DETERMINATION OF CONDITIONS FOR OYE IN WHICH THERE IS NO ION PRESENT IN THE ACTIVE SITE

By

Melissa J. Morris

****

Submitted in partial fulfillment

of the requirements for

Honors in the Department of Chemistry

UNION COLLEGE

June, 1996

ABSTRACT

MORRIS, MELISSA Determination of conditions for OYE in which there is no ion present in the active site. Department of Chemistry, June 1996.

Old Yellow Enzyme (OYE) has been the subject of extensive research since the early 1930's. However, the structure of OYE was not determined until 1994, and its physiological function still remains a mystery. Investigations into the function of OYE involve uncovering the details of protein-substrate interactions through the use of x-ray crystallography. OYE is known to oxidize NADPH, but attempts to crystallize the OYE-NADPH bound complex have been hampered by the presence of chloride in the active site of OYE. This competition prevents an accurate assessment of the structure of OYE bound to NADPH. This study will examine the possibility of determining crystallization or soaking conditions for OYE in which there is no chloride in the active site.

Three experiments were employed with this design. First, we attempted to grow OYE crystals in the absence of chloride. Unfortunately there were no positive signs of the ability to grow crystals of OYE without chloride. The second experiment involved transferring crystals to increasingly dilute solutions containing $MgCl_2$. This was only effective until a concentration of 0.16 M $MgCl_2$ at

which point the crystals cracked. In the final experiment, crystals were transferred into a solution identical to the reservoir solution in which the 0.2 M $MgCl_2$ had been replaced with 0.2 M $MgSO_4$. Although the crystals survived this transfer, our data still shows the presence of chloride in the active site. This technique is still being actively investigated.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my appreciation to a number of people for their assistance in this research. First, I would like to thank my advisor, Dr. Kristin Fox, for her patience and enthusiasm in introducing me to the world of biochemistry. Without her continual support and direction, this project would not have been possible. I would also like to recognize my lab partner Jeffrey Weinstein for his constant encouragement, willingness to selflessly lend a helping hand or mind at any instant, and his daily concerts which never failed to bring a smile to my face. I would also like to thank Chia-En Chen for her support throughout the project. There are also a number of people outside of our laboratroy who deserve recognition. Andy Karplus and Zhan Deng from Cornell University supplied samples of purified OYE, saving me a great deal of time and aggravation. Patrick VanRoey from the Wadsworth Center allowed me to use his laboratory to obtain x-ray crystallographic data essential to our research. The faculty of both the chemistry and biology departments at Union College offered their support and wealth of knowledge. Also, the department of chemistry at Union College for the opportunity to perform this research. Finally, I would like to thank my family for their support, encouragement, and computer tips. They have taught me by example that with enough dedication and confidence, almost any project is possible.

TABLE OF CONTENTS

LIST OF FIGURES

TABLE OF ABBREVIATIONS

BED --------- ß-estradiol

BSA --------- bovine serum albumin

(c-THN)TPN -- O_2-6B-cyclo-1,4,5,6 - tetrahydro-nicotinamide adenine dinucleotide phosphate

EDTA -------- ethylenediamine tetraacetic acid

FMN --------- flavin mononucleotide

HEPES ------- N-(2-hydroxyethyl) piperazine-N'-(2-ethane-sulfonic acid)

kD ---------- kiloDalton

$NADP^+$ ------- nicotinamide adenine dinucleotide phosphate, oxidized form

NADPH ------ nicotinamide adenine dinucleotide phosphate, reduced form

OYE -------- Old Yellow Enzyme

PEG -------- polyethylene glycol

PHB -------- p-Hydroxybenzaldehyde

SDS -------- sodium dodecyl sulfate

INTRODUCTION

Background

Old Yellow Enzyme (OYE) was first isolated from brewer's bottom yeast (Saccharomyces carlsbergensis) in the early 1930's by Warburg and Christian. At that time, little was known about the relationship between enzymes and proteins. When Theorell purified OYE to electrophoretic homogeneity in 1934, analysis of the purified form revealed a one-to-one ratio between the protein and a yellow flavin cofactor. Although the removal of this cofactor from the enzyme resulted in loss of activity, the fact that their recombination was enough to regain the activity served as strong evidence for the theory that protein is an important element of an enzyme (as reviewed by Schopfer and Massey, 1991).

The discovery of the flavin cofactor led to extensive experimentation on OYE. OYE was commonly used as a means of studying flavins for a couple of reasons. First, it is relatively easy to obtain large amounts of purified OYE from brewers' bottom yeast. Another characteristic which makes OYE an important experimental agent is the ease with which the flavin mononucleotide (FMN) can be removed from and returned to the enzyme.

The extensive research performed on OYE led to the identification of FMN as the phosphoric acid ester of

riboflavin (vitamin B2), but the details of the structure of OYE remained a mystery for quite some time. Finally, this puzzle was completed in 1994 when Fox solved the structure. However, the unveiling of OYE's physiological function remains to be accomplished.

Physical Properties

OYE exists as a dimer of identical subunits (Figure 1), each of which contains 399 amino acids and has a molecular weight of approximately 45,000 daltons. One flavin mononucleotide cofactor (Figure 2) is non-covalently bound to each subunit (Karplus, Fox, and Massey, 1995). The dissociation constant for this interaction was calculated using a 0.1 M phosphate buffer at a pH of 7.0 at 25°C, and found to be approximately 10^{-10} M (Schopfer and Massey, 1991).

Crystallization

A considerable amount of information concerning protein function can be gathered by uncovering the details of protein-substrate interactions. This is accomplished by obtaining detailed pictures of the protein bound to a variety of ligands. The complexes are then analyzed according to data obtained through x-ray crystallography.

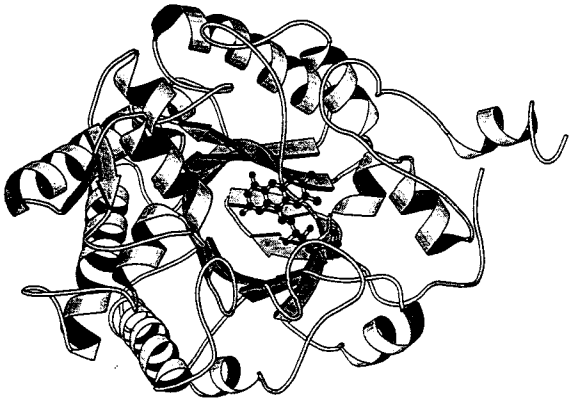

Figure 1. Structure of OYE. Above is a ribbon diagram of the subunit of OYE with the flavin shown in the center.

