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The crystallation of estrogen-binding protein from Candida albicans

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The Crystallization of Estrogen-Binding Protein from *Candida albicans*

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ABSTRACT

CHIA-EN CHEN The crystallization of estrogen-binding protein from *Candida albicans*. Department of Chemistry, June 1997.

In the past decade scientists have discovered that the pathogenic fungus *Candida albicans* contains a highly specific estrogen-binding protein (EBP). Since it is known that the use of estrogen-containing compounds causes one to be more prone to infection by this organism, it is hypothesized that this estrogen system may have a role in infection. Understanding the structure and function of EBP would be helpful in developing treatments for infection by *C. albicans*. One way to approach the structure and function question of any protein is to crystallize the protein and solve its structure using X-ray diffraction.

In the past few months, crystals of EBP have been produced using the hanging drop method of vapor diffusion. These crystals are yellow and "spiky" in appearance and grow as clusters within two weeks at 4°C in a solution containing the following: 0.1 M sodium cacodylate, pH 6.5; 0.1 M sodium acetate; and 25% polyethylene glycol (PEG) 8000. Crystals of similar appearance have been grown in less than one week using the technique of microseeding. Because neither the unseeded nor the seeded crystals grow singly, they cannot be subjected to X-ray diffraction in order for structural data to be taken. Since the jagged appearance of these crystals may be due to some microheterogeneity in the EBP sample used, I believe that the future work on this project include an attempt to grow diffraction-grade EBP crystals with a more homogeneous EBP sample. Additionally, since other conditions have been found in which EBP crystallizes, I believe that these conditions should be explored to determine whether or not sharper crystals can be grown.

Acknowledgements

I would like to thank Professor Kristin M. Fox for introducing me to this area of biochemistry and for all of her help and insight in this project. I would also like to thank Dr. Susan Miller and James Buckman at the University of California, San Francisco for providing me with the samples of Estrogen-Binding Protein. And finally, I would like to thank my family for all of their support and encouragement. Without them, I would not be where I am today.

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Introduction

Fungi generally are thought to be harmless organisms that occasionally cause annoying infections such as athlete's foot. In recent years though, many fungi have been shown to cause localized and systemic infections; depending on the severity of the systemic infection, the patient is faced with the possibility of death. While natural events, such as earthquakes, can cause endemic outbreaks of fungal infections by disseminating the spores of soil fungi, many infections are contracted in hospitals (Sternberg, 1994; Pfaller and Wenzel, 1992). Among the most common infectious fungus is *Candida albicans*, a yeast that causes various forms of candidiasis.

Those who fall victim to the various candidiases caused by *C. albicans* in hospital settings are usually in intensive care units, immunocompromised, undergoing or have undergone organ or bone marrow transplantation, infected with cancer, or are premature infants (Cabezudo *et al.*, 1989; Pfaller and Wenzel, 1992). Some symptoms of fungal infections include curd-like patches on the tongue, clouding of the eyes and blindness, fever, heart failure, shock, and even disseminated intravascular coagulation which causes sudden and immediate death (Sternberg, 1994).

In recent years, the number of hospital-related fungal infections has nearly doubled -- an alarming discovery for doctors and other medical workers because effective treatments for these fungal infections are lacking. Effective treatments have not been found due to some of the following reasons: lack of drugs, increasing fungal resistance, increasing number of fungal pathogens, and slow research (Sternberg, 1994; Pfaller and Wenzel, 1992). However, some headway has been made in the case of *C. albicans*.

In the past decade, studies of *C. albicans* have shown that it contains a highly specific estrogen-binding system which utilizes a protein called Estrogen-Binding Protein (EBP) (Powell *et al.*, 1984; Skowronski and Feldman 1989; Madani *et al.*, 1994; Zhao *et al.*, 1995). Although *C. albicans* had been studied in the past, the presence of EBP was only discovered when the yeast was grown under specific conditions, suggesting that its concentration may be regulated by various growth conditions (Powell *et al.*, 1984; Skowronski and Feldman, 1989; Madani *et al.*, 1994). Madani *et al.* and Zhao *et al.* noted that elevated levels of EBP appeared in *C. albicans* during the early growth phase while Skowronski and Feldman demonstrated that estrogen binding increases during this same period (1994,1995).

