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Molecular Modeling in Drug Design for the Development of Organophosphorus Antidotes/Prophylatics

Final Report

Wah Chiu, Ph.D.

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University of Arizona
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This contract is aimed at solving the 3-dimensional structure of acetylcholinesterase. The structural data will facilitate the drug design against the acetylcholinesterase inhibitors by molecular modeling and theoretical prediction in the action of drugs. In the first year, we implemented the biochemical procedures for purifying acetylcholinesterase from electric eel. We had preliminary success in making small crystals from the purified proteins. Computer programs for reconstructing three-dimensional structure from electron images were developed. In the second year, we focused our effort on recording electron images of the isolated acetylcholinesterase molecules and of the thin crystals of acetylcholinesterase. Electron images of these specimens at various angles of tilt have been recorded for use in reconstructing the enzyme's 3-dimensional structure. In the third year, we succeeded in crystallizing the acetylcholinesterase suitable for X-ray diffraction analysis to 4Å resolution.

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Summary

This contract is aimed at solving the 3-dimensional structure of acetylcholinesterase. The structural data will facilitate the drug design against the acetylcholinesterase inhibitors by molecular modeling and theoretical prediction in the action of drugs. In the first year, we implemented the biochemical procedures for purifying acetylcholinesterase from electric eel. We had preliminary success in making small crystals from the purified proteins. Computer programs for reconstructing three-dimensional structure from electron images were developed. In the second year, we focused our effort on recording electron images of the isolated acetylcholinesterase molecules and of the thin crystals of acetylcholinesterase. Electron images of these specimens at various angles of tilt have been recorded for use in reconstructing the enzyme's 3-dimensional structure. In the third year, we succeeded in crystallizing the acetylcholinesterase suitable for X-ray diffraction analysis to 4 Å resolution.

Foreword

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Final Report

Contract No. DAMD 17-84-C-4110

Name of Contractor: Arizona Board of Regents
University of Arizona, Tucson, AZ 85721

Project Title: Molecular Modeling in Drug Design for the Development of Organophosphorus Antidotes Prophylactics

Term of Contract: May 1, 1984 - April 30, 1987

Principal Investigator: Dr. Wah Chiu
Telephone No. (602) 621-7524

Introduction

Acetylcholinesterase is an enzyme which plays an essential role in the function of cholinergic synapses. This enzyme catalyzes the hydrolysis of the neurotransmitter acetylcholine to choline and acetic acid. The most studied form of this enzyme has been that isolated from electric organs of Torpedo and Electrophorus (1). Recently the gene of this enzyme from Torpedo was sequenced and the amino acid sequence has thus been deduced (2). However, no spatial structural information on this enzyme has yet been derived. The most powerful biophysical technique to study its detailed structure is X-ray crystallographic analysis (3). This technique requires large crystals. An alternative approach to obtain its 3-dimensional structure is electron diffraction and imaging (4). This technique can work with isolated molecules or with thin crystals of the protein. It is not yet well enough developed to provide the same level of structural detail as that determined by X-ray diffraction. Under some conditions, the low resolution structure can be useful for preliminary work on molecular modeling in drug design. Moreover, the low resolution 3-dimensional structure determined from electron microscopy can shorten the time required for subsequent X-ray diffraction analysis at higher resolution. We have been able to record the low resolution structural data of the acetylcholinesterase molecule and crystal by electron microscopy (5). We also put in an effort to prepare a X-ray quality crystal to be suitable for diffraction analysis at atomic resolution. (13)

Experimental Methods

We have set up a routine biochemical procedure of purifying the 11S form of acetylcholinesterase from electric eel. As described below:

The major electroplax organs from live Electrophorus electricus (sedated using MS222 and low temperature) were excised, cut into small pieces and quickly frozen in liquid nitrogen. These pieces were then stored at -80°C until further use. Purifications were routinely performed on 1 kg lots of this tissue. The frozen tissue was warmed to -20°C and diced into approximately 1 cm^3 pieces. Solubilization of the enzyme is similar to that reported by Lwebuga-Mukassa et al. (6) and Lee et al. (7). The tissue was homogenized in a Waring blender in 7/5 (vol/weight) ice cold 0.1M NaCl containing 10mM Tris-HCl and 10mM NaN_3, pH 8.0 (buffer A). This suspension was centrifuged for 30 minutes at 10,000 rpm in a Sorval RC2B centrifuge (GSA rotor, corresponding to 16,300 x g) and the supernatant liquid was discarded. The volume of the pellet was estimated and the material was homogenized in a Waring blender in 7/5 (vol/vol) ice cold 2M $MgCl_2$ containing 10mM Tris-HCl and 10mM NaN_3, pH 8.0 (buffer B). This material was centrifuged for 30 minutes at 10,000 rpm in a Sorval RC2B centrifuge (GSA rotor, correponding to 16,300 x g) and the supernatant liquid as retained. The remaining pellet was subjected to a second cycle of homogenization in buffer B and centrifugation. This second supernatant solution (containing approximately 10% of the acetylcholinesterase activity found in the first solution) was combined with the first and subject to a high speed centrifugation step in a Sorval RC2B centrifuge using a SS34 rotor (60 minutes at 17,500 rpm, corresponding to 37,000 x g). The supernatant liquid was filtered through cheesecloth and dialyzed at 4°C against 0.3M NaCl containing 10mM Tris-HCl and 10mM NaN_3, pH 8.0 (buffer C). Typically, four changes of buffer were made over a 48 hour period. To the dialysate was added 250 ml m-(carboxyphenyl) ethyldimethyl ammonium derivatized Sepharose. This suspension was stirred constantly for approximately 2 hours (until at least 75% of the enzyme activity had been bound) and then allowed to settle overnight. Liquid containing unbound acetylcholinesterase activity was aspirated off and the resin was resuspended in approximately 1 l buffer C. This suspension was gravity packed into a 2.2 x 90 cm column and washed with buffer C until the A_{280} fell below 0.01. At this point, the enzyme activity was eluted using a step gradient of buffer B. The enzyme elutes as a single peak at the column's void volume. The eluted enzyme was dialyzed at 4°C against buffer A. Typically, four changes of buffer were made over a 48 hour period. Aliquots (100 μl) of this material were treated with varying amounts of trypsin for 15 minutes at 37°C and the proteolytic conversion was stopped by the addition of a two-fold excess of soybean trypsin inhibitor. The enzyme concentration was estimated from activity assays assuming a value of 16,000

A_{412} per min per mg active acetylcholinesterase. Typically, trypsin: acetylcholinesterase ratios ranging from 1:200 to 1:1 were tested. These samples were analyzed by sucrose density gradient centrifugation to determine the minimal dose of trypsin which converts all of the enzyme activity into the 11S form. Doses in the range of 1:200 to 1:50 were usually sufficient. The remaining enzyme was treated with the appropiate amount of trypsin for 15 minutes at 37°C and the reaction was stopped by the addition of trypsin inhibitor. The enzyme was adsorbed onto 50 ml of the procainamide Sepharose. Essentially all the enzyme activity (>99%) binds to the resin at this step. This resin was gravity packed into a 2.2 x 11 cm column and washed with buffer A until the A_{280} fell below 0.01. The enzyme was eluted with a 500 ml linear gradient of buffer A to buffer B. A further wash with buffer B followed completion of the gradient. The enzyme elutes as a sharp peak at the end of the salt gradient, with small amounts of enzyme activity eluting before the peak. The peak, however, accounts for at least 60% of the activity which elutes off the procainamide column.

The eluted enzyme was dialyzed at 4°C against buffer A and concentrated to a small volume (corresponding to 5-20 mg/ml) using Centricon-30 or Centriprep-30 centrifugal concentrators. The concentrated protein solution was stored at 4°C until further use.