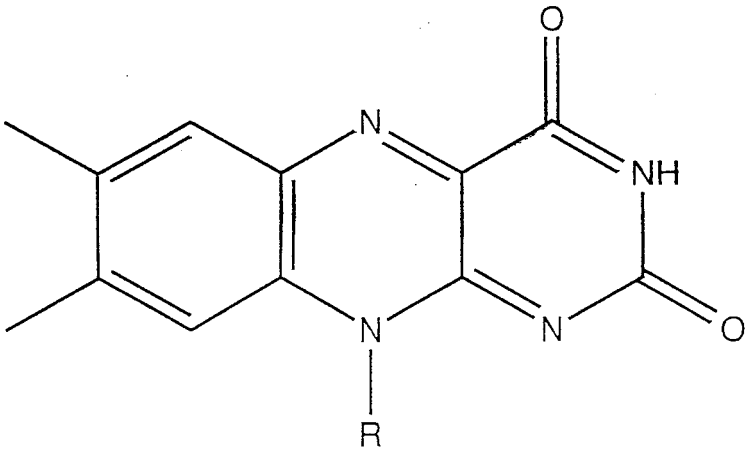

Figure 2. OYE's flavin cofactor. Above is the structure of the yellow flavin cofactor which is necessary for enzymatic activity.

There are two major obstacles which must be overcome in order to obtain useful x-ray crystallographic data. The first, which will be covered in this section, is the formation of crystals which are suitable for x-ray crystallography. The second is the successful binding of sufficient quantities of the various ligands. This will be discussed in the next section.

One technique for growing crystals is the hanging-drop method (Figure 3). This involves preparing a drop of less than 25 ul containing equal volumes of a solution of purified protein and a reservoir solution. The drop is mixed on a cover slip, which is then inverted so that the drop is hanging from it, and then is sealed on the top of a well containing the reservoir solution.

The theory behind this technique is that vapor diffusion results in a net transfer of water from the protein solution to the reservoir solution until the concentration of the precipitant is the same in both solutions. In order for crystals to grow in this situation, it is necessary to use a reservoir solution which is at a concentration that results in a concentration of precipitant in the drop at equilibrium which is optimal for crystal growth. Once these conditions have been determined, small variations can be made until an appropriate rate of crystal growth is achieved. This rate should be relatively slow in order to avoid the formation of clusters of crystals which

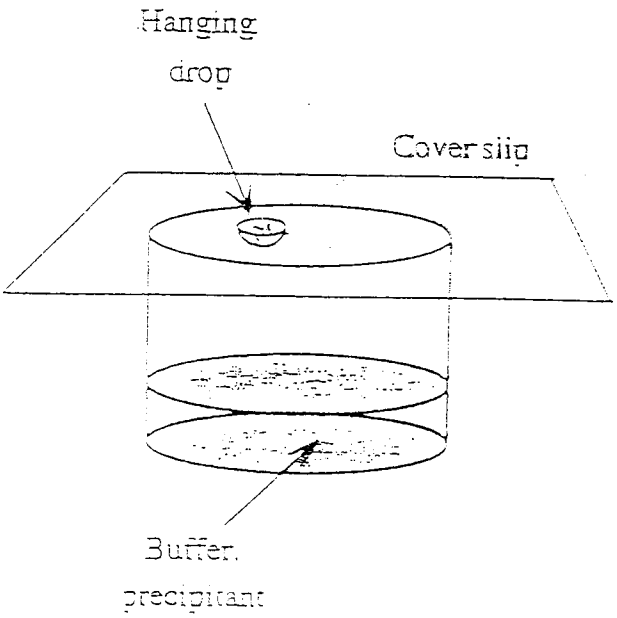

Figure 3. Hanging-drop method of crystallization. The crystallization technique utilized in the project was the hanging-drop method. Shown above is a schematic of the technique (Rhodes, 1993).

will deplete the concentration of protein before the crystals become large enough to be useful (Rhodes, 1993).

In order to examine protein functions, it is necessary to compare the x-ray crystallographic data obtained from crystals of pure protein to that recorded for crystals of protein-ligand complexes. There are two popular techniques which are used to form crystals of protein-ligand complexes. In the first method, called cocrystallization, the protein and ligand are crystallized together. This method is useful in the production of crystals of proteins which are bound to large ligands. The second method, termed soaking consists of soaking the protein crystals in a mother liquor which contains the desired ligands. These ligands can then diffuse through channels of solvent in the crystal to the binding sites of the protein. The preformed crystals soaked with ligands are more likely to be of the same form and unit-cell dimensions as the pure protein. Therefore, this method is better for obtaining crystals which are to be used for the comparison of the structure of the pure protein to that of a protein-ligand complex. (Rhodes, 1993).

Ligand Binding

OYE has been found to bind simple anions, aromatic hydrocarbons, and pyridine nucleotides. Additional research has shown that these ligands compete with one another in their attempt to bind to OYE. This finding led to the hypothesis that the ligands bind at overlapping sites.

Evidence concerning the location of the binding site was gained through the examination of visible absorption spectral data. This testimony came in the form of two types of changes which occurred in the flavin absorption spectrum upon ligand binding (Figure 4), indicating that the binding site is near the flavin. The first alteration was witnessed in the two absorption peaks which normally appear at 462 and 380 nm. The perturbation brought about by the binding of various ligands was a shift of up to 20 nm in these two peaks, along with a reduction of their height by between 2 and 20%. The second change in the spectrum was the appearance of a broad band in the region between 500 and 800 nm and another lying between 300 and 425 nm. The fluctuations from the normal absorption spectrum of the flavin suggest that the ligand binding site is located in the vicinity of the flavin (as reviewed by Schopfer and Massey, 1991).

