified to homogeneity by successive chromatographies (10-13). In one case, the amino acid sequence of the single polypeptide chain, consisting of 133 amino acid residues ($M_r$, 15,500), was determined by fast atom bombardment mass spectrometry (13) and turned out to be the same as that deduced from the above-mentioned nucleotide sequences. Furthermore, no functional differences have been detected between the native and recombinant IL-2s (10, 14), though a small difference in the primary structure has been noted (15).

In the present laboratory, the IL-2 protein was accumulated at high concentrations in the cytoplasmic inclusion bodies of E. coli (20). The expressed protein was dissolved and quantitatively refolded into a biologically active form through a specific procedure devised recently (21). The correctly refolded protein was purified to homogeneity by successive chromatographies. Now we report on the crystallization and preliminary x-ray studies of human recombinant IL-2.

MATERIALS AND METHODS

Preparation of Recombinant Human IL-2 (20, 21)—cDNA coding for IL-2 of the human leukemic T-cell line JURKAT 111 was cloned and expressed in E. coli (7). IL-2, as accumulated in the inclusion bodies, was produced in E. coli that was appropriately constructed and cultured. The cells were collected by centrifugation and homogenized by lysozyme treatment and sonication at 4 °C. The resultant lysate was centrifuged at 10,000 × g for 5 min, and the pellet-like precipitate was collected. The pellet was dissolved in a minimum amount of 0.1 M Tris-HCl buffer (pH 8.0) containing 6 M guanidium chloride. The solution was diluted to an IL-2 concentration of 0.1 mg/ml with 0.1 M Tris-HCl buffer (pH 8.0) and treated with a solution containing 10 mM reduced glutathione, 1 mM oxidized glutathione, and 2 M guanidium chloride (pH 8.0). After standing for 16 h at room temperature, the pH of the solution was adjusted to 6.0 with acetic acid, and the solution was concentrated by a factor of approximately 15 with Pellicon cassettes (Millipore, cut-off 10,000). The concentrated IL-2 solution was purified by successive chromatographies using Sephadex G-25, CM-Sepharose, YM-30 (Pharmacia P-L Biochemicals), and Mono-S (Pharmacia P-L Biochemicals). The sample of IL-2 thus obtained was proved to have a purity of more than 99% by means of RP-HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses.

Crystalization—The optimum conditions for crystallization of IL-2 were established by vapor diffusion in the hanging drop mode (16). General settings were as follows. Hinging drops of 10 μl each were equilibrated with reservoir solutions of 1.0 ml containing one precipitant such as ammonium sulfate, sodium chloride, sodium citrate, 2-methyl-2,4-pentanediol, and polyethylene glycol. Each hanging drop contained 15-20 μg of IL-2 dissolved in an appropriately buffered solution containing the same (but at a lower concentration) precipitant as used for the reservoir solution. The reservoir solution was buffered at the same pH as, or at the slightly lower pH than, the droplet.

Identification of Crystals as IL-2 by RP-HPLC and Bioassay—The crystals that appeared in the droplets were collected and washed 3 times with the corresponding reservoir solutions to remove excess mother liquor and redisolved for identification by RP-HPLC and bioassay. In a typical case, about 300 μg of crystals were dissolved in 10 μl of 0.2 M sodium phosphate buffer containing 6 M guanidium chloride and 0.03 M EDTA (pH 7.0). The resultant solution was immediately diluted with 90 μl of 0.2 M sodium phosphate buffer (pH 7.0). A 10-μl sample of this solution was injected onto a YMC-Pack AP-202 column (4.6 × 150 mm, Yamamura Chemicals) and eluted with a linear gradient of 48-76% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min for 35 min using a Hitachi 655 high performance liquid chromatography system. The eluate was monitored by measuring the absorbance at 280 nm. The protein concentration was determined by measuring peak area at 280 nm previously calibrated by amino acid analyses. The activity of crystals was assayed

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1 The abbreviations used are: IL-2, interleukin-2; RP-HPLC, reversed-phase high performance liquid chromatography; V, the unit cell volume ($A^3$); Vn, the ratio of unit cell volume to molecular weight ($A^3$/dalton).
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by measuring $[^3]$Hthymidine incorporation into an IL-2-dependent cytotoxic T-lymphocyte line (5, 6).

X-ray Diffraction Studies—Crystals were mounted in thin-walled 1.0-mm fused quartz capillaries. X-ray photographs were taken on a Nonius precession camera at room temperature using either graphite-monochromatized or nickel-filtered Cu-Kα radiation generated by a Rigaku RU-200 rotating anode generator operated at 50 kV and 90 mA. The focus size was $3 \times 0.3$ mm.

