Kinetics and Equilibria in Flavoprotein Systems

IV. The Standard Potential of the Old Yellow Enzyme of Yeast

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As part of a continuing study of the old yellow enzyme (OYE) of brewer's yeast by Theorell and co-workers, a re-determination of the standard potential of the highly purified enzyme has been made. The average value of E'_0 for OYE at pH 7 and 30° was found to be -0.123 ± 0.006 V. In the pH range, 4.8–8.9, the system showed a $\Delta E'_0 / \Delta \text{pH}$ slope of -0.06 . Reductive titrations were made with reduced Safranin T as a reagent. The use of indigo disulfonate as a mediator led to acceptable stability of potentials. In all cases a flattening out of the titration curves was encountered after approximately 50 % reduction.

In addition, some slightly modified details of the isolation of crystalline OYE have been worked out.

In 1934 Theorell¹⁻³ reported the isolation of crystalline OYE by a procedure which involved the early steps of the methods of Warburg and Christian⁴, followed by prolonged preparative electrophoresis. Characterization of this material⁵ indicated one residue of riboflavin phosphate (FMN) per molecular weight of 75 000 g. It was possible to dissociate the FMN from the apoenzyme by acidification in the presence of ammonium sulfate and to reform the OYE by recombining the FMN with OY apoenzyme^{3,5}. Since that time there have been surprisingly few studies of the metabolic importance of OYE. However, along with the recent upsurge of interest in metalloflavoproteins^{6,7} a careful re-appraisal of OYE has been instituted⁸⁻¹⁰.

EXPERIMENTAL

Isolation of crystalline OYE. The section to follow describes in detail the modified isolation procedure which led to a largely crystalline product which was approximately 85 % pure as shown by electrophoresis. This material was used in the potentiometric experiments. A complete analysis for metal ions and a re-evaluation of the extinction coefficients were not done. These matters will need to be cleared up in studies of pure OYE.

The procedure to be described involved the early steps of the Warburg and Christian procedure⁴ and the alumina adsorption step of Weygand and Hocker¹¹. Careful ammonium sulfate fractionation then led to a largely crystalline material.

Fresh bottom brewer's yeast from the Hamburger Bryggeriet, Stockholm, was washed several times by decantation with cold running tap water in a large sink. After washing, the yeast slurry was transferred to the cold room (5° C), chilled, and then centrifuged in an International Serum centrifuge for one hour at approximately 2 300 RPM. The thick gummy yeast was then air-dried after spreading on filter paper.

From 3 kg of fresh dried brewer's yeast, a Lebedev juice was prepared by adding 9 liters of distilled water at 37° C. Hand mixing was followed by slow mechanical stirring for 3 hours at 37° C. The container was then transferred to a refrigerated cooling bath at -15° and stirred rapidly until the temperature of the autolysate was 0 to -5°. The system was centrifuged for 1 ½ hours to yield 4 200 ml of a brown extract which was used immediately. The precipitation of the lead salts of proteins was accomplished by adding slowly with mechanical stirring at 0° 400 ml of lead subacetate solution (D.A.B. VI) per liter of Lebedev juice. Stirring was continued for one hour, and the preparation was usually stored overnight in the cold room. Centrifugation at 2 300 rpm separated lead proteinates. The excess lead was removed by adding at 0° with mechanical stirring 200 ml M/2 phosphate, pH 7, per liter of original Lebedev juice. After stirring for one hour the preparation was centrifuged to remove lead phosphates.

Acetone precipitations were carried out in the temperature range, 0 to -15° C. The supernatant liquid from above (volume, 5 300 ml) was cooled to 0°, and then one-half volume of cold acetone was added with mechanical stirring during one hour, and the temperature was allowed to fall to -15° as rapidly as possible. The rather copious light precipitate was allowed to settle for one hour, and then filtered by gravity at -15° to yield about 6 liters of a clear yellow filtrate. CO₂ was then bubbled in rapidly for one-half hour, following which 0.7 volume of acetone (referred to the lead phosphate supernate) were added slowly with mechanical stirring at -15°. After standing for one-half hour in the cold most of the yellow supernatant liquid could be decanted from the rather oily precipitated slurry of OYE. Centrifugation was followed by dissolving the crude OYE in about 500 ml cold distilled water. Considerable mixing of the oily, red-yellow material was required to effect solution. Centrifugation at low speed yielded a clear yellow solution.

