6-1996

The crystallization of estrogen-binding protein

Jeffrey R. Weinstein
Union College - Schenectady, NY

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THE CRYSTALLIZATION OF ESTROGEN-BINDING PROTEIN

By

Jeffrey R. Weinstein

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Submitted in partial fulfillment

of the requirements for

Honors in the Department of Chemistry

UNION COLLEGE

June, 1996

ABSTRACT

Weinstein, Jeffrey The Crystallization of Estrogen-Binding Protein. Department of Chemistry, June 1996.

Candida albicans is a yeast that is known to target humans with weakened immune systems. The growth rate and invasiveness of *C. albicans* is stimulated in the presence of estrogens. Experimental evidence suggests that the estrogen-binding protein (EBP) from *C. albicans* may be involved in the estrogen-induced increase in growth rate. In order to investigate the possible involvement of EBP in this process, work is underway to determine the atomic structures of EBP alone and bound to estrogen. Protein crystals of EBP have been grown at 4°C in the presence and absence of estrogen using vapor diffusion techniques from a solution of 2% Polyethylene glycol, 2.0M ammonium sulfate, 0.1M sodium HEPES at pH 7.5, and a protein concentration of 5.0 mg/ml. Once large, diffraction quality crystals have been grown, x-ray diffraction data will be collected from the crystals and used to solve the three dimensional structure of EBP. The structure will be useful in answering questions about how estrogen interacts with EBP and possibly understanding how estrogen induces the growth rate of *C. albicans*.

ACKNOWLEDGEMENTS

I would like to thank Dr. Patrick VanRoey from the Wadsworth Center for helping us collect x-ray diffraction data and Dr. David Feldman from Stanford University for sending the purified EBP samples.

I would like to thank Prof. Kristin Fox for all of her help throughout the project. I would also like to thank Melissa Morris for putting up with my singing in the lab and for making research more fun. I would like to thank the Union College Chemistry Department for giving me the opportunity to do this research.

Finally, I would like to thank my parents, grandparents, Amy, and Brian for their continual love and support.

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LIST OF ABBREVIATIONS

β-ED	β-estradiol
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
C. *albicans*	*Candida albicans*
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EBP	estrogen-binding protein
EDTA	ethylenediamine-tetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane-sulfonic acid)
kDa	kilodaltons
MPD	2-methyl-2,4-pentanediol
MRE	morphinone reductase
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NADP+	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form

NMR	nuclear magnetic resonance
OYE	old yellow enzyme
PEG	polyethylene glycol
Tris	Tris(hydroxymethyl)aminomethane

Introduction:

Candida albicans is known to be the pathogen responsible for causing the common yeast infection. Until recently, the common yeast infection was considered to be only a nuisance, because it was not life-threatening. However, *C. albicans* has begun targeting patients suffering from impaired immune systems. These impaired immune systems are due to AIDS, cancer chemotherapy, and drugs used by patients after organ transplantation in order to prevent organ rejection. To patients with weakened immune systems, *C. albicans* is potentially lethal.

Candida albicans is a yeast that is able to exist in two different yeast forms. The first is the common yeast form, which is relatively harmless. The second form is the invasive hyphal form, which can infect patients with weakened immune systems. The dimorphic transition from the common yeast form to the more virulent hyphal form was found to be stimulated by estrogen. It was also found that the growth rate of *C. albicans* is induced in the presence of estrogen. It is possible that if the mechanism by which estrogen induces this transition and growth can be understood and inhibited, then this transition to the hyphal form can be blocked.

In the past fifteen years, scientists have learned much information about *C. albicans* (Chan et al., 1995; Artico et al., 1995). An estrogen-binding protein (EBP) was first discovered in *C. albicans* by Powell, Frey, and Drutz in 1984. This suggested that there was a protein that could be responsible for observed growth rate induction in the presence of estrogen

The function of the EBP in *C. albicans* is still uncertain, but there are five important characteristics that are known about EBP. It is known that the yeast *C. albicans* undergoes a dimorphic transition to the hyphal form in the presence of estradiol (Madani et al., 1994). Therefore, people with high levels of estrogen, such as women on estrogen-containing birth control pills, run a higher risk of contracting a fungal infection. It is known that EBP binds estrogens (especially 17β-estradiol and estrone) with high affinity, but does not bind tamoxifen or corticosterone (Skowronski and Feldman, 1989). The dissociation constant for 17β-estradiol bound to estrogen-binding protein is 6.2 nanomolar. It was recently discovered that EBP is located in the vacuoles of the cell (Zhao et al., 1996). It is also known that there is a high level of EBP mRNA expression during the lag and early logarithmic stages of growth (Madani et al., 1994). Finally, it is known that EBP exhibits an oxidoreductase activity, in which one of the energy carriers in the cell, NADPH, is oxidized to NADP+. Madani et al.

determined that EBP demonstrated high oxidoreductase activity in the presence of an electron acceptor, such as cyclohex-2-ene-1-one, which was inhibited by nearly seventy percent in the presence of 17β-estradiol.