Because of this response, several studies were performed to characterize EBP. From these studies, it has been established that EBP is approximately 46,000 Daltons and only binds specifically to 17 α- and β-estradiol, estrone, and estriol (Figure 1). EBP does not bind estrogen antagonists like mammalian estrogen receptors do, nor does it bind other classes of human hormones or various yeast hormones (Skowronski and Feldman, 1989; Zhao *et al.*, 1995). Skowronski and Feldman demonstrated that each EBP molecule binds only one molecule of estrogen and hypothesize that EBP has a spherical, symmetrical shape due to the calculated Stokes' radius and the measured sedimentation coefficient (1989).

Since it has a high specificity for certain estrogens, it originally was thought that EBP would be related to the mammalian estrogen receptor (Skowronski and Feldman,

1989). However, computerized sequence comparisons of both showed that they are not

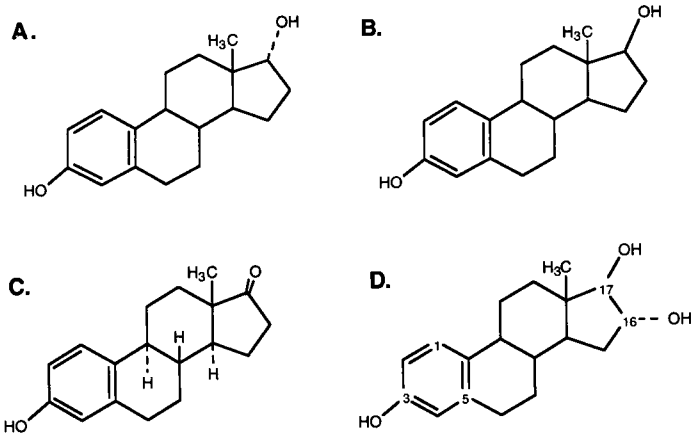

Figure 1. Structure of Various EBP Substrates. a) 17 α-estradiol b) 17 β-estradiol c) estrone d) estriol

related. Instead, EBP shows similarity to the family of FMN-binding α/β barrel proteins, especially Old Yellow Enzyme (OYE) produced by *Saccharomyces cerevisiae* -- 45% identical and 64% similar (Figure 2); both exhibit oxidoreductase activity and bind phenolic molecules (Madani *et al.*, 1994; Zhao *et al.*, 1995). Additionally, the flavoproteins in OYE's subfamily, along with EBP, contain a highly conserved sequence of amino acids which may contribute to their activity. However, EBP contains a cysteine residue, Cys-225, that the other flavoproteins lack in this conserved sequence (refer to Figure 3). Madani *et al.* suggest that Cys-225 may be critical to estrogen binding in EBP (1994).

```
      1              15 16             30 31             45 46             60
1 EBP MTIESTNSFVVPSDT KLIDVTPLGSTKLFQ PIKVGNNVLPQRIAY VPTTRFRASKD-HIP
2 OYE -----------MPFV KDFKPQALGDTNLFK PIKIGNNELLHRAVI PPLTRMRAQHPGNIP

      61             75 76             90 91            105 106           120
1 EBP S-DLQLNYYNARSQY PGTLIITEATFASER GGIDLHVPGIYNDAQ AKSWKKINEAIHGNG
2 OYE NRDWAVEYYAQRAQR PGTLIITEGTFPSPQ SGGYDNAPGIWSEEQ IKEWTKIFKAIHENK

      121            135 136           150 151           165 166           180
1 EBP SFSSVQLWYLGRVAN AKDLKD-----SGLP LIAPS-AVYWDENSE KLAKEAGNELRALTE
2 OYE SFAWVQLWVLGWAAF PDTLAR-----DGLR YDSASDNVYMNAEQE EKAKKANNPQHSITK

      181            195 196           210 211           225 226             240
1 EBP EEIDHIVEVEYPNAA KHALEAGFDYVEIHG AHGYLLDQFLNLASN KRTDKYGCGSIENRA
2 OYE DEIKQYVK-EYVQAA KNSIAAGADGVEIHS ANGYLLNQFLDPHSN NRTDEYG-GSIENRA

      241            255 256           270 271           285 286           300
1 EBP RLLLRVVDKLIEVVG ANRLALRLSPWASFQ GMEIEGEE----IHS YILQQLQQRADNGQQ
2 OYE RFTLEVVDAVVDAIG PEKVGLRLSPYGVFN SMSGGAETGIVAQYA YVLGELERRAKAGKR

      301            315 316           330 331           345 346           360
1 EBP LAYISLVEPRVTGIY DVSLK-DQQGRSNEF AYKIWKGNFIRAGNY TYDAPEFKTLINDLK
2 OYE LAFVHLVEPRVTNPF LTEGEGEYNGGSNKF AYSIWKGPIIRAGNF ALHPEVVREEVKDP-

361          375 376           390 391           405 406           420 421
1 EBP NDRSIIGFSRFFTSN PDLVEKLKLGKPLNY YNREEFYKYYNYGYN SYD-ESEKQVIGKPL A-
2 OYE --RTLIGYGRFFISN PDLVDPLEKGLPLNK YDRDTFYKMSAEGYI DYPTYEEALKLGWDK N-
```