The crystal structure was useful in the confirmation of this suspicion. The x-ray crystallographic data obtained from the oxidized enzyme in a solution containing chloride shows the presence of a chloride ion in a pocket 3.5 Å above the flavin. The position of this substrate is stabilized by hydrogen bonds from His and Asn. The study of oxidized OYE crystals which had been soaked in p-hydroxybenzaldehyde, ß-estradiol, or (c-THN)TPN (an NADPH analog) further supports

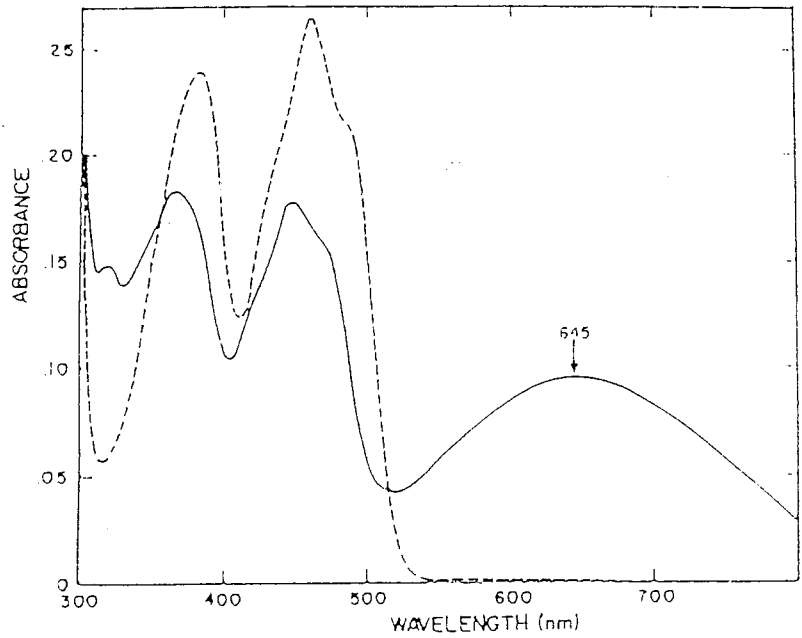

Figure 4. Absorbance spectrum of OYE. The dotted line represents OYE alone. The solid line represents OYE bound by p-Cl-phenol (Schopfer and Massey, 1991).

this finding. Each of these ligands was found to displace the chloride and hydrogen bond to His and Asn 3.2 to 3.5 Å from the flavin (Figure 5) (as reviewed by Karplus, Fox, and Massey, 1995).

Simple Anions

Titration experiments have indicated that certain monovalent anions have the ability to bind to OYE. Azide, acetate, and chloride were the most successful of the anions examined, although studies by Rutter and Rotlander in 1957 show all of the halogens, nitrate, carbonate, formate, phosphate, sulfate, and EDTA to be effective inhibitors. Cations did not bind to OYE. The spectral changes mentioned above which occurred upon the binding of these simple anions came in the form of slight peak shifts to lower wavelengths along with a decrease in the height of the peaks by up to 10% (Fox, 1994).

The conditions which have been determined for effective crystallization of OYE include the presence of 0.2 M $MgCl_2$. Therefore, the structure which is obtained through the interpretation of x-ray crystallograpic data includes chloride in the active site. This is a fairly stable complex, as chloride has a dissociation constant of 8 mM.

Aromatic Hydrocarbons

Experiments on the spectral effects of aromatic hydrocarbons revealed that the most dramatic changes occurred upon the binding of phenols to OYE. These

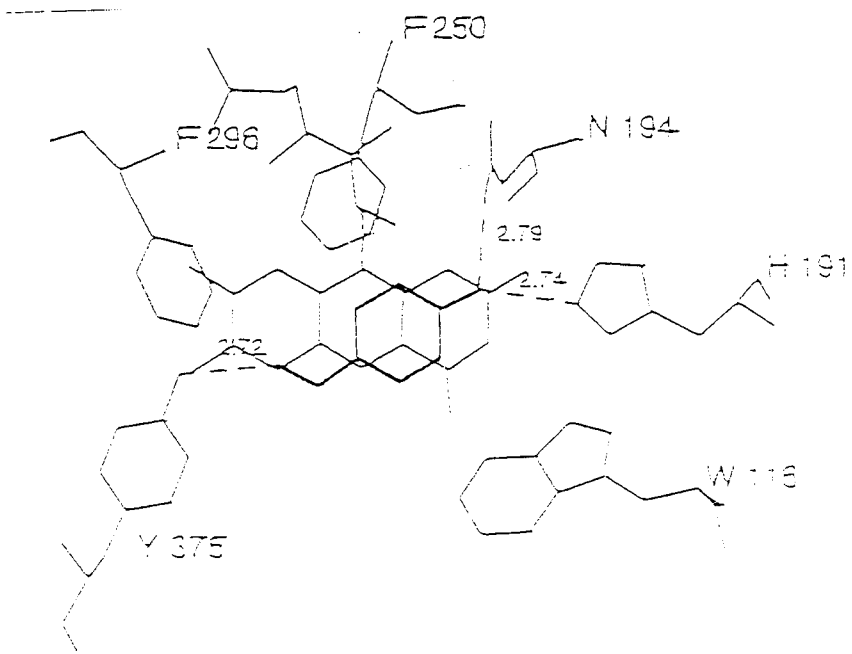

Figure 5. PHB bound in the active site of OYE. The phenol, which is represented by the thick lines, forms hydrogen bonds with H 191, N 194, and Y 375 (Fox, 1994).

alterations consisted of significant shifts in the maximum absorbance and the formation of additional absorbance bands between 500 and 800 nm as well as in the region between 300 and 425 nm. The manifestation of the long wavelength peak upon the addition of phenols was traced to its ionizable hydroxyl group. An explanation for this proposal was provided in 1976 by Abramovitz and Massey who claimed that this spectral perturbation was the result of a charge-transfer interaction in which the bound phenolate served as the charge transfer donor and the electron-deficient oxidized flavin played the role of the charge transfer acceptor. Although controversy surrounds this theory, it has prevailed as the generally accepted explanation behind the appearance of the new peak upon interactions of phenols with OYE. Although phenols bind more tightly to OYE than do simple anions, no further evidence has been found which would indicate that any of the phenols are the true substrate.

In 1994, Fox achieved a detailed structural determination of the binding of phenols to OYE. This was accomplished by examining the interaction which occurs between p-Hydroxybenzaldehyde (PHB) and OYE. This reaction took place when OYE was in the crystalline form, and it resulted in an alteration in the color of the OYE crystals from yellow to a dark shade of green. An investigation of this compound at a resolution of 2 Å revealed that the

chloride ion was displaced from the active site by the phenol ring which binds above the si-face of the flavin so that the planes of the two rings are nearly parallel. It appears as though the phenolate oxygen is bound by hydrogen bonds from His 191 and Asn 194, while a hydrogen bond forms between the hydroxyl of Tyr 375 and the aldehyde carbonyl oxygen of the PHB.