At the present time, there are three hypotheses for the function of EBP in *C. albicans*. The first hypothesis is that EBP binds estrogens and converts them into other compounds (Madani et al., 1994). The justification of this hypothesis is that EBP is known to bind estrogens and analogous steroids and convert them into other compounds, in vitro. However, studies showed that there was no major increase in this conversion, in vivo, for a clone with an over-expressed estrogen-binding protein. The second hypothesis is that EBP has a significant function in the growth regulation of *C. albicans*. This hypothesis is supported by the fact that there is the highest level of EBP expression during the lag and early logarithmic phases of growth. The third hypothesis is that the binding of estradiol to EBP regulates the oxidoreductase property of EBP in *C. albicans* (Madani et al., 1994). If this hypothesis is correct, then the transformation of *C. albicans* from the yeast to the invasive hyphal form may involve the oxidoreductase activity of EBP.

The ultimate goal of the research presented here is to determine the three-dimensional structure of EBP in *C. albicans*. This structure would be useful in many ways, such as understanding the function of

EBP and learning at which amino acids estrogen binds EBP. If this information were known, it might be possible to construct a compound that could bind EBP with a higher affinity than estrogens, thereby blocking the binding of estrogens to EBP. Since we have evidence that estrogens stimulate the conversion of *C. albicans* from the yeast to the more virulent, hyphal form, it is possible that a compound that prevents estrogens from binding to EBP could prevent this conversion. Therefore, a structure of EBP could lead to the production of a compound that would effectively inhibit the growth of fungal pathogens.

As stated above, there might be great benefits of knowing the structure of EBP in *C. albicans*. Currently, the most widely used method for solving the three-dimensional structures of macromolecules is x-ray crystallography. X-ray crystallography is a technique in which the diffraction pattern of x-rays from a crystal is interpreted. A well-ordered crystal is required because it contains many identical molecules in the same exact orientation. This yields a strong diffraction pattern because the x-rays are all diffracted identically (Rhodes, 1993). The only other method of determining the structures of macromolecules is nuclear magnetic resonance (NMR). There are two major disadvantages of NMR in structure determination of proteins. The first disadvantage is that you cannot use information

from the structure of one protein to interpret the structure of a similar protein. In x-ray crystallography, a known structure can be used to help explain the diffraction patterns of similar structures. Therefore, any proteins of known structure, that are similar to the protein being studied, can aid in the analysis of diffraction data. The second disadvantage of NMR in protein structure determination is that you cannot easily use the known structure of a protein to help determine the structures of protein-ligand complexes.

In 1994, Madani et al. were able to isolate this estrogen-binding protein from *C. albicans*. From this isolated EBP, they were able to determine the 407 amino acid sequence (See Figure 1), from which they deduced the molecular weight to be approximately 46kDa. The amino acid sequence of EBP in *C. albicans* displays a significant sequence similarity to many other flavoproteins. There is a forty-six percent identity between the sequences of the Old Yellow Enzymes found in *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* (OYE1 and OYE2, respectively). Furthermore, there is a fifty-one amino acid region in EBP (amino acids 183-233) that is sixty-eight percent identical and eighty percent similar to OYE1 and OYE2 (See Figure 2). The structure of OYE1 is known (Fox, K.M. and Karplus, P.A., 1993) and this level of similarity suggests that EBP will have a similar structure.