Figure 2. Overlay of Estrogen-Binding Protein of *C. albicans* and Old Yellow Enzyme of *S. cerevisiae*. Residues in magenta are a region of well-conserved residues. Blue denotes cys-225 and light green denotes the N-linked glycosylation residues.

Although much has been learned about this protein, EBP's function still remains unknown. The most recent characterization study performed by Zhao *et al.* attempted to elucidate this question by examining the cellular localization of EBP in *C. albicans*. Via immunochemistry and electron microscopy, they learned that EBP is associated with the inner tonoplast of *C. albicans* vacuoles. Sequence studies corroborated this observation because EBP contains an N-linked glycosylation site at residues 331-333; many vacuolar

proteins contain this signal which causes them to be sent to the vacuoles. However, EBP lacks any apparent hydrophobic signal sequences, membrane-spanning domains, or signal sequences needed to cross membranes (1995). While this information is useful, it does not clarify the EBP function question any further.

Scientists are interested in learning the function of EBP because it has been shown that *C. albicans* is stimulated to convert from its yeast form to its invasive hyphal form in the presence of estrogens (Madani *et al.*, 1994; Zhao *et al.*, 1995); the role of EBP in this process is unknown. What is known about *C. albicans* is that its invasive hyphal form causes the various cadidiases seen and that the medical world has generally accepted that pregnancy and the use of birth control pills increase the risk of *Candida* infections (Skowronski and Feldman, 1989). Since the dissociation constant of EBP with the above-mentioned specific estrogens is lower than that found for mammalian estrogen receptors, it is highly probable that EBP can interact appreciably with physiological levels of unconjugated estrogens in the blood (Powell *et al.*, 1984).

One way to approach the question of EBP's function is to study its three-dimensional structure. By examining its structure, one can learn how the protein interacts with its substrate and if this interaction leads to the transition from yeast to hyphal form. Once this is known, scientists can create drugs that more effectively target *C. albicans*. In order for these three-dimensional studies to be done, EBP must be crystallized and have its three-dimensional structure solved.

Crystallization of proteins is termed a "black box" process because there are no set guidelines for what conditions should be used for various proteins. To aid in a protein's crystallization process, there should be one or more precipitants in solution with the protein. Because of the delicate nature of proteins, these precipitants must be chosen so that their presence does not cause the protein to denature. The two most common precipitants are polyethylene glycol and ammonium sulfate (McPherson, 1982; Rhodes, 1993).