Pyridine Nucleotides

In 1985, Massey and Schopfer studied the interactions between OYE and pyridine nucleotides through the use of steady state kinetics, rapid reaction kinetics, and equilibrium binding. This led to the discovery of OYE's only known catalytic action which is the oxidation of NADPH by molecular oxygen. Further experimentation showed that α-NADPH, β-NADPH, and the acid breakdown products of NADPH all oxidize OYE. Of these compounds, (c-THN)TPN, a derivative of α-NADPH, was found to bind the most tightly (Schopfer and Massey, 1991).

The best attempt at determining the structure of OYE-pyridine nucleotide bound complexes was accomplished by soaking OYE crystals in a saturated solution of (c-THN)TPN (Fox and Karplus, 1994). This study showed that when the (c-THN)TPN binds in the pocket near the flavin, it results in the movement of the peptide chain near residues 290-300. This effect was not witnessed upon the binding of any other ligands. In this complex, the nicotinamide ribose end of

(c-THN)TPN fits reasonably well into the active site pocket while the adenine portion of the ligand remains unbound.

As with simple anions and aromatic hydrocarbons, the binding of pyridine nucleotides causes changes in the flavin absorption spectrum. In this case, the peak occurring at 462 nm is shifted to 473 nm while the peak which normally lies at 380 nm is shifted slightly to the red.

Investigating the Active Site

The search for the true substrate of OYE has led to an increased knowledge of the phenol binding site. Since this is believed to be the enzyme's active site, the hope is that the continuation of such research will lead to the discovery of OYE's physiological function. A great deal of progress has been made in this area through studies involving the binding of NADPH as well as ß-estradiol (BED). The binding of BED results in the movement of the side chain of Tyr 375 by approximately 3 Å. This occurs in preparation for hydrogen bonding. The binding of BED also causes Phe 296 to twist 120° in order to make room for BED's B ring.

The oxidation of NADPH was OYE's first known catalytic action. Since that time, OYE has also been found to catalyze many α,β-unsaturated carbonyl compounds. For example, in 2-cyclohexenone, OYE catalyzes the reduction of the olefinic bond.

Such studies have already fed some of our curiosities concerning the active site. It has now been hypothesized that both the nicotinamide and the electron acceptors bind in the same pocket. This conclusion was based on the parallel Lineweaver-Burk plots which resulted from varying the concentrations of NADPH and the acceptor. These plots are characteristic of a ping-pong mechanism which involves the $NADP^+$ leaving the enzyme before the acceptor reacts (Karplus, Fox, and Massey, 1995).

It is our hope that the continuation of this examination of the binding of NADPH to OYE will provide more information concerning the active site of OYE and eventually lead to an understanding of OYE's physiological function. One obstacle which has been encountered in the past during such studies is the competition between Cl^- and NADPH for occupancy of the binding site. The strength of the interaction which Cl^- has with OYE in the crystals which are grown makes it extremely difficult to replace the Cl^- with enough NADPH to attain a sufficient structure of OYE bound to NADPH. Although Cl^- has a dissociation constant of 8 mM as compared to that of NADPH which is ~70 uM, the Cl^- binds to OYE much more successfully because it is present in a crystal at a concentration of 0.4 M. Since NADPH is not very soluble, we are unable to get it to a high enough concentration to displace enough Cl^- from the active site of OYE to achieve sufficient structural studies. We are aiming to find a set of conditions in which NADPH is the most

competitive ligand. It is hoped that the information which can be gained through the attainment of a clearer picture of the OYE-NADPH complex will assist in the discovery of the physiological function of OYE.

METHODS

Gel Electrophoresis

Prior to performing experiments on the samples of OYE, it was necessary to determine their purity. The first assay employed for this objective was that of color. Since OYE is known to display a yellow color, any change in this appearance is indicative of an impurity. However, in order to attain a more accurate assessment of protein purity, additional measures must be taken. One more informative technique is that of gel electrophoresis. This method does not rely on the imperfect ability of the human eye to discern changes in color.

Gel electrophoresis separates proteins based on their size and charge. In the procedure, the protein samples are placed in small wells which have been preformed in a gel. An electrical current is then passed through the gel, and the protein samples migrate according to their size and charge. Since our purpose of running the gel was not to separate, but to assess purity, we were able to simplify the factors determining the distance run by the sample. This

was accomplished by heating the sample in a water bath at 90°C for five minutes (in order to break hydrogen bonds), adding ß-mercaptoethanol (to break disulfide bonds), and finally adding SDS. The SDS serves a number of functions. It causes the samples to achieve a rod-like shape; it coats the samples, thus preventing renaturation; and it masks the charges of the samples. The final result of these additions is the formation of samples with equal charge-to-mass ratios, and similar shapes. Having this accomplished, the only remaining factor which determines the distance the sample will run is molecular weight. Since this property has previously been determined for OYE (~45kD) it provides an accurate means of determining the purity of a sample.

The electrophoresis unit on which this procedure was performed is the SE 250-Mighty Small II Slab Gel Electrophoresis Unit (Hoefer Scientific Instruments). Since OYE is a relatively small protein, the pores of an acrylamide gel were of a suitable size. The details of the solutions which were prepared can be found on pages 15-17 of the instruction manual for the SE 250-electrophoresis unit.

Concentration Determination

Another important step in the characterization of new samples of OYE is the determination of concentration. This is accomplished through the use of a protein assay. Bio-Rad Coomassie Blue is used as the protein assay since its

binding to proteins results in increased absorbance of the dye at 596 nm. This was first allowed to react with 100 ul BSA samples as well as with 100 ul samples of OYE. Seven BSA samples were prepared in duplicate at concentrations ranging from 0.2 mg/ml to 1.4 mg/ml. Three OYE samples were also prepared in duplicate at concentrations which were estimated to cover the same range as the BSA samples. Each solution was then mixed with 5 ml of the Bio-Rad protein assay which had been diluted 1:5. The samples were incubated for 30 minutes at room temperature. The absorbances of the samples were measured at a wavelength of 596 nm on the Hewlett Packard 8452A diode array spectrophotometer. These results were used to plot a standard curve for BSA on Cricket Graph III. Finally, concentration values were calculated for OYE by multiplying the data obtained from the Microsoft Excel Least Squares Program by the dilution factor.

