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Protein microheterogeneity and crystal habits: the case of epidermal growth factor receptor isoforms as isolated in a multicompartment electrolyzer with isoelectric membranes

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Abstract

A purified, soluble form of the epidermal growth factor receptor (sEGFR) was found, by isoelectric focusing in immobilized pH gradients, to consist of three major isoforms (with p*I* values 6.45, 6.71 and 6.96, respectively) and ca. a dozen minor components. This wild-type sEGFR, while producing crystals, has so far defied any attempt at decoding the structure, due to the very poor diffraction pattern. When the wild-type sEGFR was purified in a multicompartment electrolyzer with isoelectric Immobiline membranes, it yielded the three major isoforms as single-p*I* components, collected in three separate chambers of the recycling electrolyzer. The p*I* 6.71 and the p*I* 6.96 isoforms produced large crystals of apparent good quality. However, while the former produced a high-quality diffraction pattern, which may lead to decoding of the three-dimensional structure, the p*I* 6.96 produced crystals which did not diffract at all. It is concluded that, in the case of "tough" proteins (large size, heterogeneous glycosylation, high water content of crystals), purification to single-charge components might be an essential step for growing proper crystals. The unique advantage of purification via isoelectric membranes is that the protein is collected both isoelectric and isoionic, i.e. uncontaminated by soluble buffers (such as the carrier ampholytes used in conventional focusing).

1. Introduction

The epidermal growth factor (EGF) receptor mediates the biological effects of polypeptide mitogens such as EGF and TGF-α, playing an important role in normal and pathological growth control. The receptor is an M_r 170 000 membrane glycoprotein consisting of three functional domains: an EGF binding cell surface domain which is heavily glycosylated, a short transmembrane region, and a cytoplasmic domain with tyrosine kinase activity (for reviews, see [1] and [2]).

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Since overexpression of EGF receptors has been observed in many types of human tumors, structure-based drug design for therapeutic modulation of receptor functions would be of high medical interest. Crystallization of membrane proteins like the EGF receptor, however, is a formidable problem, mainly due to the high extent of glycosylation and to the hydrophobicity of membrane spanning domains. In the case of the EGF receptor one of these problems could be overcome by crystallizing a secreted form of the receptor ("sEGFR") which is produced by a human tumor cell line [3]; it represents the M_r 100 000 external domain of the EGF receptor with functionally intact ligand binding. Crystallization of this hydrophilic receptor ectodomain had been accomplished in the presence of the ligand EGF [4]. Diffraction of these crystals, however, had been only about 10 Å; data collection using these crystals had not been possible so far.

A possible explanation for the poor quality of initial sEGFR crystals may come from the striking charge heterogeneity of the protein: more than 10 components are separated by isoelectric focusing. Since it is known that the presence of several isoforms can induce perturbation of crystal growth, we purified single-p*I* species of the sEGFR protein by using a multicompartment electrolyzer with buffering, isoelectric membranes [5,6]. In this system, the protein is always kept in a liquid vein (thus it is not lost by adsorption onto surfaces, as customary in chromatographic procedures) and it is trapped into a chamber delimited by two membranes having p*I* values encompassing the p*I* value of the protein being purified. Thus, by a continuous titration process, all other impurities, either non-isoelectric or having different p*I* values, are forced to leave the chamber, in which the protein of interest will ultimately be present as the sole species, purified from both macromolecular contaminants and buffering and salt ions as well. This technique has been applied to the purification of single EGF receptor isoforms with remarkable results on the quality of the crystals obtained.

2. Experimental

2.1. Materials

Acrylamide, N,N′-methylenebisacrylamide (Bis), N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium persulphate (APS) were from Bio-Rad, Hercules, CA, USA. The following Immobiline species: p*K* 3.6, p*K* 4.6, p*K* 6.2, p*K* 7.0 and p*K* 8.5 were from Pharmacia-LKB Biotechnology, Uppsala, Sweden. N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES) and L-histidine (free base) were from Sigma, St. Louis, MO, USA.