In order to find the proper crystallization conditions, one needs to test a great variety of conditions. One of the most efficient methods is vapor diffusion via a hanging drop. In this procedure, a drop of protein solution on a silicon-coated slide is inverted and sealed over a reservoir of aqueous solution. The reservoir contains various precipitants while the drop contains the protein and a lower concentration of the precipitants. Since the concentration of precipitants is lower in the drop, water will diffuse out of the drop until the concentration of precipitants in the reservoir and drop are the same. If these final conditions are optimal, protein crystals will form because the precipitant concentrations cause the protein concentration to go to saturation (Rhodes, 1993; McPherson, 1982).

The hanging drop method is normally chosen over other procedures because it requires relatively small amounts of the sample protein, is fairly inexpensive, and allows one to screen many crystallization conditions simultaneously. Other methods of crystallization, like the batch method and free interface diffusion, can be used more effectively to grow larger quantities of crystals once optimal conditions have been established. These methods are not initially utilized because they require large quantities of protein and can be expensive and labor intensive (McPherson, 1982).

Once large enough crystals are grown, they can be subjected to X-ray diffraction so that data can be collected to solve the structure. This technology came about because scientists knew that in order to see an object, it must diffract light. Due to their size, atoms cannot diffract visible light; however, they can diffract the smaller waves that X-rays produce. Thus, if an X-ray is directed at a crystal, the individual molecules that comprise the crystal will diffract the X-ray identically creating a pattern. This pattern can be captured on film and the direction and intensity of the diffracted X-rays can be used to generate an electron density image of the molecule. Fairly large, ordered crystals are need for X-ray diffraction because single molecules of interest do not diffract X-rays as strongly as the ordered array of molecules packed into a crystal. Additionally, many diffraction-grade

crystals are needed because the crystals are destroyed when subjected to X-rays (Rhodes, 1993 ; McPherson, 1982).

Because of the growing urgency to understand the function of Estrogen-Binding Protein in the growth of *C. albicans*, the focus of this project will be to find the optimal conditions in which EBP will crystallize. While some general conditions were found to have produced EBP crystals, these crystals lacked an ordered structure and were small in size. Experiments will continue to determine if these conditions can be optimized so that larger, well-ordered crystals can be formed. If such crystals can be produced consistently, then the structure of EBP can be determined using X-ray diffraction.

Methods

Protein Information

The EBP sample obtained for crystallization was purified by James Buckman at the University of California at San Francisco. The volume of protein received was 6.0 ml and at a concentration of 1.5 mg/ml in a 100 mM KPi (potassium phosphate) solution, pH 7.3. Although protein concentration was determined with a Bio-Rad assay, we chose to use the concentration J. Buckman determined via Abs 466 nm, the absorbance of flavin, because this method is more accurate than the Bio-Rad protein concentration assay.

Determination of EBP Sample Purity via Gel Electrophoresis

To determine the purity of the EBP sample obtained from J. Buckman, SDS-polyacrylamide gel electrophoresis was performed using the Bio-Rad Mini-PROTEAN® Dual Slab Cell. A 12% discontinuous polyacrylamide gel was cast according to instructions given in the instruction manual of the kit, with changes in the gel recipe in Table 1. Samples to run on the gel were prepared as follows: A marker solution was made by combining 10 µl of Promega Mid-Range Molecular Weight Markers with 10 µl of 2X treatment buffer (prepared according to the Bio-Rad Mini-PROTEAN® Dual Slab Cell instruction manual) while the EBP sample was prepared by combining 7 µl of EBP solution, 3 µl distilled water, and 10 µl of 2X treatment buffer. Both were incubated at 90°C for 3 minutes. The procedure used for running the gel along with making the appropriate running buffers were as given in the instruction manual. The gel was run for approximately 30 minutes at 200 volts.

The gel was removed and stained for a half an hour in 0.1% Coomassie Blue R-250 in a 40% MeOH/10% HOAc fixative. Once the stain was poured off, the gel was destained with 40% MeOH/10% HOAc for at least one hour. The gel was dried overnight.