Crystallization

Once the purity and the concentration of the OYE samples had been determined, we were able to begin experimentation on the protein. This generally began with an attempt to crystallize OYE. The hanging-drop method described previously was the technique used to achieve this purpose. Conditions of crystallization were determined prior to this project (Fox and Karplus, 1994). These conditions entailed a reservoir solution of 0.1 M HEPES at a

pH of 8.3, 35% PEG 400, and 0.2 M $MgCl_2$. The OYE utilized existed at a concentration of approximately 30-40 mg/ml, and the drops were stored at a temperature of 4.0°C. Since these conditions were determined with solutions prepared in another laboratory using different instruments, my first step was to prepare a tray in which the conditions included slight variations of those determined previously. In this tray, the reservoir solutions contained 0.1 M HEPES with pH's of 7.9, 8.1, 8.3, 8.5, and 8.7, and PEG 400 at 31%, 33%, 35%, and 37%. The concentration of the $MgCl_2$ (0.2 M) was not altered. The volume of reservoir solution used in each well was 500 ul and the drops each contained 5 ul of OYE and 5 ul of the reservoir solution.

While optimal conditions for crystal growth in the above mentioned solutions were being determined, we also began to search for conditions which do not contain chloride. It was our hope that if OYE crystals could grow under these circumstances, NADPH would be a more competitive substrate, allowing for a more complete structure determination of the OYE-NADPH bound complex. In an attempt to identify such a condition, a screen of 50 different reservoir solutions was used. The solutions came from Hampton Research's Crystal Screen.

Ridding OYE of Cl^-

Along with growing crystals in the absence of chloride, attempts were also made to remove the chloride from crystals which had previously been grown. Two experiments were performed with this design. The first involved transferring a crystal of OYE which contained chloride into solutions containing decreasing concentrations of $MgCl_2$. The first solution into which the crystal was transferred consisted of 0.2 M $MgCl_2$, 35% PEG 400, and 0.1 M HEPES at a pH of 8.5. These conditions were kept constant among all of the solutions with the exception of the concentration of $MgCl_2$ which decreased by 0.01 M from one solution to the next. The crystal was kept in each solution for 5 minutes before being transferred to the next solution.

The second experiment performed in the attempt to obtain crystals which did not contain chloride involved soaking crystals which had been grown in $MgCl_2$ in a solution containing 0.2 M $MgSO_4$, 35% PEG 400, and 0.1 M HEPES at a pH of 8.5. Prior to transferring the crystal into this solution, it was soaked in a solution containing 0.2 M $MgCl_2$, 35% PEG 400, and 0.1 M HEPES. The crystal was then transferred into the solution containing $MgSO_4$. After equilibrating in this solution, the crystal was transferred into an identical solution of $MgSO_4$, PEG 400, and HEPES. The crystal was soaked in this solution, and then transferred to the final solution of $MgSO_4$, PEG 400, and HEPES.

Titration of OYE $\cdot SO_4^{2-}$ Complex with p-Cl-phenol

The binding ability of SO_4^{2-} to OYE was assessed through the titration of the OYE$\cdot SO_4^{2-}$ complex with p-Cl-phenol (Massey and Schopfer, 1986). For our purposes, 0.4 M K_2SO_4 was substituted for KN_3.

RESULTS

Gel Electrophoresis

In order to assess the purity of the samples of OYE, each concentration was run in two lanes on the gel (Figure 6). One lane contained 10 ug of OYE at the given concentration, and the other lane contained 100 ug of OYE. The estimated concentrations which were run were 26 mg/ml, 40 mg/ml, and 70 mg/ml. When 10 ug of OYE was present, the 26 mg/ml sample and the 70 mg/ml sample showed no significant bands other than that at approximately 45 kD which corresponds to the molecular weight of OYE. The 40 mg/ml sample produced a major band at the molecular weight

Figure 6. A gel of OYE. The lanes are as follows from left to right: 10 ug of 26 mg/ml, 10 ug of 40 mg/ml, 10 ug of 70 mg/ml, molecular weight marker, 100 ug of 26 mg/ml, 100 ug of 40 mg/ml, 100 ug of 70 mg/ml.

of OYE, and a small band at approximately 31 kD. When 100 ug of OYE sample was run, a small band appeared at around 100 kD for the 26 mg/ml and 70 mg/ml samples. These samples also produced a series of faint bands directly below the OYE band. The 100 ug of the 40 mg/ml sample produced the band at 31 kD that also appeared when 10 ug of the sample was run along with three very faint bands which were found immediately below this band.

Concentration Determination

The samples of OYE at the three different concentration were each assayed to more precisely determine their concentration. The absorbance of the sample estimated to be at a concentration of 40 mg/ml was measured in triplicate at dilution factors of 50X, 100X, and 200X. The samples showed average concentrations of 47.1 mg/ml, 56.7 mg/ml, and 68.2 mg/ml respectively for an overall average concentration of 57.3 mg/ml. The sample at an estimated concentration of 26 mg/ml was tested in duplicate at dilution factors of 23X, 50X, and 100X. The average concentrations calculated for these samples were 31.8 mg/ml, 42.8 mg/ml, and 60.45 mg/ml respectively, yielding an overall concentration of 35.9 mg/ml. The sample approximated to be at a concentration of 70 mg/ml was prepared at dilution factors of 50X and 100X. The absorbances of these samples were measured in duplicate and yielded average concentrations of 88.2, and 80 mg/ml respectively for an overall average of 84.1 mg/ml.

Crystallization

The conditions which had been reported as being optimal for growing OYE crystals proved to be effective in our laboratory. These conditions are 0.1 M HEPES at a pH of 8.3, 35% PEG 400, and 0.2 M $MgCl_2$. Examples of crystals which were grown under these conditions can be seen in Figure 7. Although more trials are necessary to optimize the size of these crystals, this reservoir solution did yield single crystals which were used to obtain x-ray crystallographic data.

Some broad screens were set up in an attempt to improve the quality of crystal being grown. These looked at both the high and the low ends of the spectrum of the pH of HEPES and the percentage of PEG 400 present using the conditions determined as being optimal previously as the standard. Although crystals were produced in the trays exploring both ends of this spectrum, the conditions which had been determined previously appeared to yield the best crystals in general.