Acetone precipitation was repeated *twice* more as follows: To the OYE solution at 0° 0.6 volume cold acetone was slowly added with careful mixing as the temperature was dropped rapidly to -15°. The OYE separates as a red-yellow oil, and the rather turbid supernatant liquid can be decanted away. The OYE was dissolved in 200 to 400 ml cold distilled water and then centrifuged to remove light insoluble material.

These steps were followed by an adsorption on alumina C-γ¹². To the OYE solution at 0° were added 1.5 volumes of alumina. After careful mixing and standing for about 30 minutes at 0°, the preparation was centrifuged. If the supernate was yellow in color, more alumina was added. About three or four additions of alumina were required. When all the OYE was adsorbed, the alumina was washed twice in the centrifuge at 0° with cold distilled water. Elution was accomplished at 0° with dilute NH₃. (NH₄)₂HPO₄ (33 ml concentrated NH₄OH + 10 g (NH₄)₂HPO₄, diluted to 500 ml). Three or four elutions with about 2 volumes of eluant per volume of alumina were required. Most of the pink color remained on the alumina. The OYE solution was dialyzed in the cold room for 12 hours against several changes of distilled water.

Ammonium sulfate fractionation of the above solution was carried out in several ways, one of which went as follows: The pH was adjusted to 5.5 with 10 % acetic acid, and the system was brought to 0.45 saturation with solid ammonium sulfate, added at 0°. After standing for a short time a small amount of precipitate was centrifuged out. The system was then brought to 0.5 saturation and re-centrifuged. If any yellow material was precipitated, it was saved and re-used. Further fractionation in the range, 0.5 to 0.65 saturation was carried out by "dialyzing in" the ammonium sulfate. The calculated amounts of ammonium sulfate were slurried in water into a cellophane tube which was then placed in the OYE solution and gently rocked to equilibrium at 0°. In this way preparations which were largely crystalline were obtained. One such preparation was about 85 % pure as shown by electrophoresis in phosphate buffer at pH 7.8.

In two preparations carried out as described yields of 517 mg and 576 mg OYE were obtained. In another preparation a lower yield resulted (281 mg), presumably because of the quality of the dried yeast.

The recovery of OYE and its purity can be followed during isolation by determining the optical density at 280 $m\mu$ and 465 $m\mu$. After one ammonium sulfate fractionation a given preparation showed an O.D. 280 $m\mu$ /O.D. 465 $m\mu$ ratio of 18.8, while an earlier preparation at the same stage showed a ratio of 35.7. The best OYE preparation obtained showed a ratio of 12.7 after five recrystallizations. Dry weight analysis suggested a purity of 96.8 % based on the extinction coefficient value⁸, $E_{465\text{ }m\mu} = 1.098 \times 10^4 \text{ cm}^2/\text{mole}$. However, on electrophoresis this material was approximately 85 % pure. The implication is that a re-evaluation of the extinction coefficient is needed.

The OYE was stored in 0.7 saturated ammonium sulfate at 0°. About 12 hours before use, portions were dialyzed against water or buffer at 0° and then diluted to desired concentrations based on the value of $E_{465\text{ }m\mu}$ given above and a molecular weight of 75 000.

POTENTIOMETRIC EXPERIMENTS

The only reported value for E'_0 of OYE is that of Kuhn and Boulanger¹³. In this report exact details of the purity of the OYE were not given, and the major interest was in the E'_0 values for a number of synthetic riboflavin analogs. Two reductive titrations of OYE with $\text{Na}_2\text{S}_2\text{O}_4$ were presented, and no indication of a sluggish behavior of OYE at smooth platinum electrodes was given. Hence, it seemed desirable to re-examine the potentiometric behavior of OYE, since a reliable E'_0 value is a requirement for calculations of the free energy change for interaction with other oxidation-reduction systems.

In kinetic studies of the reaction:

Theorell and Nygaard⁸⁻¹⁰ have evaluated the dissociation constant, K_0 , for this reaction under a variety of conditions.

$$K_0 = \frac{[\text{FMN}] [\text{Protein}]}{[\text{OYE}]}$$

An approximate figure for K_0 of 10^{-8} indicates a rather tight binding of FMN to the apoenzyme. As can be seen from the following equation¹⁴:

$$E'_{0(\text{OYE})} = E'_{0(\text{FMN})} + \frac{RT}{nF} \ln \frac{K_0}{K_r}$$

an indication of the tightness of binding of FMNH_2 by apoenzyme could be arrived at if the standard potentials of OYE and FMN were well established. It would be extremely difficult experimentally to measure the dissociation of FMNH_2 from OYEH_2 if the binding is such that K_r would be about 10^{-12} .