Table 1. Appropriate Volumes of Components for a Discontinuous Gel

Solution	12% Gel	Stacking Gel
Distilled Water	4.35 ml	6.42 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	none
0.5 M Tris-HCl, pH 6.8	none	2.5 ml
10% (w/v) SDS stock	100 µl	100 µl
40% Acrylamide/Bis Stock	3.0 µl	980 µl
10% Ammonium Persulfate (made fresh daily)	50 µl	50 µl
TEMED	5 µl	10 µl

Switching of EBP Storage Buffer

The buffer in which EBP was stored was changed to 10 mM TRIS, pH 7.4, by the following method. Two Centricons® (Amicon Co.), molecular weight cutoff of 10,000 Daltons, were rinsed and spun with de-ionized water to remove the ethanol storage solution from them. The EBP solution was thawed and divided evenly between two Centricons®. Both Centricons® were centrifuged simultaneously for approximately 1.5 hours until almost all of the original buffer had passed through to the waste chamber. The waste chambers of the Centricons® were removed to dispose of the KPi buffer in them and then replaced. Following this, equal amounts of 10 mM TRIS, pH 7.4, were added to the remaining volume of EBP protein solution; they were spun for 1.5 hours. The addition of the 10 mM TRIS and subsequent centrifugation was performed 4 more times to remove any residual KPi buffer in which EBP was originally stored.

After the final spin, the waste chambers were removed, and the Centricons® were inverted and spun for 1 minute to force the concentrated EBP solution into the conical collection cup on the end of the centricon. The volume of EBP collected was brought up to 6 ml with 10 mM TRIS, pH 7.4 to restore the solution to the original concentration of 1.5 mg/ml. 500 µl aliquots were put into microfuge tubes, labeled, and immediately frozen.

General Crystallization Procedure

To screen many crystallization conditions, the technique of vapor diffusion of a hanging drop was used. The materials used to create the diffusion chambers were Linbro 24-well culture plates, 22 mm siliconized circular glass slides, clay, and vacuum grease. Small balls of clay were pressed into each corner of the lids of the tissue culture plates. The rims of the wells each plate were coated with vacuum grease. Each well was filled with a standard volume, usually 500 µl, of either pre-made solution or the appropriate volumes of various stock solutions needed to create a standard volume of reservoir solution. 2 µl of reservoir solution was removed from a well and put in the middle of a dust-free cover slip. 2 µl of 1.5 mg/ml EBP in 10 mM, TRIS, pH 7.4 was carefully added to the drop. The cover slip was quickly inverted and placed over the well from which the reservoir solution came. Each well was sealed by pressing the cover slip around the greased rim of the well (Figure 3). The plates were incubated at 4°C. Plates periodically were viewed under 40X magnification for crystal growth.

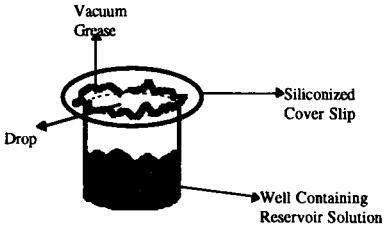

Figure 3. Well Set-Up for the Hanging Drop Method

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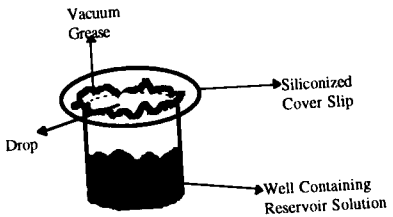