2.2. Biologicals

EGF ligand was prepared from adult mouse submaxillary glands [7]. A monoclonal anti-sEGFR E30 antibody was developed using immunoaffinity purified protein as antigen [8] (this antibody is now commercially available from E. Merck, Darmstadt, Germany). Monoclonal antibody E30 recognizes a non-carbohydrate epitope located between residues 332 and 589 of the EGF receptor in its native as well as in its denatured conformation.

2.3. Preparation of de-sialo sEGFR

Serum-free medium (Dulbecco's modified Eagle's medium + Ham's F12 medium, mixed 1:1) was conditioned by confluent cultures of a special A431 variant cell line selected for maximal biosynthesis of EGF receptor; supernatants were freed from cellular debris, stabilized with 1 m*M* EDTA, 100 U/ml Trasylol (Bayer) and concentrated by ultrafiltration prior to immuno affinity chromatography [9]. sEGFR was eluted from the immuno adsorbent with 0.1 *M* acetic acid, 0.05 *M* sodium chloride and immediately neutralized with trisodium phosphate. Purified sEGFR (30 mg, 8 ml) was then dialyzed against 50 m*M* sodium acetate pH 5.5, in presence of 4 m*M* calcium chloride, and digested for 3 days at room temperature with 1 unit of neuraminidase (1 mg/ml, from *Vibrio cholerae*; Boehringer

Mannheim) immobilized on 0.3 ml of tresyl chloride-activated agarose (Pharmacia). Dialysis against 10 m*M* Tris acetate buffer, pH 7.4, added with 15% glycerol represented the final step prior to the immobilized pH gradient (IPG) separation.

2.4. Preparation of analytical IPG gels

These gels were of 25 × 10 cm size and 0.5 mm thick. An IPG pH 5.0–8.0 was set in a 5% T, 4% C polyacrylamide matrix[1] (the recipe can be found in Ref. 10). Note that, after preparing the two limiting, acidic and basic mixtures, they are titrated (with a weak acid and a weak base) to pH values close to neutrality. This is important in order to ensure uniform polymerization and efficient monomer conversion throughout the preformed pH gradient. Upon gel washing (4 × 30 min) in distilled water, all added titrants (as well as catalysts and ungrafted monomers) are efficiently removed. The gels are then equilibrated for 30 min in 2% glycerol solution, dried in air and reswollen in 15% (v/v) glycerol solutions. The protein samples (ca. 50 μg in 20 μl) are usually applied in surface wells both close to the anode and to the cathode. Focusing is continued (at 5000 V after an initial 1 h period at 500 V) for 6 h at 10°C. Staining is carried out in Coomassie Brilliant Blue R-250 in presence of Cu^{2+} according to Righetti and Drysdale [11].

2.5. Preparation of isoelectric immobiline membranes

After determining, in the above analytical pH 5.0–8.0 gels, the precise p*I* values of the sEGFR isoforms, seven isoelectric membranes are made having the following p*I* values: 5.90, 6.34, 6.63, 6.76, 6.92, 7.05 and 7.35. The first and last membranes, being adjacent to the anolyte and catholyte compartments, respectively, are made in a 10% T, 5% C matrix, whereas the other five are polymerized in a 5% T, 4% C polyacrylamide. The membranes have a diameter of 4.7 cm and a thickness of ca. 1 mm. Note that the membranes are supported by glass fibre filters (see Ref. [5] for a detailed description of their properties). After washing and equilibrating the membranes in 15% (v/v) glycerol, the multicompartment apparatus is assembled and the entire protein amount (40 mg) equally distributed into the two sample chambers closer to the anodic reservoir. In order to avoid sample dilution, no reservoirs have been connected to the six recycling chambers, so that the total sample volume has been limited to 39 ml total (6.5 ml per chamber). After an initial, low-voltage run (500 V) for eliminating excess salt in the sample, purification has been achieved at 2500 V (over a 12 cm electrode distance) in less than 10 h. The anolyte was 52 m*M* HEPES (pH 5.27, conductivity: 9.1 μS) and the catholyte 14 m*M* L-histidine (pH 7.56; conductivity 11.5 μS). The supporting solution in all chambers was 15% (v/v) glycerol. No circulating coolant was utilized and joule heat was dissipated in air in a cold room (5°C). Under the above conditions, the temperature rise in the liquid in the electrolyzer, at steady state, was only 3°C.

