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Crystallization and preliminary X-ray diffraction studies of human epidermal growth factor

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Human epidermal growth factor (hEGF), a 6.2 kDa protein of 53 amino acids with three internal disulfide bridges, has been crystallized by the hanging-drop method. hEGF crystallizes in space group $P3_121$ (or $P3_221$) using $MgCl_2$ as precipitant, with unit-cell parameters $a = b = 61.4$, $c = 87.0$ Å. Another type of crystal, obtained using NaCl as precipitant, belongs to a tetragonal point group and has unit-cell dimensions $a = b = 102.5$, $c = 166.6$ Å. The trigonal crystals with the smaller unit cell diffract X-rays better and a native data set from a single crystal has been collected to 3.0 Å resolution.

1. Introduction

Epidermal growth factor (EGF) is a protein that plays an important role in the molecular mechanism controlling mammalian cell growth, oncogenesis and wound healing (Carpenter & Wahl, 1991). For this reason, considerable attention has been directed towards the structural elucidation of EGF in order to clarify the relationship between its structure and function. In this respect, progress has been made recently in understanding the molecular basis for the recognition of EGF by its receptor. The three-dimensional structures of mouse EGF and of a biologically active derivative of human EGF have been determined by a combination of high-resolution ^{1}H NMR and computational techniques (Cooke *et al.*, 1987; Montelione *et al.*, 1987). In addition, the secondary structure of rat EGF (Mayo *et al.*, 1989) and various structures of human tumor growth factor a (TGFa) have also been reported (Kohda *et al.*, 1989; Kline *et al.*, 1990; Harvey *et al.*, 1991). This structural knowledge and the information available from comparison of the amino-acid sequences of the proteins with growth-factor activity led to a proposal for residues important for receptor recognition (Campbell *et al.*, 1990). However, since the NMR structures were obtained from solutions at acidic pH (lower than physiological pH), it is not possible to support the assertion that they reflect the structure of EGF in its native form. This can be confirmed by the fact that EGF has a maximum receptor-binding activity in the pH range 7–9, about 50% activity at pH 6 and no activity below pH 5 (Massagò, 1983). The NMR structures of EGF at pH 6.8 have been reported (Kohda & Inagaki, 1992), but they did not converge as well as those obtained at pH 2.0, mainly owing to the disappearance of NOE cross peaks involving the resonance of some residues at this pH value. The crystal structure of EGF in the physiological pH range would be an important step forward in obtaining more detailed information on its molecular properties and the molecular interaction of EGF with its receptor. Higuchi *et al.* (1988) reported the crystallization of hEGF using PEG 6000, but only data to 6.0 Å were collected. Here, we report a preliminary X-ray crystallographic analysis of hEGF, in which diffraction data to 3.0 Å were collected.

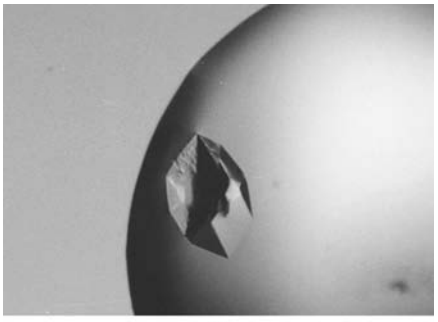
2. Crystallization

hEGF was expressed and purified as described previously (Huang *et al.*, 1998). The purified protein, usually having a purity of ∼95% after threefold chromatography, was concentrated for crystallization experiments. Crystallization of hEGF was achieved by the hanging-drop method using Linbro tissue-culture plates. We could not obtain crystals of hEGF using the reported crystallization conditions (Higuchi *et al.*, 1988), for which trivial differences in the samples may be responsible. Preliminary crystallization trials were conducted at 291 K using Crystal Screens I and II (Hampton Research). Typically, 1.2 µl of protein solution containing 10 mg ml^{-1} hEGF and 0.1% Na_3N were mixed in a 1:1 ratio with reservoir solution and the resulting 2.4 µl drops were incubated at 291 K. Crystal screen II condition 47 (2.0 *M* $MgCl_2$, 0.1 *M* Bicine pH 9.0) produced a large number of the first type of microcrystals within one week. After many refinements to the crystallization conditions, we were able to obtain larger crystals, usually having a size of 0.4 × 0.3 × 0.3 mm (Fig. 1*a*), after about two months. The best crystals were obtained from a solution containing 0.9 *M* $MgCl_2$, 0.1 *M* Bicine (pH 8.1) and 3.5 m*M* CYMAL-3 (cyclohexyl-propyl-β-D-maltoside), 0.1% Na_3N and 50 mg ml^{-1} hEGF. This crystal type can be obtained over a