In these studies it was also desired to investigate the effect of chloride ion on a partially reduced OYE system¹⁰. If chloride ion acts to dissociate FMN and/or FMNH_2 , a striking effect on the potentiometric behavior should be noted.

For the potentiometric measurements a titration vessel similar to that used by Rodkey and Ball¹⁷ was constructed. It is shown in Fig. 1, in which drawing only one smooth platinum electrode is included. The jacket temperature of the cell was maintained at $30 \pm 0.05^\circ$ by means of a circulating pump

Fig. 1. Vessel for potentiometric titration of OYE.

and a water thermostat. A thermometer was placed in the line immediately adjacent to the water inlet of the cell jacket. Tank argon of high purity was passed through two bubble towers containing hypovanadous acid¹⁶ to remove any traces of O_2 and then through a water tower. Rubber connections were avoided as much as possible and — where used — were painted with General Electric Glyptal Red Enamel, No. 1201. The bubble towers were glass-sealed to each other, and copper tubing was soldered to the argon tank valve and the joint painted with Glyptal. A painted rubber connection joined one end of the gas train to the copper tubing and the other end to the titration cell gas inlet. In use the cell was gassed at a rate of 2 to 3 bubbles per second for about one hour before titration was begun. The contents were stirred continuously with a magnetic stirrer. The inlet tubes were placed in carefully bored holes in a Plexiglas stopper ground to fit a 24/40 standard taper joint. The holes were countersunk to a depth of about 5 mm from the top so that thin gum rubber tubing sleeves could be placed on the glass tubes. Effective gas seals were made in these wells with a drop of water or glycerol.

The glass electrode was a new one (Catalog No. 120 B) ordered from Radiometer, Copenhagen. It had a 3 mm stem and bulb diameter and was the seventh electrode tested before satisfactorily reproducible potentials were obtained. It is the opinion of the author that commercial glass electrodes more than a year or two old cannot be expected to perform properly. The use of the glass electrode eliminated liquid junction potentials from consideration but necessitated the use of an electronic potentiometer. The instrument used was the Electronic Potentiometer, Type PHM 3a made by Radiometer. It was found desirable not to use the pH scale and temperature compensator circuit, but rather to make all measurements at 30° with the millivolt scale of the instrument. In this way potential readings on standard buffers made with the glass electrode *versus* a saturated calomel electrode agreed within 0.001 V from day to day.

To check the performance of the glass electrode and to make possible accurate pH readings of titration systems, three standard reference solutions were prepared with the greatest care¹⁷: (1) 0.100 M HCl, pH 1.084, 30°; (2) 0.05 M

Fig. 2. Reference potentials. Solid lines are drawn with theoretical slopes of -0.06 V/pH unit. Open circles represent potentials measured with the glass electrode vs. a saturated calomel electrode. Solid circles: glass electrode vs. quinhydrone electrode.

potassium acid phthalate, pH 4.015, 30° ; 0.025 M KH_2PO_4 — 0.025 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, pH 6.848, 30° . When the glass electrode was checked against the saturated calomel electrode at these pH's and against the quinhydrone electrode at pH 4.70 and 6.85, its behavior was essentially perfect, in accordance with the expected $\Delta E_h/\Delta \text{pH}$ value of -0.06 V per pH unit at 30° . It is estimated that recorded pH values measured at 30° were accurate to ± 0.03 pH unit. The potential scale of the potentiometer allowed readings accurate to ± 0.0001 V.

The microburette was the Agla Micrometer Syringe, Burroughs-Welcome and Co., London, which had a capacity of 0.5 ml and could be read to ± 0.0005 ml. The microburette was fitted with a tip about 10 cm long, finely drawn out at the end. The drawing indicates that the end of the tip was placed below the surface of the liquid in the cell. The tip of the microburette was inserted through a moist gum rubber sleeve which formed a gas-tight seal under the 2 cm hydrostatic head maintained in the gas exist water trap.

The gas tight characteristics of the system were demonstrated on numerous occasions when partially reduced $\text{OYE} \cdot \text{H}_2$ systems were maintained for 3 to 4 hours at essentially constant potential. In pilot experiments leucomethylene blue was also successfully held at constant potential for several hours. On the admission of air to such systems the potential quickly shot up about 300 mV to values near the initial ones.