2.6. Crystallization and X-ray investigation

Crystals were grown at a controlled temperature of 20°C by the hanging as well as sitting drop vapour diffusion technique [12]; droplets of 20 μl sEGFR (15 mg/ml) complexed with equimolar amounts of EGF were mixed with 7 μl precipitant (1.95 *M* ammonium sulphate or sodium phosphate, pH 7.5) and exposed to 5 ml reservoir solutions (same as precipitant); crystals grew within variable time spans (> 1 week). Crystals were analyzed on the X11 synchrotron beam-line in the EMBL Outstation at DESY (Deutsches Elektronen-Synchrotron, Hamburg, Germany). The storage ring was operated in main user mode with 4.465 GeV and 20–45 mA. Rotation images were recorded on a MAR 300 mm image plate scanner at room temperature. Exposure times were set to about 6 min for 1° rotation images using a wavelength of 0.92 and a crystal-to-plate distance of 500 mm. Images were

[1] T = (g acrylamide + g Bis)/100 ml solution; C = g Bis/%T.

processed with a version of the XDS integration package [13]. Some crystals were analyzed using a GX21 rotating anode generator (Nonius, Delft, Netherlands) operating at 40 kV and 75 mA (we acknowledge the assistance of Drs. R. Hilgenfeld and H. Bertchold for these experiments).

3. Results

Affinity-purified EGF receptor ectodomain (sEGFR) exhibits one single band in electrophoresis on sodium dodecyl sulphate gels; however, >25 bands can be separated by isoelectric focusing (not shown). Since the charge heterogeneity is mainly caused by variable presence of terminal sialic acid residues on numerous oligosaccharide side chains (11 N-glycosylation sites), the receptor protein was extensively digested with neuraminidase. Desialylation resulted in an increment of p*I* values by ca. 0.5 pH units and a reduction of microheterogeneity, but not on its elimination. Fig. 1 shows the results of an analytical Immobiline gel (pH 5–8) of de-sialo sEGFR: three major isoforms are well separated (with p*I* values of 6.45, 6.71 and 6.96) together with about a dozen minor components. In order to ascertain the origin of these bands, they were blotted and stained with monoclonal anti-sEGFR antibodies (alkaline phosphatase detection). It is seen that all the different bands are isoforms of the sEGFR.

Fig. 2 shows the results of a preparative run in the multicompartment electrolyzer (mounted with six sample chambers plus the two electrodic reservoirs) for the purification of sEGFR. It is seen that the unfractionated sample is composed of three major isoforms and a number (>6) of minor components. Upon purification, we could collect, as single bands, six isoforms, with the three major components collecting in chambers 2, 3 and 5 (p*I* values 6.45, 6.71 and 6.96, respectively).

As the p*I* 6.71 and 6.96 isoforms represented the most abundant components, and were in a state of high purity, attempts were made at crystallizing them. Fig. 3A shows a crystal of the p*I* 6.71 isoform. This crystal could be grown to