FIGURE 1. The amino acid sequence of the estrogen-binding protein in Candida albicans.

```
1          10          20          30          40          50
TIESTNSFVVPSDTKLIDVTPLGSTKLFQPIKVGNNVLPQRIAYVPTTRF

51         60          70          80          90          100
RASKDHIPSDLQLNYYNARSQYPGTLIITEATFASERGGIDLHVPGIYND

101        110         120         130         140         150
AQAKSWKKINEAIHGNGSFSSVQLWYLGRVANAKDLKDSGLPLIAPSAVY

151        160         170         180         190         200
WDENSEKLAKEAGNELRALTEEEIDHIVEVEYPNAAKHALEAGFDYVEIH

201        210         220         230         240         250
GAHGYLLDQFLNLASNKRTDKYGGGSIENRARLLLRVVDKLIEVVGANRL

251        260         270         280         290         300
ALRLSPWASFQGMEIEGEEIHSYILQQLQQRADNGQQLAYISLVEPRVTG

301        310         320         330         340         350
IYDVSLKDQQGRSNEFAYKIWKGNFIRAGNYTYDAPEFKTLINDLKNDRS

351        360         370         380         390         400
IIGFSRFFTSNPDLVEKLKLGKPLNYYNREEFYKYYNYGYNSYDESEKQV

401    406
IGKPLA
```

Figure 2. The aligned amino acid sequences of EBP and Old Yellow Enzyme

```
      1              15 16              30 31              45 46              60 61              75 76              90
EBP1: TIESTNSFVVPSDTK LIDVTPLGSTKLFQP IKVGNNVLPQRIAYV PTTRFRASKD-HIPS -DLQLNYYNARSQYP GTLIITEATFASERG
OYE1: -----------SFVK DFKPQALGDTNLFKP IKIGNNELLHRAVIP PLTRMRALHPGNIPN RDWAVEYYTQRAQRP GTMIITEGAFISPQA
                  * *        ** * ** * ** *** *  *      * ** **      **   *    **  * * * ** **** * *

      91            105 106            120 121            135 136            150 151            165 166            180
EBP1: GIDLHVPGIYNDAQA KSWKKINEAIHGNGS FSSVQLWYLGRVANA KDLKDSGLPLIAPS- AVYWDENSEKLAKEA GNELRALTEEEIDHI
OYE1: GGYDNAPGVWSEEQM VEWTKIFNAIHEKKS FVWVQLWVLGWAAFP DNLARDGLRYDSASD NVFMDAEQEAKAKKA NNPQHSLTKDEIKQY
      *     **      *    * **  ***   * *  **** **  *      *   **      *   *  *   *  ** * *    **  **

      181           195 196           210 211           225 226           240 241           255 256           270
EBP1: VEVEYPNAAKHALEA GFDYVEIHGAHGYLL DQFLNLASNKRTDKY GCGSIENRARLLLRV VDKLIEVVGANRLAL RLSPWASFQGMEIEG
OYE1: IK-EYVQAAKNSIAA GADGVEIHSANGYLL NQFLDPHSNTRTDEY G-GSIENRARFTLEV VDALVEAIGHEKVGL RLSPYGVFNSMSGGA
        **  ***    * * * **** * ****  ***   ** *** * * *********  * * ** * *  *   . * **** *  *

      271           285 286           300 301           315 316           330 331           345 346           360
EBP1: EE----IHSYILQQL QQRADNGQQLAYISL VEPRVTGIYDVSLK- DQQGRSNEFAYKIWK GNFIRAGNYTYDAPE FKTLINDLKNDRSII
OYE1: ETGIVAQYAYVAGEL EKRAKAGKRLAFVHL VEPRVTNPFLTEGEG EYEGGSNDFVYSIWK GPVIRAGNFALHPEV VREEVKDK---RTLI
      *         *    *   **  *  **   * ******              * ** * * *** *  *****                *  * *

      361           375 376           390 391           405 406           420 421           435 436           450
EBP1. GFSRFFTSNPDLVEK LKLGKPLNYYNREEF YKYYNYGYNSYDESE KQVIGKPLA-    406
OYE1: GYGRFFISNPDLVDR LEKGLPLNKYDRDTF YQMSAHGYIDYPTYE EALKLGWDKK    399
      *  *** ******   *  * *** * *  * *      **  *   *
```

Another protein that has a significant similarity to EBP is morphinone reductase, from the bacteria *Pseudomonas putida* (French and Bruce, 1995). The amino acid sequences of EBP and morphinone reductase are thirty-eight percent identical (See Figure 3) , but the structure of morphinone reductase is unknown. Also, there is no visible similarity between the sequence of EBP in *C. albicans* and the sequence of the human estrogen receptor, suggesting that the EBP structure will not be similar to that of the human estrogen receptor (Madani et al., 1994). Therefore, the structure of OYE1 may be useful in the interpretation of x-ray diffraction data from EBP.

In order to use x-ray crystallography to determine the structure of EBP, it must be crystallized. Therefore, the first part of our research will be focused upon discovering the conditions necessary to grow diffraction grade crystals of EBP.