In Fig. 2 are presented the calibration data for the glass electrode, which carries the number 7 to indicate that its six predecessors had failed. The solid lines are theoretical lines drawn with a -0.06 slope, and the circles are experimental. The value for the saturated calomel electrode at 30° was taken to be $+0.242$ V. It is obvious that at pH 6 no corrections of the potentials shown by the glass electrode were required to convert them to E_h values.

REDUCTIVE TITRATIONS OF OYE WITH REDUCED SAFRANINE T

In preparing to carry out reductive titrations some thought was devoted to appropriate negative dyes as reducing agents. Through the kindness of Professor W. Mansfield Clark, The Johns Hopkins University, Baltimore, several valuable dyes were made available for this study. They included Safranine T, Safranine B, Induline Scarlet, Rosindone Sulfonic Acid $\neq 6$, Rosinduline GG, phthiocol and several others. From this group phthiocol (2-hydroxy-3-methyl-1,4-naphthoquinone; E'_0 , pH 7 and $30^\circ = -0.180$ V) and Safranine T (a phenazonium dye as shown in the formula; E'_0 , pH 7 and $30^\circ = -0.289$ V) were selected ¹⁷.

Safranine T

Several pilot titrations were done with reduced phthiocol, following which the results to be presented were obtained with reduced Safranine T. The dyes were reduced immediately before use in a small two-vessel apparatus so arranged that tank H_2 could be bubbled through the two vessels to out-gas the system thoroughly, following which a bit of platinized asbestos was quickly admitted to the first vessel. As soon as reduction was complete (several minutes), partial closing of a by-pass valve between the two reducing vessels forced the nearby colorless reduced dye through a sintered glass filter stick into the second vessel, where it was kept under H_2 flow. The tip of the Agla microburette could then be inserted and the syringe rinsed several times with reduced dye solution before being filled for use in titration. Reduced Safranine T solutions seemed to be nearly stable for about an hour, after which they began to develop a very noticeable yellow fluorescence which undoubtedly changed their reducing titer.

In early experiments to test the apparatus reductive titrations of $K_3Fe(CN)_6$ and methylene blue were performed with reduced phthiocol as reductant. It appeared necessary to "titrate out" residual traces of dissolved O_2 by adding small amounts of reduced dye and waiting for several minutes while the potentials fell rapidly about 200 mV or so. In the titrations of OYE, solutions of a final concentration of about 5×10^{-5} M were used. On the basis of a final volume of 7.0 ml and a molecular weight for OYE of 75 000, this amounted to about 20 mg per titration. Two things soon became apparent: 1) The OYE was electromotively sluggish at a smooth Pt electrode; and 2) reduced phthiocol appeared not to be "negative" enough to reduce the system completely. A few attempts to use de-aerated solutions of $Na_2S_2O_4$ as a reducing agent led to

Fig. 3. Titration of OYE with reduced Safranine T. Potassium indigodisulfonate as mediator. OYE = 3×10^{-5} M. Mediator = 1.5×10^{-6} M. 0.05 M Phosphate buffer, pH 6.98. Argon atmosphere. 30°. Circles are experimental points. Curve is theoretical for two-electron change.

nothing simulating either stable potentials or theoretical behavior. It is true that reduction could be accomplished (accompanied by bleaching of the yellow color), but a satisfactory titration curve could not be obtained.

Accordingly, the use of potassium indigo disulfonate (E'_0 at pH 7, 30° = -0.125 V) as a mediator at 1/20 of the molar concentration of OYE was tried, and reduced Safranine T was used as reductant. In Fig. 3 is shown an experiment which is quite typical of those titrations in which acceptably stable potentials were achieved (drifts less than 0.0001 V per minute). In every case the early potentials after gassing for about one hour were about 200 mV more positive than those reached rather quickly after the first small additions of reduced Safranine T. It was possible to detect the end of the "titrating out" of residual O_2 by noting the arrival of stable potentials and agreement between the two Pt electrodes.

The curve in Fig. 3 is a theoretical curve for a 2-electron change. It can be seen that the points were reasonably well behaved until about 50 % reduction was reached, at which point a very considerable flattening out was repeatedly encountered, even though the reduced Safranine T should be plenty "negative" to allow the completion of the titration. It soon became apparent that the E'_0 values were going to be lower by about 0.07 V. than those previously reported by Kuhn and Boulanger¹³ and that a distinctly nontheoretical behavior was being manifest. Analysis of the titration curves by the method of Reed and Berkson¹⁸ as modified by Clark and Perkins¹⁹, which involves the rearrangement of the equation for the potential into a linear slope — intercept form, revealed a profound deviation from ideal behavior.