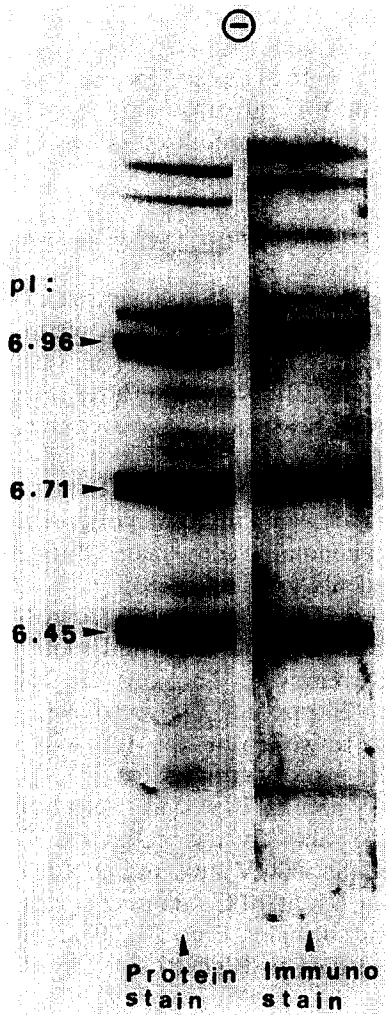


Fig. 1. Analytical isoelectric focusing gel of affinity-purified sEGFR. The gel contained an immobilized pH gradient (IPG, pH 5–8) grafted on a 5% T, 4% C matrix. The protein samples (ca. 50 μg in 20 μl) were applied in surface wells at the cathode. Focusing was at 10°C for 6 h at 5000 V. Staining with Coomassie Brilliant Blue in presence of Cu^{2+} (left gel strip). The right strip is a blot on cellulose nitrate followed by immuno fixation with monoclonal anti-sEGFR antibodies and alkaline phosphatase detection.

the remarkable size of $1.3 \times 0.5 \times 0.3$ mm^3. The p*I* 6.71 isoform exhibited the best diffraction ever achieved with this protein (see Fig. 3B). It allowed data collection up to 6 Å for the first 10 images; then diffraction patterns decreased to 10 Å during the following 10 exposures because of increasing radiation damage. A number of 2942