There are many known methods that have been successfully used to crystallize proteins in the past. Among the popular crystallization methods are bulk crystallization, batch method, dialysis techniques, sequential extractions, free interface diffusion, and vapor diffusion (McPherson, 1982). Vapor diffusion using hanging drops was chosen for this situation for a few reasons. First of all, only a few microliters of protein solution is necessary for each well. Also, the materials needed are inexpensive. Finally, the procedure is easy and

Figure 3. The aligned amino acid sequences of EBP and Morphinone Reductase

```
        1              15 16             30 31             45 46             60 61             75 76             90
EBP1    TIESTNSFVVPSDTK LIDVTPLGSTKLFQP IKVGNNVLPQRIAYV PTTRFRASKD-HIPS -DLQLNYYNARSQYP GTLIITEATFASERG
MRE     --------------- -MPDTSFSNPGLFTP LQLGSLSLPNRVIMA PLTRSRTPDS--VPG -RLQQIYYGQRAS-- AGLIISEATNISPTA
                           *       ** *    *   ** *     * ** *       *    **  **  *       *** ***  *

        91             105 106            120 121            135 136            150 151            165 166            180
EBP1    GIDLHVPGIYNDAQA KSWKKINEAIHGNGS FSSVQLWYLGRVANA KDLKDSGLPLIAPS- AVYWDENSEKLAKEA GNE-------LRALT
MRE     RGYVYTPGIWTDAQE AGWKGVVEAVHAKGG RIALQLWHVGRVSHE LVQP-DGQQPVAPS- ALKAEGAECFVEFED GTAGLHPTSTPRALE
              ***  ***    **   ** *  *      ***  ***          *    ***  *             *  *           ***

        181            195 196            210 211            225 226            240 241            255 256            270
EBP1    EEEIDHIVEVEYPNA AKHALEAGFDYVEIH GAHGYLLDQFLNLAS NKRTDKYGCGSIENR ARLLLRVVDKLIEVV GANRLALRLSPWASF
MRE     TDEIPGIVE-DYRQA AQRAKRAGFDMVEVH AANACLPNQFLATGT NRRTDQYG-GSIENR ARFPLEVVDAVAEVF GPERVGIRLTPFLEL
          **  ***  *  * *  *  **** ** *  *   *  ***     * *** ** ****** **  * ***   **  *  *   ** *

        271            285 286            300 301            315 316            330 331            345 346            360
EBP1    QGMEIEGEE----IH SYILQQLQQRADNGQ QLAYISLVEPRVTGI YDVSLK-DQQGRSNE FAYKIWKGNFIRAGN YTYDAPEFKTLINDL
MRE     FGLTDDEPE---AMA FYLAGELDRRG---- -LAYLHFNEPDWIGG DITYPE----GFR-E QMRQRFKGGLIYCGN YDAGRAQARLDDNTA
         *      *       *    *  *       ***    **   *              *   *       **  *  ** *           *

        361            375 376            390 391            405 406            420 421            435 436            450
EBP1    KNDRSIIGFSRFFTS NPDLVEKLKLGKPLN YYNREEFYKYYNYGY NSYDE-SEKQVIGKP LA    406
MRE     ----DAVAFGRPFIA NPDLPERFRLGAALN EPDPSTFYGGAEVGY TDYPFLDNGHDRLG- --    377
                  *   **** *   **  **       **     **   *
```

fast, allowing hundreds of different conditions to be tried in a short period of time (McPherson, 1982).

The theory behind the hanging drop method of vapor diffusion is that the reservoir solution contains conditions that are optimum for precipitating the protein. However, when the protein solution is mixed with the same solution for the hanging drop, the hanging drop has a concentration of precipitating solution that is lower than that of the reservoir solution. Therefore, water will be distilled out of the small hanging drop and into the vapor form, until it goes into the reservoir solution. Because the reservoir solution has a much larger volume than the hanging drop, the concentration of the reservoir solution will not change significantly. Therefore, the hanging drop is able to slowly change its concentration to the best precipitating conditions, which in many cases will yield protein crystals. As these protein crystals are forming, all of the molecules of protein are losing kinetic energy, which enables them to be held together by intermolecular forces, such as hydrogen bonds, between the protein molecules and intervening water molecules. These intermolecular forces are what hold the molecules together in a low entropy system, such as a crystal (Rhodes, 1993; McPherson, 1982).