A summary of data from eight acceptable titrations is presented in Table 1. The rather large deviations are indicative of the difficulty of establishing equilibrium between oxidized and reduced OYE at an inert electrode. The

Table 1. Old Yellow Enzyme. Calculation of E'_0 values at pH 7, 30°.

pH	E'_0 (V)	E'_0 at pH 7 (V)
4.80	+0.0168	-0.1152
6.00	-0.0747	-0.1347
6.22	-0.0856	-0.1324
6.75	-0.1033	-0.1183
6.98	-0.1227	-0.1239
7.00	-0.1206	-0.1206
8.15	-0.1919	-0.1229
8.90	-0.2335	-0.1195
Average		-0.1234 ± 0.0064 V.

average value, - 0.123 V is 0.064 V lower than that previously reported. The effect is to bring the OYE system somewhat closer to the pyridine nucleotides.

In Table 2 are presented several E'_0 values for tentative reference. It can be seen that the effect of binding FMN to its apoenzyme is quite comparable to that of binding DPN⁺ to yeast alcohol dehydrogenase, as calculated by Theorell and Bonnichsen²⁰.

Several reductive titrations were also carried out with riboflavin and FMN. Here, substantial agreement with Kuhn and Boulanger was noted. In Fig. 4 the E'_0 values obtained for riboflavin and FMN are shown, and the E'_0 -pH relationship for OYE is given. The open circles represent experiments in which potassium indigo disulfonate was used as a mediator. The solid circle is a single experiment in which no mediator was used. These results indicate clearly that no proton dissociation is involved in the oxidation-reduction of OYE in the pH range, 4.7 to 8.9. The "flattening out" of the titration curves was also encountered in titrations of riboflavin and FMN, but stable potentials were rapidly attained indicating that the "sluggishness" exhibited by OYE is probably due to steric influences of the large protein moiety.

It is not possible at this time to assess properly the significance of the failure of OYE, FMN and riboflavin to be completely reduced. It can be suggested that the "too positive" potentials were indicative of an essential instability of the reductants, making the term, $0.03 \log[\text{oxidant}] / [\text{reductant}]$, larger numerically than it should be. Clark and Perkins¹⁹ and Stiehler *et al.*²¹ noted an

Table 2. E'_0 values at pH 7, 30°.

O ₂	+0.81 V	
Cytochrome <i>c</i>	+0.262 V	
Cytochrome <i>b</i>	0	
OYE	-0.123 V	> 0.063 V
FMN	-0.186 V	
ADH-DPN ⁺	-0.240 V	> 0.078 V
DPN ⁺	-0.318 V	
H ₂	-0.420 V

Fig. 4. E'_0 -pH Curve for OYE. 30°. Acetate, phosphate, glycine or Veronal buffers: 0.05 M. Open circles with 1/20 molar equivalent of potassium indigodisulfonate. Solid circle without mediator. E'_0 for riboflavin (-0.185 V, pH 7) and for FMN (-0.190 V, pH 7).

apparent similar instability of the reduced forms of the phenazine dyes, neutral red and several of the safranines. It may be that the cell maintains its flavin materials in the oxidized state to avoid such inherent instability.

EFFECT OF CHLORIDE

Recently Theorell *et al.*¹⁰ have suggested an interesting possible effect of chloride ion with respect to the dissociation of certain coenzymes from their apoenzymes.

It appeared possible in these potentiometric studies to reduce an OYE system partially and then add O_2 -free saturated KCl with the microburette. When this was done at pH 4.77, an immediate effect was noted. The potentials became erratic, and disagreement between the Pt electrodes developed. Presently a turbidity was noticed accompanied by a rapid negative drift of about 0.06 V. After an hour the system contained a considerable quantity of precipitated protein, and the solution was a different shade of yellow-red, suggesting that FMN had been dissociated from its apoenzyme by the action of chloride ion. The final Cl^- concentration in this experiment was 0.39 M. At pH 9.35 a similar effect of Cl^- was observed. It seems clear that these experiments demonstrated a dissociating effect of Cl^- on OYE.

If E'_0 values for OYE and FMN of -0.123 V and -0.190 V are used and a value of 10^{-8} M is taken for K_0 , one arrives at a value for K_r of 6×10^{-11} M. This approximate value of course indicates an extremely tight binding of $FMNH_2$ to its apoenzyme, a situation which is probably duplicated by $FADH_2$ -apoenzyme complexes.

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