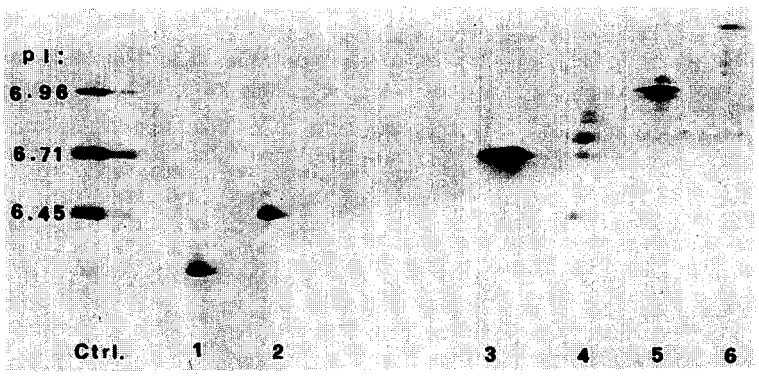


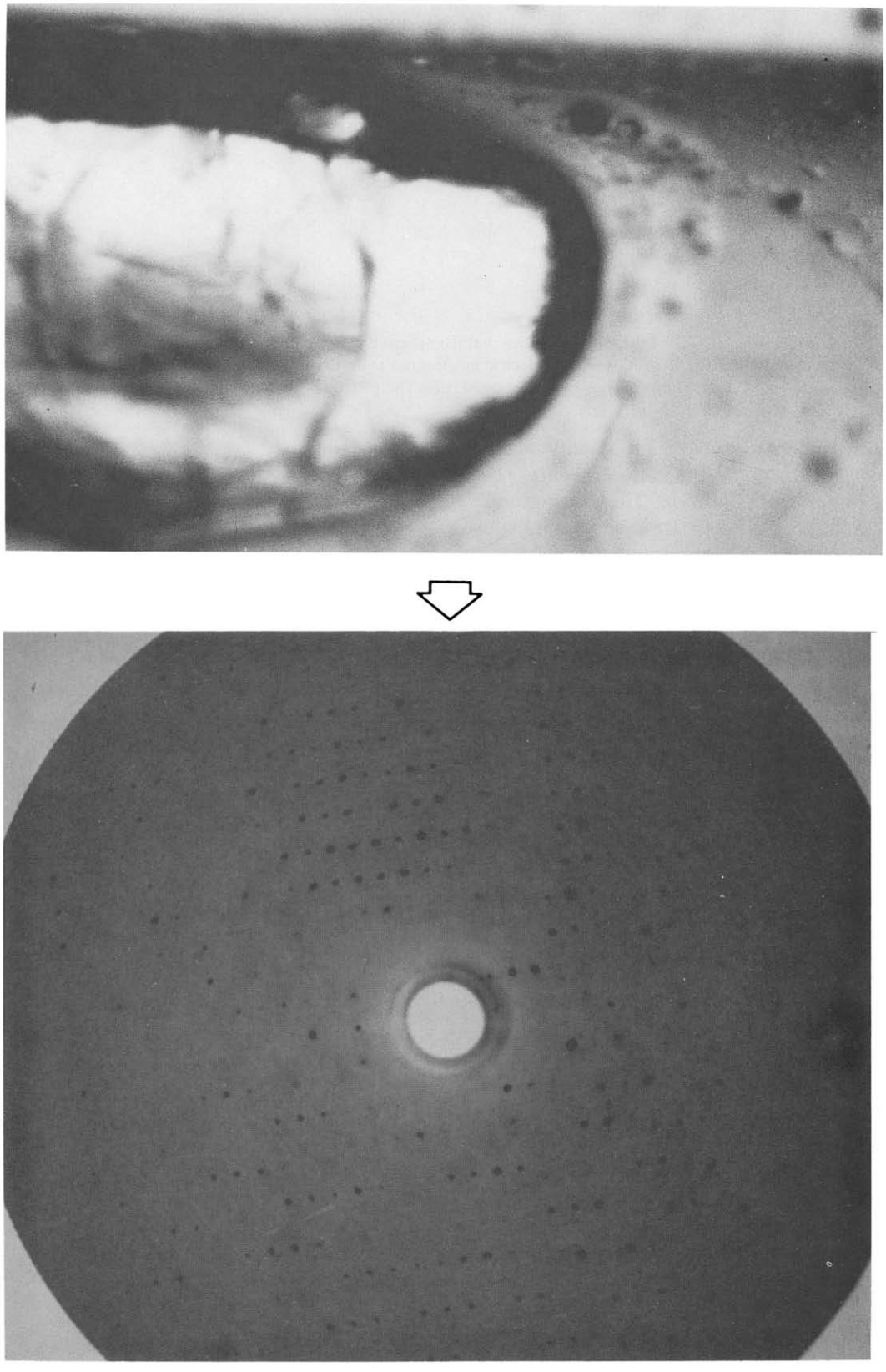
Fig. 2. Analytical IPG run (pH 5–8) of the content of the multicompartment electrolyzer. Lanes 1–6: content of each of the six chambers of the electrolyzer assembled with seven isoelectric membranes with p*I* values as given in the Experimental section. All other conditions as in Fig. 1. Ctrl. = control, unfractionated sEGFR. The p*I* values of the three major isoforms are given on the left side. Note the high purity of each isoform.

reflections was merged to yield a reliability factor (R. symm) of 5.4%. The reduced data set showed a completeness of 26%. The space group was assigned to be orthorhombic $P2_122$ or $P2_12_12$ with pseudo-tetragonal unit cell parameters of $a = 116.3$ Å, $b = 119.5$ Å and $c = 204.5$ Å. These values yield a unit cell volume of $2.8 \cdot 10^6$ Å^3 and a packing parameter V_M of 3.58 Å^3/u assuming two molecules each of receptor and ligand in the asymmetric unit. The fractional volume occupied by solvent was calculated to be 65% which is higher than found in most other proteins (40–60%) but observed also for virus crystals. This fact indicates a loose arrangement of the molecules in the crystal lattice and may explain the fast radiation decay. Additional crystals of the p*I* 6.71 isoform could be grown from a different sEGFR protein batch. These crystals of 1.2 mm diameter were used to test whether the crystal quality would allow data collection with a conventional X-ray source (as opposed to synchrotron radiation). Even under these far-from-optimal conditions, using a rotating anode generator, data collection was possible to about 7 Å, thus helping in refining the cell dimensions. Interestingly, several crystals could be grown also from the p*I* 6.96-sEGFR isoform (Fig. 4) and were analyzed by synchroton radiation; although these crystals had a comparably well-shaped morphology and remarkable size, they did not diffract at all (in Fig. 4 the diffraction pattern is substituted by a question mark).