The search for crystallization conditions was initially conducted using the hanging drop vapor diffusion method. Both polyethylene glycol (PEG) and ammonium sulfate were used as precipitants. The conditions tested covered a range of pH from pH 4 to pH 9, NaCl concentrations ranging from 10 mM to 1.5 M and protein concentrations ranging from 1.5 ato 25 mg/ml. Citrate buffers were used between pH 4 and pH 7 and Tris was used between pH 7 and pH 9. Borate buffers were also tested between pH 8 and pH 9. Trials were done at room temperature (25°C) and at 4°C. After conditions for crystal growth were determined by vapor diffusion, microdialysis was also used successfully to produce crystals. Light and electron microscopy were used to monitor the growth of these crystals (8). Electron images of acetylcholinesterase and thin crystals of acetylcholinesterase were recorded with the Philips 420 electron microscope at 100 KV. X-ray diffraction patterns were recorded on an Elliott GX-20 rotating anode X-ray generator operated at 35 KV and 40 mAmps from large 11S acetylcholinesterase crystal kept at 4°C.

The recorded images were evaluated for their quality in an optical diffractometer. The selected micrographs were digitized with the Perkin-Elmer microdensitometer and analyzed with the computer program which has been available in our laboratory. For images of single acetylcholinesterase molecules, we used the SPIDER program (9) to sort out the images of the particles with high similarity, and to average them in order to enhance the signal-to-noise ratio of the particles. For images of the thin acetylcholinesterase crystal, we performed a Fourier transform of

the digitized images from which we extracted the structural data from the reciprocal lattice points. We have developed an algorithm to measure the lattice distortion in the image of the crystal by correlation analysis and to correct this distortion by a real space interpolation procedure. This algorithm was tested with data of other proteins with known structures and has been used in processing our crystal data of the acetylcholinesterase. The outcome of the processed image of the crystal would provide a set of amplitudes and phases from which a density map can be calculated (4). The X-ray diffraction patterns were evaluated visually for their resolution and crystal space group.

Results

Table 1 is a summary of the results from three typical purifications. This procedure proved reproducible and reliably allowed purification of 10-20 mg of 11S acetylcholinesterase. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the purified enzyme shows a major band at M_r 70,000 (Figure 1). A band at M_r 140,000 is also apparent. This band is presumed to represent unreduced dimer. Reduced samples also show traces of some lower molecular weight bands at M_r 50,000, 27,000, and 23,000. The apparent molecular weight of these bands correspond well with proteolytic fragments of the acetylcholinesterase observed by other investigatiors (11). This is not unexpected since our purification involves a deliberate proteolysis. Traces of these bands were also observed from redissolved crystals, again suggesting that these low molecular weignt bands are fragments of the acetylcholinesterase.

One approach that we have taken is to obtain the structural data of the acetylcholinesterase without the crystal by electron imaging. These data can be used to confirm and/or supplement the crystal structure data. Figure 2 is a density map of the 11S acetylcholinesterase molecules in tetrameric form obtained by the method described above. This is an averaged map from 20 images of the single particles. We have now recorded sufficient electron images with which we can process more than 200 particles. The striking result from this preliminary analysis is the lack of four fold symmetry in the tetramer. This spatial asymmetry is consistent with the biochemical prediction on the basis of non-equivalent disulfide linkage among the monomers in the tetrameric enzyme (12).

Among 50 experimental conditions under which we have tried to crystallize the acetylcholinesterase, we have found a number of cases that would give us small and thin crystals. Figure 3 shows an electron micrograph of such thin crystal of acetylcholinesterase. The periodic features are visible in the image. We have recorded 10 tilt series from this type of crystal, and have accumulated over 100 electron images of the acetylcholinesterase crystals at various tilt angles. We have

calculated the amplitudes and phases from the images of the crystals at zero degree tilt. We are at the stage that we can combine the data at high tilt angles in order to calculate the amplitudes and phases in the third dimension with which we will be able to generate the 3-dimensional coordinates of the acetylcholinesterase. Among the latest crystallization attempt, we were able to grow some crystal which could diffract to 4Å resolution by X-ray as shown in figure 4. We subsequently determined the space group to be F222 and the unit cell spacings of the crystal are a =141.7Å, b=202.6Å and c=235.5 Å (13).