Ridding OYE of Cl^-

In the first attempt to produce crystals which do not contain any chloride in the active site, a screen of 50

different conditions for crystal growth was performed. Of these 50 conditions, the following results were recorded. Solution 4 containing 0.1 M Tris HCl pH 8.5 and 2.0 M Ammonium sulfate produced yellow precipitate. Solution 9 which contained 0.2 M Ammonium acetate, 0.1 M Na Citrate pH 5.6, and 30% w/v PEG 4000 produced a lot of very small, frayed crystals. Solution 17 containing 0.2 M Lithium sulfate monohydrate, 0.1 M Tris HCl pH 8.5, and 30% PEG 4000 resulted in the formation of very thin small crystals which were quite well defined. Solution 18 which consisted of 0.2 M Magnesium acetate tetrahydrate, 0.1 M Na Cacodylate pH 6.5, and 20% PEG 8000 produced a yellow, brown crystal like precipitate. Solution 39 which was a mixture of 0.1 M Na Hepes pH 7.5 and 1.4 M Sodium citrate dihydrate resulted in the formation of thin, yellow, hairlike precipitate. Finally, solution 46 which contains 0.2 M Calcium acetate, 0.1 M Na Cacodylate pH 6.5, and 18% w/v PEG 8000 produced clusters of very small yellow crystals.

In an attempt to remove the chloride from the active sites of crystals grown in a solution containing 0.2 M $MgCl_2$, two experiments were performed. In the first, crystals grown in the chloride solutions were transferred to solutions containing decreasing concentrations of $MgCl_2$. This experiment was run two times. In the first trial, the crystal was not harmed by the solutions containing 0.2, 0.19, or 0.18 M $MgCl_2$. However, when it was transferred

form the 0.18 M solution to a solution containing 0.16 M $MgCl_2$, a small crack developed across the bottom of the crystal. At a concentration of 0.14 M $MgCl_2$, the crystal broke in two at this crack. As the top part of the crystal was transferred to solutions containing 0.14, 0.13, 0.12, and 0.11 M $MgCl_2$, it was allowed to equilibrate for 15 minutes instead of just 5 in the hope that it would adapt to the new conditions. However, the small cracks which formed in the 0.14 M $MgCl_2$ solution continued to grow and new ones formed with each transfer. Finally at a concentration of 0.11 M $MgCl_2$, the trial was terminated.

In the second trial, the crystal was transferred from 0.2 to 0.19 to 0.18 to 0.17 and finally to 0.16 M $MgCl_2$ and allowed to equilibrate for 10 minutes in each solution. Again, the crystal remained in tact until it was immersed in the solution containing 0.16 M $MgCl_2$. At this point it developed a slight crack during the first 5 minutes and more severe cracks by the end of 10 minutes.

The final experiment involved soaking crystal grown in the presence of chloride in a solution identical to the reservoir solution except that the 0.2 M $MgCl_2$ had been replaced with 0.2 M $MgSO_4$. The crystal did not attain any cracks and it kept its original color throughout the transfers. Following the transfer into the third consecutive solution containing 0.2 M $MgSO_4$, x-ray crystallographic data were collected from the crystal.

Titration of OYE $\cdot SO_4^{2-}$ Complex with p-Cl-phenol

In an attempt to determine whether or not SO_4 binds in the active site of OYE, an experiment was adapted from Massey and Schopfer (1986). In this experiment, a sample of OYE alone was titrated with 2 ul portions of p-Cl-phenol and the absorbance was measured from 300 to 800 nm following each addition. The experiment was then repeated after making one change. In the second trial, K_2SO_4 was added to the solution of OYE prior to titrating with p-Cl-phenol. Again, the absorbance was measured followed each addition of p-Cl-phenol (Figure 8). The concentration of p-Cl-phenol required for a maximum change in absorbance was then measured for each solution. The solution containing the OYE alone required 1.6 mM p-Cl-phenol to achieve this maximum absorbance change. This quantity was also calculated for the solution containing the mixture of OYE with K_2SO_4. Again, 1.6 mM of p-Cl-phenol was found to be necessary to reach this maximum change in absorbance.

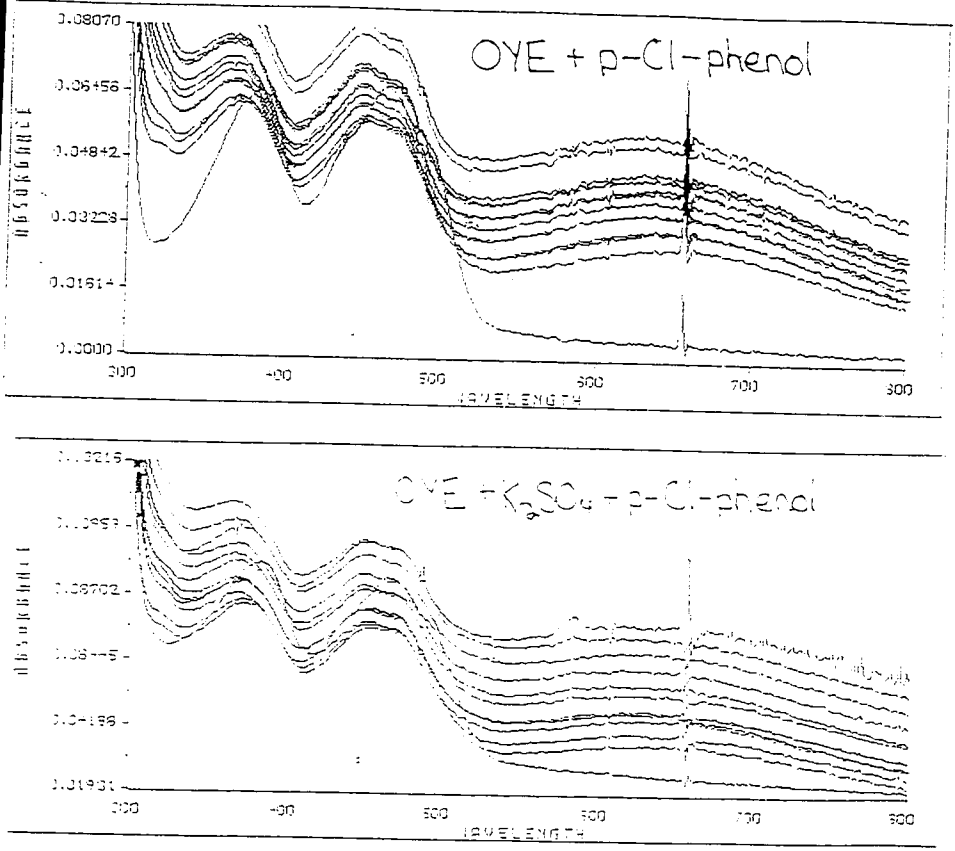

Figure 8. Absorbance spectra of OYE alone being titrated with p-Cl-phenol (top), and OYE and K_2SO_4 being titrated with p-Cl-phenol (bottom).