4. Discussion

4.1. Protein isoforms and crystal habits

Our data clearly prove that the crystal quality of protein isoforms may differ significantly. In our particular case, we could grow crystals from both isoforms, highly purified in the multicompartment apparatus. However, while the crystals produced by the p*I* 6.71 isoform gave a good diffraction pattern, the crystals of the p*I* 6.96 band did not diffract at all. This might explain the failure at decoding the structure of such crystals so far, since they were up to the present time grown from the entire spectrum of different isoelectric forms. The p*I* 6.71 crystal allowed, for the first time, collection of a partial diffraction data set. The present crystal quality, therefore, is sufficient, in combination with cryotechniques, to search heavy atom derivatives (it should be noted that at the same time a sEGFR crystal of comparable quality was grown also from the wild-type protein during a space shuttle flight; apparently the microgravity conditions could compensate for the negative influence of charge heterogeneity of the protein which —in this study— is eliminated by preparative isoelectric focusing). Purified receptor isoforms tend to form crystals with sharper edges (as opposed to the more roundish forms of the wild-type protein, i.e. the protein still containing all the different isoforms). But —most important— they



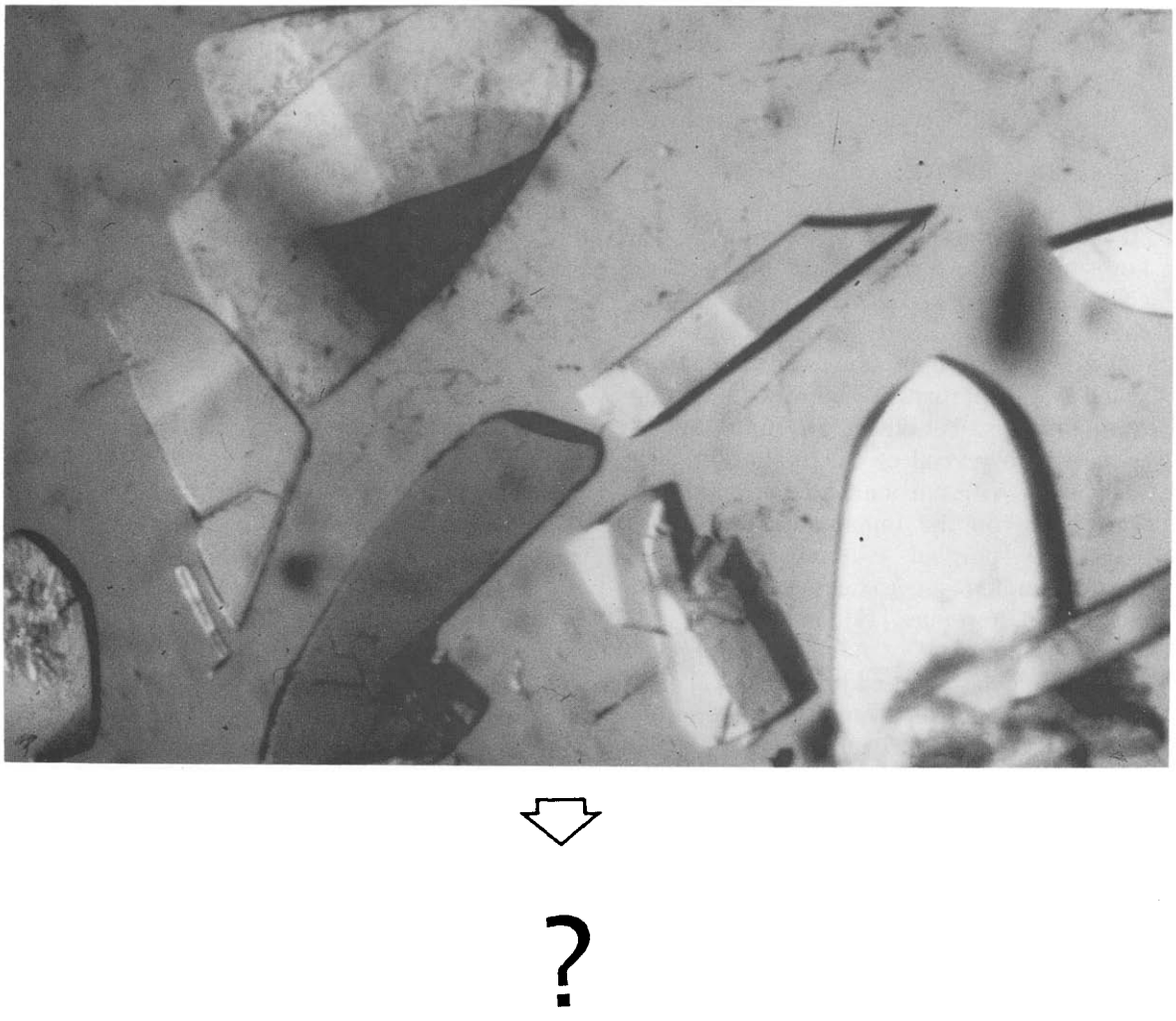


Fig. 4. Picture of some crystals of the p*I* 6.96 isoform as recovered from chamber 5 of the multicompartment electrolyzer. Also this isoform gave large-size crystals, but no one of them produced a diffraction pattern (as indicated by the question mark in the lower part of the figure). Scale: 1.2 cm corresponds to 0.1 mm.

tend to grow to a larger size. The p*I* 6.71 crystal had the surprising size of $1.3 \times 0.5 \times 0.3$ mm^3, which had never been obtained before. For the crystallography of such "tough" glycoproteins like EGFR with their high solvent content and loose crystal lattice the crystal volume can be regarded as the limiting parameter. Therefore; since the higher homogeneity achieved by isoform separation seems to favour growth to a large crystal volume, the isoelectric focusing in

Fig. 3. (Top) Picture of the crystal of the p*I* 6.71 isoform as recovered from chamber 3 of the multicompartment electrolyzer. The crystal has a size of $1.3 \times 0.5 \times 0.3$ mm^3 and is shown mounted in the glass capillary of the synchrotron beam line. (Bottom) Picture of the diffraction pattern, showing the high quality of the diffracting crystal. Scale: 1.7 cm corresponds to 0.1 mm.

the multicompartment electrolyzer reveals to represent an ideal or even an essential step in crystallographic projects.

4.2. Chromatography vs. electrophoresis