Conclusion

We have established a procedure of purifying acetylcholinesterase from electric eel and making a small crystal of this protein. We have demonstrated the feasibility of using electron microscopy to record structural data from individual acetylcholinesterase molecules and from thin crystals of this enzyme. Three-dimensional data sets have been collected from these specimens. Computer processing of these data has been started. The reconstructed asymmetry in the tetrameric form of the enzyme. These data can be used to supplement the structural data from the small crystal. We also demonstrated the feasibility of obtaining an X-ray diffraction analysis of the acetylcholinesterase crystal to 4Å resolution. This suggests the likely prospect of solving this crystal structure where the polypeptide backbone can be traced from the X-ray diffraction data. These data can also reveal the conformation of the active site.

Military Relevance

When we complete the three-dimensional reconstruction of the acetylcholinesterase from images of single molecular form and of crystal, we will be able to determine the structural coordinates of this protein. This structural information can be displayed in a color graphics display system with which we can contemplate the possible strategy of drug design against the enzyme inhibitors. The crystal which is large enough to provide high resolution X-ray diffraction intensities can be used to derive the useful structural information for the stated objective.

References

(01) Massoulie, J. and Bon, S. (1982) Ann. Rev. Neurosci. 5: 57-106.

(02) Schumacher, M. Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S. S., Friedman, T. and Taylor, P. (1986) Nature 319: 407-409.

(03) Blundell, T. L. and Johnson, L. N. (1976) Protein Crystallography, Academic Press, N.Y.

(04) Unwin, P. N. T. and Henderson, R. (1975) J. Mol. Biol. 94: 425.

(05) Morgan, D. G., Schrag, J. D., Olson, S., Schmid, M. F. and Chiu, W. (1986) Proc. Electron Microscopy Soc. Amer., in press.

(06) Lwebuga-Mukassa, J. G., Lappi, S., and Taylor, P. (1976) Biochemistry 15: 1425-1434.

(07) Lee, S.T., Heinemann, S., and Taylor, P. (1982) J. Biol. Chem. 257 12283-12291.

(08) McPherson, A. (1982) Preparation and Analysis of Protein Crystals, John Wiley and Sons, N.Y.

(09) Frank, J., Verschoor, A. and Wagenknecht, T. (1985) in New Methodologies in Studies of Protein Configuration, ed., T. T. Wu, Van Nostrand Reinhold Co., N.Y.

(10) Chiu, W., Schmid, M. F. and Jeng, T. W. (1986) Proc. Electron Microscopy Soc. America, in press.

(11) Powell, J.T., Bon, S., Rieger, F. and Massonlie, J. (1973) FEBS Lett, 36: 17-22.

(12) Rosenberg, T. L., Chen, Y. T. and Bock, E. (1974) Biochemistry 15: 3068-3079.

(13) Schrag, J.D., Schmid, M.F. Morgan D.G., Phillips, G.N., Chiu, W. and Tang, L. (1988) J. Biol. Chem. accepted for publication.

List of Publications

1) Chiu, W. (1986) Electron microscopy of frozen, hydrated biological specimens, Ann. Rev. Biophys. Chem. 15, 237-257.

2) Chiu, W., Schmid, M. F. and Jeng, T. W. (1986) Computer processing of high resolution images of periodic specimens, Proc. of Electron Microscopy Soc. America, 2-5.

3) Morgan, D. G., Schrag, J. D., Olson, S., Schmid, M. F. and Chiu, W. (1986) Singles particle averaging of purified 11S acetylcholinesterase, Proc. of Electron Microscopy Soc. America, 146-147.

4) Schrag, J.D., Schmid, M.F., Morgan, D.G., Phillips, G.N., Chiu, W., and Tang, L. (1988) Crystallization and preliminary X-ray diffraction and analysis of 11S acetylcholinesterase. J. Biol. Chem., accepted for publication.

