Yeast Glutathione Reductase

I. SPECTROPHOTOMETRIC AND KINETIC STUDIES OF ITS INTERACTION WITH REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE*

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SUMMARY

The interaction of the flavoprotein glutathione reductase from yeast with NADH has been studied. A reduced enzyme species resulting from addition of 2 electrons per enzyme-bound FAD is formed on addition of NADH to oxidized enzyme. This 2-electron reduced enzyme is stable toward further reduction to the 4-electron reduced state by excess NADH. Yeast glutathione reductase reduced by excess NADH has spectral properties distinct from those of yeast glutathione reductase reduced by excess NADPH, the physiological substrate, but virtually identical with the spectral properties reported by Massey and Williams (J. Biol. Chem., 240, 4470 (1965)) for yeast glutathione reductase reduced by a 526-fold molar excess of GSH. It is concluded that glutathione reductase reduced by a small molar excess of NADH is the free, 2-electron reduced enzyme with no oxidized or reduced pyridine nucleotide bound to it.

A bimolecular rate constant of 1.8 X 10^4 M^{-1} sec^{-1} was measured using stopped flow techniques for reduction of the enzyme-bound FAD by NADH. The rate of reduction of the enzyme-bound FAD by NADH is identical with the steady state rate of NADH-dependent GSSG reduction, suggesting that reduction of enzyme-bound FAD is rate limiting in the over-all reaction.

Yeast glutathione reductase (EC 1.6.4.2, NADPH:GSSG oxidoreductase) is a flavoprotein which catalyzes the NADPH-dependent reduction of oxidized glutathione. The enzyme appears to be a dimer with molecular weight of approximately 118,000 (1-3) and contains 1 FAD per monomer (2, 3).

Both a ping pong mechanism and an ordered sequential mechanism have been proposed for yeast glutathione reductase (2, 4). These two mechanisms differ basically in that the ping pong mechanism requires only binary enzyme-substrate complexes and is most simply rationalized in terms of a single substrate binding site at which one or the other substrate is bound, whereas the ordered sequential mechanism requires both substrates to bind to the enzyme simultaneously to give a ternary complex. Massey and Williams (2) have proposed a ping pong mechanism in which the oxidized enzyme, E, is reduced by NADPH to produce a species from which NADP^+ dissociates to give the free reduced enzyme species, F. F is then reoxidized by GSSG to regenerate E and produce GSH. This type of mechanism is indicated schematically in Equations 1 and 2.

\[ E + NADPH \rightleftharpoons E-NADPH \rightleftharpoons F-NADP^+ \rightleftharpoons F \] (1)
\[ F + GSSG \rightleftharpoons F-GSSG \rightleftharpoons E-(GSH)_2 \rightleftharpoons 2GSH + E \] (2)

basing their assumptions on product inhibition studies, have proposed an ordered sequential mechanism in which the complex F-NADP^+ between the reduced enzyme and NADP^+ is directly reoxidized by GSSG prior to dissociation of NADP^+. This type of mechanism is shown schematically in Equations 3, 4, and 5.

\[ E + NADPH \rightleftharpoons E-NADPH \rightleftharpoons F-NADP^+ \] (3)
\[ F-NADP^+ + GSSG \rightleftharpoons F-NADP^+ + 2GSH \] (4)
\[ E-NADP^+ \rightleftharpoons NADP^+ + E \] (5)

The difference between these two mechanisms, then, is whether NADP^+ remains bound to the 2-electron reduced enzyme species during reoxidation by GSSG. Massey has reported (5) that the over-all steady state velocity at 5° is in excellent agreement with that calculated for a ping pong mechanism based on directly measured values using stopped flow techniques for the rate of reduction of the enzyme-bound FAD by NADPH and the reoxidation of borohydride-reduced enzyme (which contains no bound pyridine nucleotide) by GSSG. This agreement suggests that the binary (ping pong) mechanism is adequate.

Massey and Williams (2) have also reported spectrophotometric studies of the enzyme bound FAD in the presence and absence of substrates. They have concluded that both the enzyme-bound FAD and a protein disulfide function as electron acceptors, requiring a total of 4 electrons to completely reduce both groups. Thus both a 2-electron and a 4-electron reduced

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enzyme can be produced. Their studies indicate that it is the 2-electron reduced enzyme that is the catalytic intermediate.