Figure 3. Well Set-Up for the Hanging Drop Method

Concentration of EBP Sample

Due to the observations made of the initial screening plates, a portion of the EBP samples were concentrated to 4.5 mg/ml and 10 mg/ml by the following method. Two Centricons®, with a molecular weight cutoff of 10,000 Daltons, were rinsed and spun with de-ionized water to remove the ethanol storage solution from them. Three 500 µl aliquots of frozen EBP solution were thawed and pipetted into a centricon tube. The other Centricon® was filled with 1.5 ml of de-ionized water to serve as a balance during centrifugation. Both Centricons® were spun at the highest setting in a clinical centrifuge until the volume of concentrated EBP in the centricon was approximately 500 µl to give a 4.5 mg/ml concentrated solution and approximately 225 µl to give a 10 mg/ml concentrated solution. The waste chambers were removed, and the Centricons® were inverted and spun for 1 minute to force the concentrated EBP solution into the conical collection cup on the end of the centricon. The concentrated EBP was pipetted into a microfuge tube, labeled, and promptly frozen.

Microseeding

In order to obtain crystals of EBP in a shorter amount of time, the technique of microseeding was used in conjunction with the hanging drop method. The seeding solution used in the drops was made by combining a drop containing crystals with a solution that was the same as the reservoir solution, except with a higher concentration of the precipitant PEG.

To make the seeding solution, the coverslip of a crystal-containing well was carefully removed. The excess grease was wiped from one edge of the coverslip with a Kimwipe. Then, the drop was washed over the grease-free edge into a microfuge tube with 50 µl of the solution. An additional 50 µl of solution was added to the microfuge

tube. The crystals were crushed using a micropestle in an up and down fashion five times and a twisting fashion two times. The following serial dilutions were made from the stock seeding solution to be used in the drops: undiluted, 1:10, 1:100, 1:1000, and 1:1000. The wells were set up as described in the general crystallization procedure except that the components of the drop were 1 µl of reservoir solution, 1 µl of seeding solution, and 2 µl of 10 mg/ml EBP solution.

Results

EBP Sample Purity

A 12% discontinuous polyacrylamide gel was run to determine the purity of the sample of EBP obtained from J. Buchman. Due to some of the volume of the sample of molecular weight standards running over into the two neighboring wells, the sample of EBP was run in lane 4 (Figure 4). There are seven visible bands for the molecular weight markers, as seen in lanes 1, 2, and 3, and only one dark and obvious band for EBP in lanes 4 and 5. The appearance of a band in lane 5 is also due to some of the volume of the EBP sample running over into lane 5.

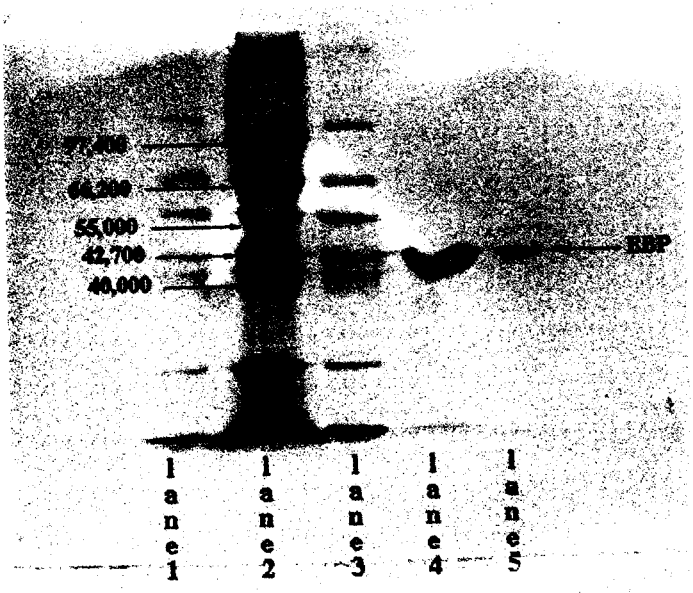

Figure 4. 12% Discontinuous Polyacrylamide Gel Run to Determine EBP Sample Purity. Lane 1, 2, 3 contain Promega Mid-Range Molecular Weight Markers while lanes 4 and 5 contain EBP.