List of Personnel Receiving Contract Support

Dr. Mike Schmid
Dr. Joe Schrag
Dr. David Morgan
Mr. Steve Olson
Departmental dishwasher (part-time worker)

Table 1

SUMMARY OF PURIFICATIONS

	total protein	% yield	specific activity units/mg	purification fold
extract	7666±2230	100	34.9±.9	-
column 1	170.3±30.7	69.3±6.0	1067±247.8	31.3±6.8
column 2	17.5±2.2	33.7±7.0	5356±897.3	159.8±40.5

Figure 1

SDS-Polyacrylamide gel of the purified 11S acetylcholinesterase. Lane 1 shows the Bio-Rad molecular weight standards and lane 2 shows 50 μg purified 11S acetylcholinesterase. The major band corresponds to M_r 70,000, the expected position of monomeric enzyme. A dimer band at M_r 140,000 is also visible. The fragments at M_r 50,000, 27,000, and 23,000 are presumed to be fragments of the enzyme.

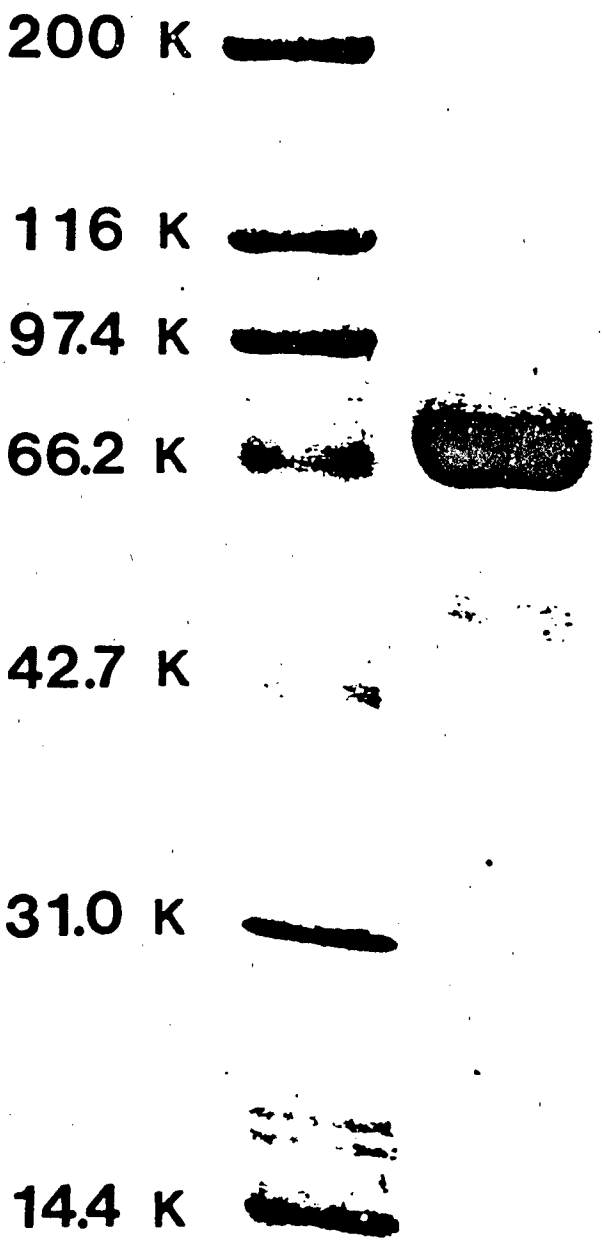

200 K
116 K
97.4 K
66.2 K
42.7 K
31.0 K
14.4 K

Figure 2.

Reconstructed density map of 11S form of acetylcholinesterase averaged from 20 images of single protein molecules.