DISCUSSION

Purity of OYE Samples

In assessing the suitability of the sample of OYE which was estimated to be at a concentration of 26 mg/ml, the following conclusions were drawn. The only extra band to be justified in this sample appeared at a molecular weight of 100 kD when 100 ug of sample were present. The presence of this band was negated in our decision to use this sample for two reasons. The first was that this band was found to appear in samples which were successfully crystallized in the past. The second factor considered was the ratio of the size of the band produced by OYE to that of the extra band. Since a mere look at the gel reveals that this is an immense quantity, the sample was deemed suitably pure for experimentation.

The second sample analyzed was that with an estimated concentration of 40 mg/ml. The decision of whether or not to use this sample was slightly more troublesome, as a band which was not produced by OYE appeared when only 10 ug of sample were present. This band was also present in the lane containing 100 ug of sample. However, this band is known to appear as a result of proteolysis in OYE. Since the sample was prepared approximately two years prior to this project and samples containing this band have been crystallized, the presence of this band was permitted. The other bands which appeared when 100 ug of this sample were present were too

small to be significant. Therefore, it was concluded that this sample was also acceptable for our purposes.

The final sample studied through the use of gel electrophoresis was that with an estimated concentration of 70 mg/ml. This sample produced a gel nearly identical to that formed by the 26 mg/ml sample except that the magnitude of the bands formed by the 70 mg/ml sample were proportionally larger than those formed by the 26 mg/ml sample. Again in this sample, the band which appeared at 100 kD was overlooked for the reason stated above. Since no other bands were large enough to be considered significant, this sample was found to be adequate for our means.

Crystallization

The variety of results recorded in the attempts to crystallize OYE make it difficult to pinpoint an optimal condition for crystal growth. Prior to this project, the hanging drop method had been used to determine the optimal conditions for growing OYE crystals (Fox and Karplus, 1994). These conditions include a reservoir solution consisting of 0.1 M HEPES at a pH of 8.3, 35% PEG 400, and 0.2 M $MgCl_2$ and a sample of OYE at a concentration between 30 and 40 mg/ml. The quantity of OYE used in the drop was found to be directly related to the size of the crystal(Fox and Karplus, 1994). Based on these findings, a tray was set up with this condition in the center and percentages of PEG ranging from 31 to 37 varying with wells containing HEPES ranging in pH

from 7.9 to 8.7. Although the majority of the wells in this tray produced crystals of OYE, the drops consisted mostly of a large number of small crystals. Since no trend was found when the percent of PEG 400 was varied or when the pH of HEPES was varied, two new trays were set up. One tray explored the high end of these conditions while the other assayed the lower end of these conditions. The tray containing HEPES ranging in pH from 8.5 to 8.9 and PEG 400 ranging in percentage from 33 to 39 yielded slightly varied results, but they were much less ambiguous than those from the initial tray. Here it was determined that the wells containing HEPES at a pH of 8.5 or 8.7 and 35% or 37% PEG were more successful in growing adequate crystals than those containing HEPES at higher pH's or higher percentages of PEG. This finding correlates with the original conditions which had been determined. The tray exploring the lower range of conditions contained solutions with HEPES ranging in pH from 7.7 to 8.1 and PEG ranging from 29% to 35%. The findings from this tray were more concrete than in the other trays. When the pH of HEPES was below 8.1, only one well produced crystals. At a pH of 8.1, the only wells which produced crystals were those with 29% and 31% PEG 400. The relation between the pH of HEPES and the growth of OYE crystals again agrees with the conditions which were determined previously as being optimal. However, the percentage of PEG 400 which was present did not relate to the expected values which would successfully precipitate

crystals. In order to reach a conclusion as to why these lower percentages of PEG 400 yielded crystals, it would be necessary to perform more trials. This was not possible during my project as we ran out of suitable samples of OYE. The conclusion which was reached as a result of each of these trials is that the conditions which had been termed optimal prior to this project generally yield the most adequate crystals in our laboratory.

Following this wide screen of the known conditions, we began to examine other factors than the pH of HEPES and the percent of PEG 400 present. For these trials we reverted back to the original conditions used in the first tray. In the first of these experiments, a new sample of OYE at an estimated concentration of 26 mg/ml. This tray yielded no crystals. In an attempt to rule out systematic error, a new tray was prepared using the same solution of OYE on a smaller scale. Again, no crystals of OYE were formed with this new solution. Since the absorbance spectrum of the sample appeared to be normal and the gel showed no signs of impurities, other factors were varied. For examples, a tray was set up using PEG 400 attained from Sigma in two drops and PEG 400 attained from Fluka in another two drops. This test was run multiple times and the only crystals to from in any of these trials were salt crystals. Finally, the same conditions were tested using the sample of OYE estimated to be at a concentration of 70 mg/ml. Despite repetitive trials varying all of the afore mentioned conditions, the

samples of OYE at concentrations of 26 mg/ml and 70 mg/ml, which were both received at a later date than the 40 mg/ml sample, did not yield any crystals. Therefore, we concluded that these samples were prepared in a different way than the original sample, and this variation prevented the growth of crystals of OYE.

Growing OYE Crystals in the Absence of Cl^-

The results of the screen of the 50 different conditions for crystal growth were discussed previously. Although the conditions mentioned did yield crystals, there was not a condition which did not contain a monovalent anion which resulted in the formation of crystals. Since these monovalent anions bind to the active site of OYE in the same manner as chloride, growth under these conditions would still leave us with the problem of removing the anion from the active site of OYE. For this reason, none of these conditions were studied in more detail.

Removing Cl^- From the Active Site of Crystals Grown in the Presence of 0.2 M $MgCl_2$

Two experiments were performed with the intent of removing chloride from the active site of OYE crystals which

had been grown in its presence. The first experiment involved transferring a crystal into solutions containing decreasing concentrations of $MgCl_2$. Prior to this project, crystals grown in the presence of $MgCl_2$ had been transferred to solutions of the mother liquor which did not contain any $MgCl_2$. This resulted in severe cracking in the crystals (personal communication). The hope for this experiment was that if this change in concentration was introduced to the crystals gradually, they would be able to adapt. The concentration of $MgCl_2$ was decreased by 0.01 M between each transfer in this experiment. The crystal being transferred was able to exist in the new solutions until it was soaked in the solution containing 0.16 M $MgCl_2$, when cracks developed. This result was recorded for two trials of the experiment despite being allowed to equilibrate for 10 minutes instead of 5 minutes during the second trial, indicating that this is not a plausible method of removing chloride from the active site of OYE.