EBP Crystallization Conditions

A kit purchased from Hampton Research called Crystal Screen I was used t perform the initial screening of fifty crystallization conditions. Once the concentration o EBP was increased to 10 mg/ml, crystals were observed to grow at 4°C in crystal screen solution #28 -- 30% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, and 0.2 M sodium acetate. The crystals produced in this condition were colored yellow and were "spiky" in appearance; they appeared to be bundles of needle-like crystals growing together (Figure 5).

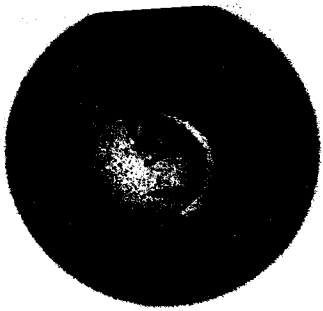

Figure 5. EBP Crystals at 40X Magnification

This condition was expanded upon in an attempt to grow X-ray diffraction-grade crystals. To do so, the concentration of PEG, average molecular weight of PEG, concentration of the sodium acetate solution, and pH of the sodium cacodylate buffer were varied; however, all of the crystallization plates were incubated at 4°C. In these expansions, crystals have been grown in mid- and high-weight PEG, 0.1 M sodium cacodylate solutions, pH 6 to 7, and 0.2 M sodium acetate solution (Table 2). The growth

of crystals in the lower molecular weight PEGs are preceded by the formation of yellow EBP precipitate, while crystal growth in the higher molecular weight PEGs is normally not

Table 2. Expanded EBP Crystal Growth Conditions at 4°C

Component	Concentration
PEG 6000, 8000, 12000, & 20000	20-40% (w/v)
Sodium Cacodylate	0.1 M, pH 6.0, 6.5 & 7.0
Sodium Acetate	0.2 M

accompanied by EBP precipitation. The time table for growth of crystals in the lower molecular weight PEGs is fairly long compared to the growth rate of EBP crystals in the higher molecular weight PEGs (Table 3). However, crystals grown under any of the expanded conditions were similar in appearance to the crystals initially grown from Crystal Screen solution #28.

Table 3. Physical Information on EBP Crystals Grown

	PEG 6000 and 8000	PEG 12,000 and 20,000
Presence of EBP Precipitate	Yes	Very rarely
Time for Crystal Growth	4 - 6 weeks	approximately 2 weeks
Appearance of EBP Crystals	yellow bundles of needle-like crystals	yellow bundles of needle-like crystals

Small crystals of EBP were observed to grow in the microseeded drops. Like their counterparts grown in the lower molecular weight PEGs, these crystals were preceded by yellow EBP precipitate; these crystals were similar in appearance to the others. However, these crystals grew within 1 week of making the drops. Finally, the number of crystals in the drops generally decreased as the seeding solution used became more dilute.

Discussion

EBP Sample Purity

As seen in Figure 4, there is only one distinct, visible band in EBP samples lanes 4 and 5. Since this is the only visible band, the sample received from J. Buckman on 8 October 1996 was homogeneous. If there was more than one band in lanes 4 and 5, as in lanes 1-3, then there would be more than one protein and/or impurities in the sample.

Additionally, the protein in the sample was verified to be EBP by determining its approximate molecular weight using the gel. As seen in Figure 4 lanes 1-3, the molecular weight standards separated in such a fashion as to produce a "ladder." Each rung of the ladder corresponds to a protein of known molecular weight. The molecular weight of the protein sample was estimated using the standards because the one band seen in lanes 4 and 5 of Figure 5 traveled roughly the same distance as the ovalbumin band in the molecular weight standard lanes. Since ovalbumin has a molecular weight of 42,700 Daltons, it was estimated that the protein in lanes 4 and 5 is approximately 46,000 Daltons, the molecular weight of EBP.