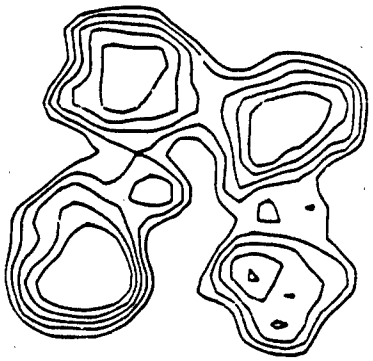
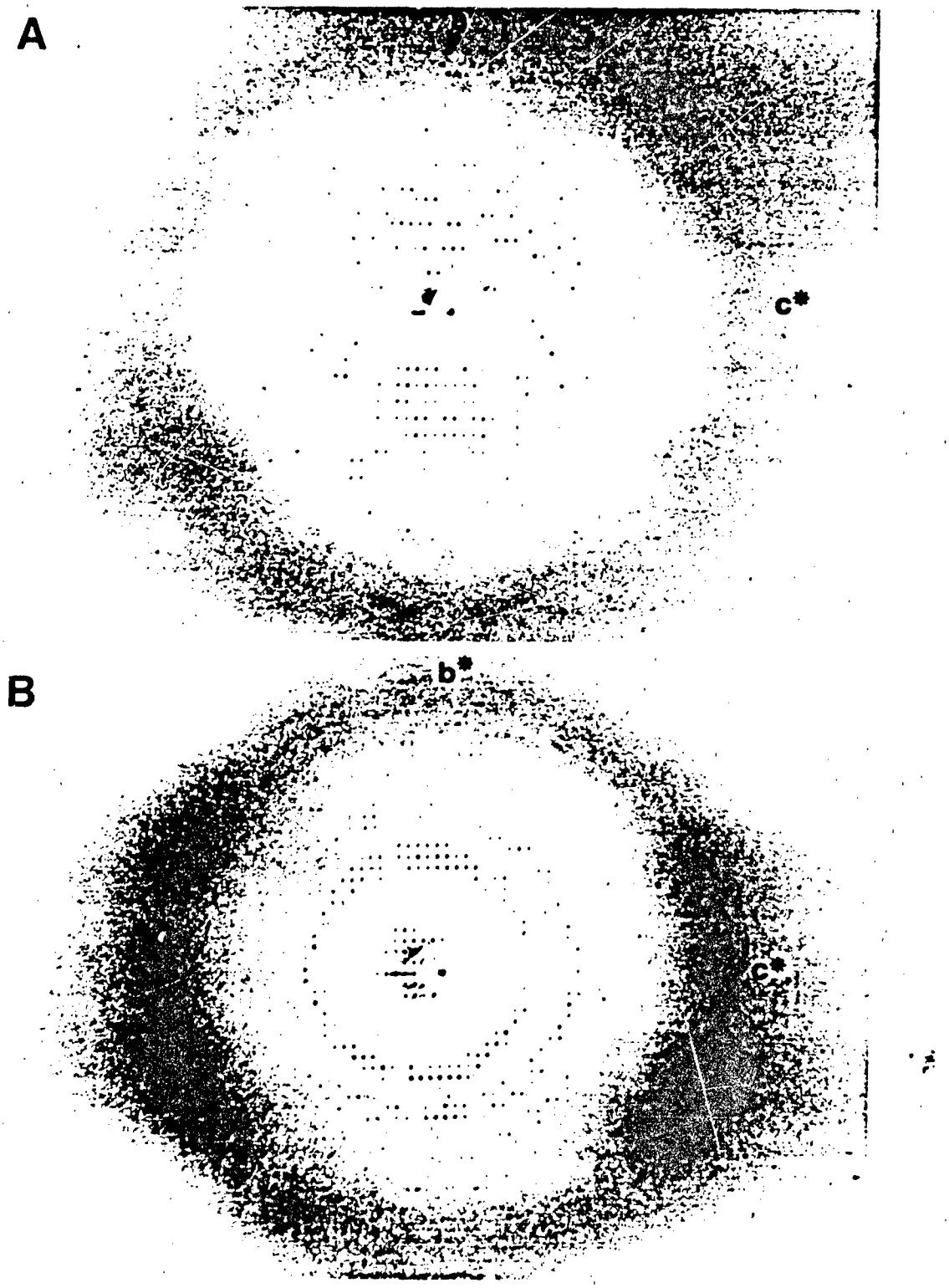


Figure 3.

Electron image of thin crystal of acetylcholinesterase where periodic features are visible.

Figure 4

2° Oscillation photographs. A) The X-ray beam was inclined about 12° from parallel to the crystallographic b axis. B) The X-ray beam was nearly parallel to the crystallographic a axis. The exposure time in each case was 16 hours. The centering of the reciprocal lattice and the anisotropic nature of the diffraction are observed in both photographs.


A
c*
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