The fact that chromatography so far has had the lion share in down-stream protein processing is well-documented in the literature [14,15]. Boschetti [16] has recently reviewed advanced sorbents for protein separation, while Narayanan [17] has covered preparative affinity chromatography of proteins. Yet, there are hints that we are witnessing a revival of electrophoretic techniques as well. With our multicompartment electrolyzer, based on the Immobiline technology, we have now purified a number of r-DNA proteins, including superoxide dismutase [18], human growth hormone [19], monoclonal antibodies against the gp41 of AIDS virus [6], eglin C [20], glucoamylase [21] and sEGFR [22]. This technique is in general very mild to proteins and allows full recovery of enzyme activities coupled to high yields (typically $>80\%$). The other unique advantage of our recycling technique is that the protein is recovered both isoelectric and isoionic, i.e. uncontaminated by any kind of soluble buffer or counterion. Conversely, in conventional isoelectric focusing in soluble, amphoteric buffers, the protein is always contaminated by the amphoteric ions cofocusing in the same (and neighbouring) region. It has been reported quite often that these amphoteric buffers, at the isoelectric state, have a tendency to adhere to the protein surface, thus paradoxically, hampering crystallization even of species purified to single-p*I* isoforms. With the Immobiline technology this situation can never occur, since the isoelectric membranes are thoroughly washed free of any leachable contaminant. Other electrophoretic techniques are emerging as well. Thus, Bier's group is now offering simple buffers for their recycling isoelectric focusing unit, instead of the ill-defined carrier ampholyte mixture [23,24]. Also preparative isotachophoresis in agarose gels [25], in sucrose density gradients [26] and in the recycling free-flow mode [27] is being evaluated and discussed with increasing frequency [28]. Additionally, a new wave of interest is rising on protein purification by continuous-flow electrophoresis (CFE; the "Hannig" technique) [29]. CFE has never become quite popular, due to a number of problems connected with sample stream deformation (sedimentation, thermal convection, electroosmosis and, most deleterious of them all, electrohydrodynamic distortion) [30]. There are now hints that the latter problem could be cured by superimposing onto the a.c. field responsible for the separation a d.c. field transverse to it and to the flow direction, with an appropriate frequency, and an effective strength equal to that of the d.c. field [31]. As operative problems in preparative electrophoresis are solved with increasing frequency, it will be of interest to see how these different modes of protein purification will develop and grow in the years ahead.

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