RESULTS AND DISCUSSION

Several different types of crystals appeared in the droplets depending on the conditions employed. For the large hexagonal bipyramidal crystals shown in Fig. 1 the concentrations of ammonium sulfate were 0.8–1.3 M and 1.5–2.5 M for the droplet and reservoir well solutions, respectively. The pH of the droplet was in the range 6.3–7.3. The crystals appeared after about 7 days at 25 °C. For large plate crystals as shown in Fig. 2, on the other hand, the concentrations of ammonium sulfate were 0.8–1.5 M and 1.5–3.0 M for the droplet and reservoir well solutions, respectively. In this case, pH of the droplets was in the range 4.5–5.5. The crystals appeared after about 5 days of incubation at 25 °C. For the latter crystal form, apparently the same type of crystals also appeared when sodium chloride or sodium citrate was used as the precipitant. Two additional types of crystals distinct from either the hexagonal bipyramidal or the plate crystals mentioned above appeared when Z-methyl-2,4-pentanediol or polyethylene glycol 4000 was employed as the precipitant. At present, however, these crystals are too small for identification and characterization.

For both the plate crystals and the hexagonal bipyramidal crystals, the crystals in the hanging drops were collected, dissolved, and subjected to RP-HPLC analyses (Fig. 3). In both cases, the retention time coincided with that of the authentic sample of IL-2 purified as described under "Materials and Methods." The mother liquor left in the hanging drops was also subjected to RP-HPLC analyses. In both the plate and hexagonal crystals almost no protein was detected. This indicates that almost all protein molecules contained in the hanging drops were incorporated into the crystals during

FIG. 1. Hexagonal bipyramidal crystals of human recombinant interleukin-2. The maximum dimensions are $0.8 \times 0.5 \times 0.5$ mm. The long axis is the c axis.

FIG. 2. Plate crystals of human recombinant interleukin-2. The maximum dimensions are $0.6 \times 0.1 \times 0.01$ mm. The long axis is the a axis.

FIG. 3. RP-HPLC analyses of (a) purified IL-2, (b) dissolved plate crystals, and (c) the dissolved hexagonal bipyramidal crystals. The column was eluted with a linear gradient made by mixing A, 0.1% trifluoroacetic acid and B, 0.1% trifluoroacetic acid in 80% acetonitrile. A small peak preceding the main peak of IL-2 is present in b and c. This peak was identified as that of modified IL-2 in which Met-104 was oxidized (details will be described elsewhere). Note that the hexagonal crystals contain larger fractions of oxidized material. This might be the reason for lower resolution of this crystal form.
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dalton respectively, defined by Matthews (17). A K2PtCl4 derivative of this crystal form showed reasonable diffraction intensity changes. A preliminary account of this crystal form has appeared (22).

The precession photographs (one of which is shown in Fig. 5) of the plate crystal, on the other hand, exhibited mm m Laue symmetry. The reflections are seen at least up to 2.5 Å. The plate crystal belongs to an orthorhombic space group P212121 with a = 47.9 Å, b = 79.6 Å, and c = 31.9 Å (V = 1.22 \times 10^6 Å³). In this case, only the Vm value of 1.96 Å³/dalton, which corresponds to the existence of 1 IL-2 molecule in the asymmetric unit, is in the acceptable range. Because of the much smaller unit cell volume and higher resolution, the plate crystal form is more suitable for the x-ray diffraction study than the hexagonal bipyramidal one.

The high purity of the present preparation of human recombinant IL-2 has been verified by RP-HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses (see “Materials and Methods”). Now this has also been confirmed by the following crystallographic facts: 1) almost all the protein in the hanging drop was found to be incorporated into the crystals; and 2) only 1 IL-2 molecule was found in an asymmetric unit of the plate crystal of IL-2. The latter fact precludes the existence of a covalently linked oligomer of IL-2, since it is highly unlikely that the “subunits” in such an oligomer, if any, should be associated through crystallographic symmetry elements. It is noteworthy that this holds despite the fact that: 1) all of the three cysteine residues in human IL-2 were found to be in free state in the original product stored as inclusion bodies in E. coli cells; and 2) these were oxidized quantitatively to a correct pattern of linkage (disulfide bridging between Cys-58 and Cys-105 and one free Cys-125 (18, 19)) through a specific procedure devised in the present laboratory (20). Further x-ray diffraction study will reveal the structure and function relationships of IL-2.

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Note Added in Proof—Heavy-atom survey for the orthorhombic crystal form has yielded some promising results.

After submission of this paper we became aware of another paper on the crystallization of human recombinant IL-2 using sodium chloride as a precipitant (Moriya, N., Yamada, T., Kato, K., and Nishimura, O. (1986) Biotechnology 4, 904–905). Although their crystals exhibit partial morphological similarity to ours, the identity of their crystals cannot be known in the absence of any description of their x-ray diffraction experiments.

REFERENCES