NADH is also a substrate, albeit a poor one, for yeast glutathione reductase (4, 6). No studies of the effect of NADH on the enzyme-bound FAD of glutathione reductase have been reported. A critical experiment to distinguish between the ping pong and ordered sequential mechanisms is to produce the 2-electron reduced enzyme in the absence of NADP+ and study the effect of added NADP+ on the kinetics of reoxidation of the 2-electron reduced enzyme by GSSG. The Michaelis constant for NADH with glutathione reductase is large in those cases in which it has been measured (7, 8). With the idea that the large Michaelis constant for NADH might be reflected in a low affinity of E and F for NADH and NAD+, the use of NADH to produce the free, 2-electron reduced enzyme in the absence of NADP+ and NADPH was investigated. In this paper we report static spectral and steady state and stopped flow kinetic studies of the interaction of yeast glutathione reductase with NADH. In the accompanying paper (9) are reported studies of the interaction of the NADH-reduced enzyme with NADP+ and NADPH, undertaken to characterize these interactions in the absence of GSSG preliminary to studies of the kinetics of reoxidation of the various species by GSSG.

**EXPERIMENTAL PROCEDURE**

**Enzymes—**Yeast glutathione reductase was obtained from Sigma Chemical Company as a purified suspension in ammonium sulfate. Enzyme from two different lots, 1078-7441 and 0022-7481, was used. The enzyme was further purified approximately 1.5-fold by chromatography on Bio-Rad hydroxylapatite. Final specific activities obtained from the two lots were 283 to 331 units per mg of protein and 237 to 272 units per mg of protein, respectively. The specific activity of our purified enzyme compares well with the values of 295 units per mg (2) and 200 units per mg (3) reported for other highly purified, homogeneous preparations. Disc gel electrophoresis at pH 8.9, according to the method of Davis (10), of a representative enzyme preparation used in these experiments showed it to be greater than 99% homogeneous. A small impurity, estimated visually to be less than 1% of the total protein, was visible when 120 μg of enzyme was applied to the gel. The enzyme was stored in the presence of 0.5 mM EDTA in 0.01 M sodium phosphate buffer, pH 7.0, at 4°. *Neurospora crassa* NAD+ glycohydrolase (EC 5.2.2.5) was obtained from Worthington Biochemical Corporation.

**Reagents—**All inorganic chemicals were of reagent grade. NADPH and NADH were obtained from either Calbiochem or Sigma Chemical Company and were further purified by DEAE-cellulose column chromatography by a modification of the procedure of Silverstein (11). In this work dibasic sodium phosphate was used as the eluent. The ratio of absorbance at 260 nm to that at 340 nm for the reduced pyridine nucleotides purified in this manner ranged from 2.36 to 2.46.

**Enzyme Assays—**Standard enzyme assays for glutathione reductase were performed at pH 7.6 under the conditions of Massey and Williams (2) except that no bovine serum albumin was present. Protein concentrations were determined at 280 nm using an E_280^1% of 18.6 (2). An enzyme unit was defined as that amount of protein catalyzing the oxidation of 1 μmole of NADPH per min under standard assay conditions.

**Stopped Flow Kinetic Experiments—**Stopped flow experiments were performed with a Durrum-Gibson stopped flow spectrophotometer with a 2-cm light path and equipped with a pneumatic drive attachment. The temperature was controlled with a Precision Scientific Lo-Temprol constant temperature unit. Changes in transmittance of light by the reaction mixture were monitored using a Techronix type 564 storage oscilloscope with a type 2A63 differential amplifier and a type 2B67 time base. The traces were recorded permanently with a Polaroid camera and kinetic parameters evaluated from the photographs.

The rate of reduction of the enzyme-bound FAD was followed at 462 nm at 25 ± 0.5°, using a slit width of 0.05 mm. One of the drive syringes contained NADH at the desired concentration in 0.06 M sodium phosphate buffer, pH 7.6, containing 3.0 mM EDTA. The other drive syringe contained enzyme (approximately 4 μM enzyme-bound FAD) in the same concentration of buffer and EDTA. These experiments were performed under aerobic conditions. It was demonstrated that reoxidation by dissolved oxygen of the reduced enzyme species produced was very slow relative to the rate of reduction. An apparent first order rate constant, k_obs was evaluated from photographs of the oscilloscope traces by converting the per cent transmittance at selected times to absorbance and plotting log (per cent unreacted) against time. From the slope of such a plot k_obs was evaluated according to the equation:

\[ \frac{d[A]}{dt} = -k_{obs} [A] \]

where A is the absorbance or absorbance change by the molar concentration of enzyme-bound FAD. The concentration of enzyme-bound FAD was determined at 402 nm using a molar absorbivity of 1.13 x 10^4 M^-1 cm^-1 (2).

For the anaerobic titrations, enzyme solutions were made anaerobic in Thunberg cuvettes as described above for anaerobic spectra. An anaerobic solution of NADH or NADPH of known concentration was then added in small aliquots through a rubber septum using a 50-μl Hamilton syringe. After each aliquot was added the enzyme solution was mixed and the per cent transmittance at 462 nm was measured using a Beckman model SRLG recorder operated in the transmittance mode. The scale on the recorder was expanded so that 50 to 100% T was full scale. The per cent T was read to the nearest 0.01% T and converted to absorbance.