EBP Crystallization Conditions

Of the many crystallization conditions tested after expanding upon the conditions of crystal screen solution #28, the crystals were observed to grow optimally and very reproducibly at 4°C in 25% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, and 0.2 M sodium acetate. The crystals grown in these conditions are crystals of EBP because they have a distinct yellow color to them, which is indicative of the flavin that is bound to each individual EBP molecule. Although the crystals grow optimally in the above-stated conditions, they have been grown reproducibly in various related conditions, as given in Table 2

The crystals grown under the other conditions described in Table 2 are also spiky and yellow. However, the growth rate of these crystals appears to be dependent on the molecular weight of the PEG used. As Table 3 indicates, the crystals grown in PEG 12,000 and 20,000 grow in a shorter amount of time than their counterparts grown in PEG 6000 and 8000. Because the area of the PEG molecules increases as the molecular weight of these molecules increases, the number of water molecules needed to solvate the larger PEG molecules increases. Thus, the higher molecular weight PEGs will compete more actively with EBP for the water molecules. Since there is also diffusion of water out of the drop so that the precipitants in the drop can be in equilibrium with the precipitants in the reservoir, there are fewer water molecules available to both the PEG and EBP. The final result of this diffusion and solvating process is that EBP is driven to its crystalline form faster in the presence of a high molecular weight PEG, as I have observed.

Since the EBP crystals grown to date lack smooth, distinct faces and orderly packing, no attempts have been made to collect any X-ray diffraction data, nor have any attempts been made to transfer any crystals to a mother liquor solution so that they may be transported to collect data. The individual molecules of EBP that create the crystals are not oriented in the same direction, as their spiky appearance indicates. Thus, any X-ray directed at these crystals would result in a "fuzzy" and weak diffraction pattern because each molecule would not diffract the X-ray in the same direction. If data from these crystals were translated into an electron density map, it most likely would result in an inferior electron density map that would not be suitable for structure solving.

The "spiky" appearance of the EBP crystals grown may be due to some microheterogeneity in the EBP sample used for crystallization. The microheterogeneity may be a result of the methods employed to purify EBP. When EBP is expressed, it is synthesized as one long polypeptide chain that has six extra amino acids at the N-terminus. These six amino acids are not found in naturally expressed EBP because the added N-terminus tail is used as a tag to purify EBP on an affinity column. Once the EBP is

released from this column, the six amino acid tail is proteolytically cleaved to yield pure EBP. However, the protease used for this cleaving does not always cut off all six amino acids. Thus, each individual protein may not be the same length, and the difference in length cannot be detected by a polyacrylamide gel because it does not have the ability to resolve proteins that differ in molecular weight by only a few amino acids. Since each protein is not the same length, each will not fold in the same manner. The slight difference in the folding of each protein will cause any crystals that form to be misshapen because they do not fit together exactly.

The shape of the crystals may also be attributed to the fact that the sample of EBP used has degraded. Although the EBP samples are kept frozen to minimize protein degradation and denaturation, thawing and refreezing normally cause proteins to eventually degrade. Due to this degradation, there is more "debris" in the protein sample that can hinder the crystallization of EBP.

Although crystals of EBP have been grown faster when microseeding was employed, the size of the seeded crystals is considerably smaller than their unseeded counterparts. The reason(s) for this observation are unknown, but may be due to the presence of more precipitate and/or debris in the seeded drops. The increase in precipitate and/or debris may result from making the seeding solution, protein degradation, or the presence of dust in or on the tools used to make it. However, microseeding does appear to be an effective method for producing crystals in a short amount of time. Since there are small EBP crystals in the microseeded drops, the EBP can crystallize at a faster rate because it does not need to overcome the initial difficulty of nucleating since there are already crystals to which the EBP can add.

The results obtained to date on the crystallization of EBP are encouraging. Although the crystals grown may not be optimal for data collection using X-ray diffraction, it may be interesting to take some of these crystals to a synchotron to determine whether or not useful data can be taken from them. However, before this method is pursued, there are

other directions that can be taken to grow diffraction-grade crystals of EBP. This can include crystallizing microhomogeneous samples of EBP under some of the conditions explored in this research; and because there are other conditions that have been found to crystallize EBP but not explored in this research, some of the research effort should be directed at exploring and expanding these conditions.

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