Candida albicans has emerged as a yeast that can undergo a transition to a harmful hyphal form, and estrogen has been shown to

play a role in stimulating this transition. Since *C. albicans* contains an estrogen-binding protein, which binds estrogens with a high affinity, we are able to focus on the protein in order to find a way to inhibit the transition. Therefore, we propose to determine the structure of EBP in *C. albicans*, and to examine the binding of estrogen in atomic detail.

Experimental:

Determination of Protein Purity by Gel Electrophoresis

The EBP solution was purified by Feldman et al at Stanford University and shipped in dry ice. The purity of EBP was checked using an SE 250 - Mighty Small II Slab Gel Electrophoresis Unit purchased from Hoefer Scientific Instruments. The instructions for setting up the unit, preparing and pouring the acrylamide separating gel and the stacking gel, and preparing the buffers and solutions were described in the instruction manual and strictly followed. The samples were prepared by pipetting the desired amount of protein (1-10μl) to a microfuge tube and adding enough distilled water to bring the volume up to 10μl. In order to determine the molecular weight of the protein sample, 5μl of a set of proteins of known molecular weights, called markers, are run on the same gel for comparison. A 2X treatment buffer was prepared according to the instruction manual, and added to bring the total volume of all tubes to 20μl. The samples were then heated for five minutes in a 90°C water bath in order to denature the proteins. The samples were pipetted into the wells, and the power

supply was turned on. The gel was run at a constant current of 40mA, with the voltage not exceeding 240 volts. After the gel was finished running, the apparatus was taken apart and the gel was removed. The gel was placed into a large glass container and a stain of Coomassis Blue R-250 was added. After at least thirty minutes, the stain was removed and a destain of methanol and acetic acid was added. At this point the gel was analyzed.

Determination of Protein Concentration

Standards of Bovine Serum Albumin (BSA) were prepared in duplicate ranging from 0.2mg/ml to 1.4mg/ml in enough distilled water to make a final volume of 100µl. EBP samples containing 30µl, 50µl, and 100µl of protein were prepared in duplicate and brought up to a volume of 100µl with distilled water. Finally, a control tube containing only 100µl of distilled water was prepared. Then, 5ml of diluted Bio-Rad Dye Reagent were added to all tubes. The tubes were vortexed and incubated for thirty minutes at room temperature. A Hewlett Packard Diode Array Spectrophotometer was used to measure the absorbance at a wavelength of 596 nanometers, and a standard curve of absorbance vs. µg of protein was created using Cricket Graph

III. Excel Least Squares Fit was used to determine the amount of EBP in the samples and the concentration of EBP was then calculated.

Crystallization Procedures

Small pieces of clay were placed in each corner of the cover of a Linbro Tissue Culture multi-well plate to prevent the cover from fully closing. Dow Corning high vacuum grease was put in a 12 ml syringe. The syringe was then squeezed slowly, in order to spread little beads of grease completely around each well. 500µl of a reservoir solution, which contained a precipitant, were pipetted into a well. A siliconized Fisherbrand Microscope Cover Glass was cleaned with a Kimwipe and a few microliters of the reservoir solution were pipetted onto the center of the coverslip. A few microliters of protein, usually the same amount as reservoir solution, were pipetted onto the bead of reservoir solution in the center of the coverslip. Finally, the coverslip was inverted and placed over the well, while slight pressure was applied in order to form an airtight seal. The plates were then incubated at either 4°C or room temperature in order to allow the crystals time to grow. The individual wells from the plates were examined at 40X magnification and the results recorded in a binder.

Concentration and Ultrafiltration of Protein

In order to change the concentration of EBP from 0.637mg/ml to 5.0mg/ml, 2ml of EBP were pipetted into a Centricon 10 Concentrator. An equal amount of distilled water was pipetted into another Concentrator, and both were spun at high speed in a centrifuge at 4°C. The protein buffer was changed from a solution that was 200mM NaCl, 20mM Tris, 1mM EDTA, 1mM DTT to a solution of 10mM Tris by repeatedly concentrating the protein and rediluting with a 10mM Tris solution. The protein solution was then spun down until the volume was less than 0.2548µl, and the Centricon 10 Concentrator was spun upside down in order to collect the concentrated protein solution into a microfuge tube. Finally, the volume was brought up to 0.2548µl with the 10mM Tris solution.