The second experiment employed in the attempt to remove chloride from OYE was the soaking of crystals grown in the presence of $MgCl_2$ in an identical solution in which the $MgCl_2$ had been replaced with $MgSO_4$. $MgSO_4$ was chosen for a number of reasons. First, it is believed that OYE crystals can not exist without the presence of Mg^{2+}, although the details behind this requirement are not fully understood. The decision to use SO_4 was slightly questionable, as there has been controversy over whether or not SO_4 binds to the

active site of OYE. The binding of ability of SO_4 was studied, and the results are explained below. Finally, the $MgSO_4$ was chosen because it is soluble. The theory behind this experiment was that we would drive the equilibrium between the chloride bound to OYE and the unbound chloride to the side of the unbound chloride by soaking the crystal in $MgSO_4$.

The mechanics of this experiment were similar to those in which crystals were transferred into decreasing concentrations of $MgCl_2$. Again, the crystal was first transferred into a solution of the mother liquor. After equilibrating in this solution, it was transferred into three identical solutions which contained $MgSO_4$ instead of $MgCl_2$. The crystal remained intact throughout all of these transfers. By the end of the third transfer, the hope was that we had a crystal of OYE which did not contain any chloride in its active site. X-ray crystallography was used to determine whether or not this was the case.

X-Ray Crystallographic Findings

In order to determine whether or not the active site of the crystal which had been soaked in $MgSO_4$ was occupied, x-ray crystallographic data were obtained on the crystal. Although the details of the structure were not very concrete, as the crystal was too small to provide a large signal to noise ratio, a comparison between this crystal and

one known to contain chloride in the active site was made. Unfortunately, no significant difference was found between the two structures. This tells us that we did not successfully drive the equilibrium between the bound and unbound chloride to the side of the unbound chloride by soaking the crystal in $MgSO_4$.

In a second attempt to accomplish this goal by these means, two adjustments will be made. In an attempt to attain a clearer picture of the crystal which we soak in $MgSO_4$, we would like to grow larger crystals prior to the soaking. This will provide a larger signal to noise ratio when the x-ray crystallographic data is obtained. Secondly, in order to drive the equilibrium further to the side of the unbound chloride, the crystal will be transferred to an increased number of solutions containing $MgSO_4$. We are currently in the process of making these alterations.

Conclusions

At this point there has been no proof that crystals of OYE can survive in the absence of chloride, thus allowing for the successful binding of ligands such as NADPH. However, progress has been made in the determination of a condition which will accomplish this goal. First of all, it has been shown to be improbable that adequate crystals of OYE can be grown in the absence of chloride. This finding has led to the study of possible ways to remove chloride

from the active sites of crystals of OYE which have been grown in the presence of 0.2 M $MgCl_2$. We have discovered that OYE crystals appear to require the presence of some type of salt since they would not survive when the $MgCl_2$ was removed or even when the concentration was decreased. However, the possibility of soaking OYE crystals in another salt to drive the equilibrium between the bound and the unbound chloride to the side of the unbound chloride looks promising. Furthermore, it has been found that crystals of OYE can survive in a solution of 0.1 M HEPES, pH 8.5, 35% PEG 400, and 0.2 M $MgSO_4$. This finding is very encouraging and will hopefully lead to the displacement of chloride from the active site of OYE.

Future Work

This research can be followed up with a great deal of future work. In the immediate future, we would like to determine a condition which will successfully remove chloride from the active site of OYE without destroying its crystals. If the technique of soaking crystals grown in $MgCl_2$ in a solution containing $MgSO_4$ does not prove to be successful, then other means of accomplishing this goal will need to be determined.

Once this task has been accomplished, the ligands which were not able to successfully compete with chloride for occupation of OYE's binding site can be bound to the crystalline form of OYE. Since OYE will not have anything

bound to its active site at this point, these ligands should bind in large quantities, allowing for much more detailed structure determinations of the various ligand-OYE complexes. These detailed structures may help in the identification of OYE's physiological function.

One ligand which will be looked at in detail if chloride can be removed from OYE's active site is NADPH. The oxidation of NADPH was OYE's first known catalytic function. It is thought that a detailed look at the binding of NADPH to OYE may lead to the discovery of the physiological function of OYE. If we are able to successfully remove chloride from the active site of OYE, this would be the next logical study.

References

Abramovitz, A. S. & Massey, V. (1976). Purification of intact old yellow enzyme using an affinity matrix for the sole chromatographic step. J. Biol. Chem. 251, 5321-5326.

Fox, K. M., (1994) Crystallographic Structure Determination of Oxidized, Reduced and Ligand-Bound Forms of Old Yellow Enzyme. Ph.D. Thesis. Cornell University.

Fox, K. M. & Karplus, P. A. (1994). Old yellow enzyme at 2 A resolution: overall structure, ligand binding, and comparison with related flavoproteins. Structure. 2, 1089-1105.

Karplus, P. A., Fox, K. M. & Massey, V. (1995) Structure-function relations for old yellow enzyme. The FASEB Journal. 9, 1518-1526.

Massey, V. & Schopfer, L. M. (1986). Reactivity of old yellow enzyme with α-NADPH and other pyridine nucleotide derivatives. J. Biol. Chem. 261, 1215-1222.

Rhodes, Gail. (1993). Crystallography Made Crystal Clear. Academic Press, Inc., San Diego.

Rutter, W. J. & Rolander, B. (1957). The effect of ions on the catalytic activity of enzymes: the old yellow enzyme, Acta Chem. Scand. 11, 1663.

Schopfer, L. M. & Massey, V. (1991). Old Yellow Enzyme. In A Study of Enzymes (Kuby, S. A., ed.), pp. 247-269, CRC Press, Cleveland, OH.

Theorell, H. (1934). Reinigung des Gelben Atmungsfermentes mettels Electrophorese. Naturwissenshaften, 22, 289-290.

Warburg, O. & Christian, W. (1932). Ein Zweites Sauerstoffübertragendes Ferment und Sein Absorptionspektrum. Naturwissenshaften, 20, 688.