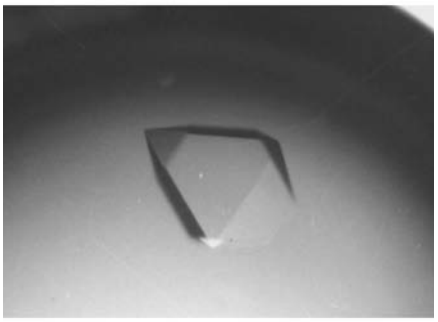
wide pH range (7.1–10.0) using $MgCl_2$ or $CaCl_2$ as precipitant; however, $MgCl_2$ usually generated larger crystals. Another crystal type with a tetragonal prismatic morphology was grown from a solution containing 1.5 *M* NaCl, 0.2 *M* $MgCl_2$, 0.1 *M* Bicine (pH 9.0). These crystals reached a size of 0.5 × 0.5 × 0.6 mm (Fig. 1*b*) in one month. This crystal type could also be obtained using 3.0 *M* NH_4Cl and the same buffer, but in this case had a smaller size.

3. X-ray analysis

Preliminary characterization of the first crystal type indicated a hexagonal or trigonal space group with unit-cell parameters $a = b = 61.4$, $c = 87.0$ Å. Data to a resolution of 3.0 Å were collected from a single crystal, using synchrotron X-ray radiation at the Advanced Photon Source, Argonne National Laboratory. A crystal of the same size (about 0.3 × 0.2 × 0.2 mm) diffracted X-rays to a resolution of 3.5 Å using a rotating-anode generator. All diffraction data were indexed, integrated and corrected for Lorentz and polarization effects using the program *DENZO* (Otwinowski, 1993). Scaling and merging of the data using *SCALEPACK* (Otwinowski, 1993) indicated the space group to be one of the enantiomorphic pair $P3_121$ or $P3_221$, with $R_{\rm merge}$ = 10.4% and a completeness of 99.0% for data in the resolution range 30.0–3.0 Å. The $I/\sigma(I)$ ratio for the highest resolution shell (3.11–3.0 Å) is 2.0. Two, three or four hEGF molecules per asymmetric unit give V_m values (Matthews, 1968) of 3.82, 2.55 or 1.91 Å^3 Da^{-1}, respectively, and solvent contents of 67.6, 51.3 or 35.1%, respectively. This crystal type diffracts X-rays weakly. The reason for this may be the high solvent content of the crystal. In this context, it is more likely that two EGF molecules exist in an asymmetric unit. Calculations of the self-rotation function using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) indicated the possible presence of a non-crystallographic twofold axis perpendicular to the *c* axis and at 30° to the *a* axis; no significant peaks were found in the $\kappa = 120°$ section. At present, neither the value of V_m nor the calculation of the self-rotation function can unanimously determine the number of EGF molecules per asymmetric unit.



(*a*)



(*b*)

Figure 1
Crystals of hEGF. (*a*) Trigonal crystals grown from 0.9 *M* $MgCl_2$, 0.1 *M* Bicine pH 8.1, 3.5 m*M* CYMAL-3; (*b*) tetragonal crystals grown from 1.5 *M* NaCl, 0.2 *M* $MgCl_2$, 0.1 *M* Bicine pH 9.0.

Limited diffraction data for the second type of crystals were collected at room temperature using Cu $K\alpha$ X-rays from a Rigaku RU-200 rotating-anode generator operating at 40 kV and 100 mA. This crystal type (approximate dimensions 0.5 × 0.5 × 0.6 mm) only diffracts X-rays to about 4.5 Å and belongs to space group *P*422, with unit-cell parameters $a = b = 102.5$, $c = 166.6$ Å.

Owing to the availability of NMR structure models for EGF and several EGF-like domains, many attempts have been made to solve the crystal structure of hEGF by the molecular-replacement method, using either models of distance-derived pseudo-*B* factors (Wilmanns & Nilges, 1996) or superimposed models with uniform *B* factors (Kleywegt, 1996), but all these efforts have failed. We think there may be two reasons for this. The first, perhaps the more important, is the differences between NMR models and crystal structure, which decrease the signal-to-noise ratios in the rotation and translation functions. The second may be the small size of EGF and its elongated shape. In molecular-replacement studies, the smaller the molecule the greater the problems (Müller *et al.*, 1995).

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