Binding β-Estradiol (β-ED) to EBP

A one hundred microliter solution of 1.0M β-ED (M=272.4g/mol) in dimethyl sulfoxide (DMSO) was prepared. In a test run, the β-ED solution precipitated upon contact with water. Therefore, the solution

was further diluted to a concentration of 0.1M β-ED in DMSO. This new solution was partially soluble in water. The effects on crystallization due to the binding of β-ED to EBP were studied by adding β-ED to a solution of concentrated EBP (5.0mg/ml). Then crystal screens were set up with this new protein solution.

Mother Liquor Preparations

A solution is needed for the crystal to be transferred into from the hanging drop so that it can be transported for x-ray diffraction. This solution, which must be able to sustain the crystal without cracking or dissolving it, is called the mother liquor. The mother liquors were prepared using the same conditions and pH as the reservoir solution for the wells with crystals in them, except for the concentration of 2-methyl-2,4-pentanediol (MPD). The concentration of MPD was varied by increments of five percent (v/v) until a solution was found in which a crystal could remain without cracking or dissolving.

Amino Acid Sequence Alignments

A search for proteins with a similar amino acid sequence was performed using Basic Local Alignment Search Tool (BLAST) (Altschul et al, 1990) using the amino acid sequence of EBP, and Swiss-Prot (Rodriquez-Tome et al, 1996) full text search using the term "estrogen." There were two different procedures that were used to check the alignments of the amino acid sequences. First, a multiple sequence alignment was performed using CLUSTAL W(1.5) (Higgins et al, 1991) multiple sequence alignment. The accession numbers of the proteins were typed into the program and the sequences were aligned and analyzed by the program. However, this program only worked for certain proteins for which the sequences were available by accession number. For the proteins that did not have a sequence available through CLUSTAL W, Swiss-Prot was used to obtain the sequences. These sequences were then pasted into CLUSTAL W, converted, and aligned. The sequence alignments were analyzed by hand after the sequences were printed out. All proteins with a twenty-five percent or greater identity were considered significantly similar.

Results:

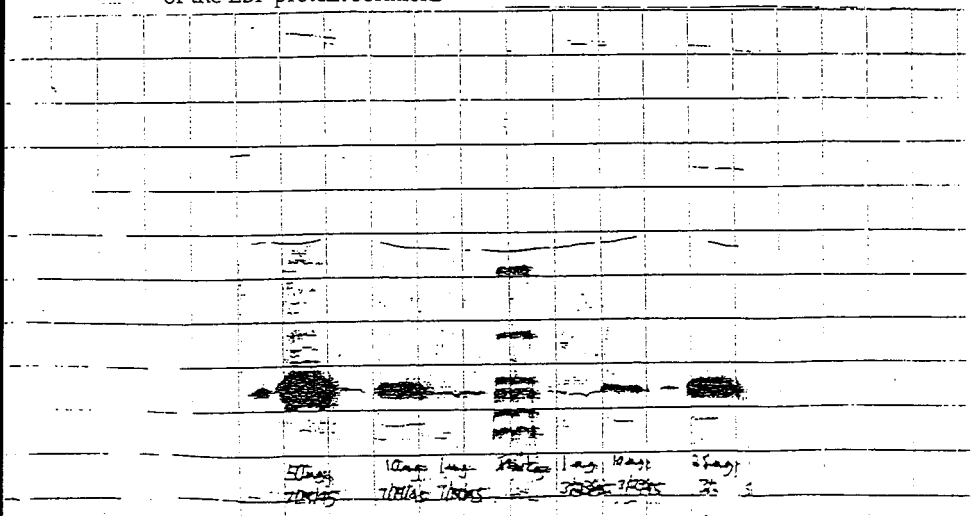
Determination of Protein Purity by Gel Electrophoresis

The Polyacrylamide Gel Electrophoresis was run for two different samples of Estrogen-Binding Protein. The sample that was received from Dr. David Feldman on July 14, 1995 was run in wells containing 1μg, 10μg, and 50μg of protein. The sample received from Dr. Feldman on March 23, 1995 was run in wells containing 1μg, 10μg, and 28μg of protein. A photocopy of this gel is shown in Figure 4.

Determination of Protein Concentration

The EBP sample that was received from Dr. Feldman on July, 14, 1995 was stated to contain EBP at a concentration of 1.26 mg/ml. A standard curve using a range of Bovine Serum Albumin (BSA) from 20μg to 140μg was set up and the absorbance at 596nm was measured in duplicate for each standard. The absorbance of EBP was measured at 596nm in duplicate for samples containing 30μl, 50μl, and 100μl. A least squares fit determined that the concentration of

Figure 4. The polyacrylamide gel used to determine the purity of the EBP protein solutions



the 30µl sample was below the standard curve, the 50µl sample had a concentration of 0.541 mg/ml, and the 100µl sample had a concentration of 0.637 mg/ml.

Crystallization Procedures

The fifty different standards in the crystal screen kit were set up under five different conditions. These conditions were at a protein concentration of 0.63 mg/ml at 4°C and at room temperature, at a protein concentration of 5.0 mg/ml at 4°C and at room temperature, and at a protein concentration of 5.0 mg/ml at 4°C with β-ED bound to the protein. The plates of wells were observed periodically in order to find crystals that appeared to be large, sharp protein crystals. These crystals were referred to as promising crystals. Promising crystals were found in seven of these wells and five of these conditions have been expanded upon. These expansions involved the varying of the pH, temperature, and concentration of precipitant in the reservoir solution. All three of these factors can have a direct correlation with the size of the crystals grown, the time it takes to grow the crystals, and the quality of the crystals. Therefore, it is essential to find the best conditions in order to grow the largest, highest quality crystals.

The first promising condition to be expanded upon was 30% MPD, 0.02M $CaCl_2$, and 0.1M Sodium Acetate at a pH of 4.5 at room temperature. Large, sharp, diffraction quality crystals were grown with 32% MPD, 0.02M $CaCl_2$, and 0.1M sodium acetate at a pH of 3.25 at room temperature. They were transferred into a mother liquor and diffraction data were collected from these crystals at the Wadsworth Center in Albany.

The second condition expanded upon was 8% Polyethylene Glycol (PEG) 4000 and 0.1M sodium acetate at pH 4.54 at 4°C. No promising crystals were grown in this expansion.

The third promising condition that was expanded upon was 2M ammonium phosphate and 0.1M Tris HCl at a pH of 8.5 at 4°C. No promising crystals were grown in this expansion.

The next two conditions that were expanded upon were 2M ammonium sulfate, 2% PEG 400, and 0.1M sodium HEPES at a pH of 7.5 at 4°C. The protein concentration was 5.0 mg/ml for both of these conditions, but one of these protein solutions had β-ED bound to EBP. Both of these expansions are beginning to show signs of promise because small, sharp crystals are starting to appear in some of the wells.

The last two promising conditions have not yet been expanded upon.

Concentration and Ultrafiltration of Protein

The procedure for concentrating and ultrafiltering the protein solution of EBP served two purposes. The first purpose was to concentrate the protein from 0.63 mg/ml to 5.0 mg/ml. This was achieved by reducing the volume from 2.0ml to 0.2548ml. The second purpose of this procedure was to change the buffer of the protein solution. The protein solution was repeatedly diluted with a solution of 10mM Tris and then reconcentrated in order to decrease the concentration of the components in the original protein solution to less than 1mM of each.

Binding β-Estradiol (β-ED) to EBP

In order to make a 100μl solution of 1M β-ED in DMSO, 0.0279g of β-ED was added to 100μl of DMSO. In a test run, this solution precipitated upon contact with water. The concentration was decreased to 0.1M β-ED in DMSO with the addition of 900μl of DMSO. After a successful test run with water, 1.25μl of this solution was added to 256μl of a 5.0 mg/ml EBP solution. The faint yellow color of the EBP

solution changed to a peach color with very little of the β-ED solution precipitating. The concentration of β-ED in the protein solution was estimated to be 0.49mM.

Mother Liquor Preparations

Mother liquors were prepared with increasing concentrations of MPD from 35% to 50% MPD. Crystals grown in 32% MPD, 0.02M $CaCl_2$, and 0.1M sodium acetate were transferred into these solutions and checked periodically under a microscope. Only crystals in the 50% MPD solution remained intact and uncracked for long periods of time.

Amino Acid Sequence Alignments

The sequence of EBP was obtained from Swis-Prot and similar sequences were searched for using BLAST. The sequences were converted and aligned using ClustalW multiple sequence alignment. The only two proteins that were found to have a high percent identity to the sequence of EBP were Old Yellow Enzyme and Morphinone Reductase. OYE was calculated to be 43.6% identical and MRE was calculated to be 36.1% identical to the sequence of EBP. These

calculations were performed by dividing the number of conserved amino acids by the total number of amino acids in the protein with the fewest amino acids.

Discussion:

Protein Preparation

The polyacrylamide gel was dried and examined for protein purity. All of the wells containing EBP showed a band representing a protein of molecular weight of approximately 46kDa. These bands are known to be EBP. Only the wells containing 10μg or more of EBP showed bands representing impurities. However, these bands were very small in comparison to those of EBP. Therefore, we concluded that both samples of EBP were more than 95% pure, which is pure enough for our needs.

The protein concentration was calculated from a standard curve of Bovine Serum Albumin. Only the 50μl and the 100μl samples of EBP fit within the standard curve. The 100μl sample was considered to be more accurate because of its greater volume and protein content. Therefore, this sample was used to calculate the protein concentration of EBP to be 0.637 mg/ml.

As previously stated, the protein sample was concentrated to 5.0 mg/ml in order to attempt to grow larger crystals. The buffer was also changed by repeatedly diluting the protein solution with a 10mM Tris

solution and then reconcentrating the protein solution until the dilution factor was over 200 times. This reduced the previous buffer to containing less than 1mM NaCl and other solutes.

Finally, enough β-ED was added to some of the 5.0 mg/ml EBP solution to make the solution 0.49mM β-ED. The color change from a yellow to a peach color indicated that the β-ED was bound by the EBP.

Crystallization Procedures

The crystal screens were examined under a microscope at least once a week and observations were recorded in a binder. The largest, sharpest crystal was found in a solution containing 30% MPD, 0.2M $CaCl_2$, and 0.1M sodium acetate at a temperature of 4°C. After expanding upon the conditions listed above, we were able to grow large, diffraction quality crystals. These crystals were transferred into different solutions in order to be transported to the Wadsworth Center for x-ray diffraction data collection. When a solution was found that did not dissolve or damage the crystals in any way, the crystals were transported to the Wadsworth Center in the solution. X-ray diffraction data was converted into a pattern that displayed the amplitude of the waves diffracted by different atoms in the crystal. This pattern

consisted of only two dots spaced far apart, which symbolizes a small closely-packed molecule, such as a salt crystal. The crystal is likely to be made up of $CaCl_2$ since this salt was present in large amounts in the growth solution. A solution of methylene blue was purchased and a small amount of methylene blue was added to the solution containing the crystals. Methylene blue is a small organic molecule that will fit inside the water channels of a protein crystal, but not inside the smaller channels of a salt crystal. The crystals that were in the solution did not take up the blue color, proving that they were indeed salt crystals.

The other solutions that contained large crystals grown under different conditions were also tested with the methylene blue solution. The crystals formed from a solution in the presence and absence of β-ED in 2% PEG 400, 2M ammonium sulfate, and 0.1M sodium HEPES took up all of the blue dye out of solution. These crystals turned a very dark shade of blue, while the solution around them turned clear. Therefore, these crystals are likely to be protein crystals because they contain water channels large enough to fit the methylene blue.

There were only two other conditions that have evidence, by this method, suggesting that the crystals are indeed protein crystals. However, these conditions have not yet been expanded upon. The current research is focused upon growing larger, diffraction quality

crystals of EBP with and without estrogen bound in order to collect x-ray diffraction data from EBP.

Amino Acid Sequence Alignments

It is generally accepted that any two proteins that have a 25% or greater amino acid sequence identity will have a similar three dimensional structure. The only two proteins that had greater than a 25% sequence identity with EBP were OYE and MRE. OYE was calculated to have a 43.6% sequence identity with EBP, while MRE was calculated to have a 36.1% sequence identity with EBP. Therefore, both of these proteins were thought to have a similar three dimensional structure to EBP. Since the structure of OYE is already known (Fox, K.M. and Karplus, P.A., 1993), molecular replacement techniques can be used to help solve the structure of EBP. In this method, x-ray diffraction data from EBP crystals will only give a pattern with information regarding amplitude, but the structure of OYE can give information regarding the phase. Together, the amplitude and the phase are enough information to construct a three dimensional electron density diagram. The atoms from EBP are then fit into this diagram in order to get a structure of the protein.

Conclusions

Protein crystals of EBP have already been grown in the presence and absence of β-ED. These crystals are currently being studied in order to grow larger diffraction quality crystals. Once these higher quality crystals are grown, they will be transferred into a mother liquor and diffraction data will be collected. These data will be analyzed using molecular replacement techniques and the structure of OYE. The three dimensional structures of EBP with and without estrogen bound will allow us to examine the binding of estrogen to EBP in atomic detail. Then, we will be able to better understand the role that EBP plays in the increase in growth rate of *C. albicans* and in the dimorphic transition of *C. albicans* from the dormant to the more invasive, hyphal form.

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