For ease of comparison between several experiments in which different enzyme concentrations were used, all absorbance measurements are converted to molar absorbivities by dividing the observed absorbance or absorbance change by the molar concentration of enzyme-bound FAD. The concentration of enzyme-bound FAD was determined at 402 nm using a molar absorbivity of 1.13 x 10^4 M^-1 cm^-1 (2).

For the anaerobic titrations, enzyme solutions were made anaerobic in Thunberg cuvettes as described above for anaerobic spectra.
0.05 ml of a suitably diluted enzyme stock solution to 0.95 ml of
spectra are not corrected for excess NADH. 1, oxidized enzyme;
2, enzyme reduced anaerobically by a 1.2-fold molar excess of
yeast glutathione reductase by
DB-G spectrophotometer equipped with a Sargent model SRLG
method of least squares. Stopped flow (kss) data, 0; steady state
rate-limiting step in the NADH-GSSG reaction of yeast gluta-
reductase in the oxidized state (Curve 1) and when reduced
anaerobically by NADH and NADPH. Curve 3 is obtained on
an aerobic addition of a 29-fold molar excess of NADH per en-
zyme-bound FAD. It can be seen that there is an increase in
absorbance at wave lengths greater than 505 nm and partial
bleaching at wave lengths between 450 and 505 nm. Curve 2
is the spectrum obtained 10 min after anaerobic addition of a
29-fold molar excess of NADH per enzyme-bound FAD in the
presence of N. crassa NAD+ glycohydrolase approxi-
ately 10 min after the initial reduction; 3, enzyme reduced anaer-
obically by a 29-fold molar excess of NADPH; 4, enzyme reduced
anaerobically by a 34-fold molar excess of NADPH. The amount
of NAD+ glycohydrolase in the experiment of Curve 2 is calculated
as described in the text.

RESULTS

In order to be able to study the spectral and kinetic properties
of the 2-electron reduced enzyme in the absence of NADPH and
NADP+, it was necessary to find a method of producing this
species without using NADPH as the reductant. Although the
specificity of glutathione reductase with respect to pyridine
nucleotides strongly favors NADPH over NADH, NADH can
function as a poor substrate (4, 6, 7, 8). If this specificity for
NADPH over NADH also manifests itself in the relative affinity
of the enzyme for binding NADH or NAD+, it would be possible
to produce the free reduced enzyme by reduction with a small
molar excess of NADH.

With yeast glutathione reductase at pH 7.6 and 25° the ob-
served turnover number at 100 μM NADH and 3.25 mM GSSG
is only 2.0 sec⁻¹, less than 1% that observed with 100 μM NADPH
under identical conditions. Fig. 1 shows the dependence of the
initial steady state turnover number (0) at 3.25 mM GSSG on the
NADH concentration out to 161 μM NADH. The turnover
number appears to be directly proportional to NADH concen-
tration in this range. The apparent direct proportionality be-
tween the steady state turnover number and NADH concentra-
tion can be described by the relationship

\[ TN = k_{ss} \text{ (NADH)} \]

where \( k_{ss} \) is an apparent bimolecular rate constant evaluated
from the steady state data. Evaluation of \( k_{ss} \) from the slope of
the line drawn in Fig. 1 gives a value of 2.0 × 10⁴ M⁻¹ sec⁻¹.
The steady state velocity of the reduction of GSSG by NADH
is also relatively insensitive to GSSG concentration. Thus with
110 μM NADH, the steady state turnover numbers obtained
with 0.02 mM GSSG and 2.5 mM GSSG are 1.8 sec⁻¹ and 2.0
sec⁻¹, respectively. This behavior strongly suggests that the rate-
limiting step in the NADH-GSSG reaction of yeast glutathione
reductase involves interaction of the enzyme with NADH, presum-
ably a reduction.

Fig. 2 shows the visible absorption spectra of glutathione
reductase in the oxidized state (Curve 1) and when reduced
anaerobically by NADH and NADPH. Curve 3 is obtained on
an aerobic addition of a 29-fold molar excess of NADH per en-
zyme-bound FAD. It can be seen that there is an increase in
absorbance at wave lengths greater than 505 nm and partial
bleaching at wave lengths between 450 and 505 nm. Curve 2
is the spectrum obtained 10 min after anaerobic addition of a
1.2-fold molar excess of NADH per enzyme-bound FAD in the
presence of N. crassa NAD+ glycohydrolase. A molar absorptivity of 6.22 × 10³ m⁻¹ cm⁻¹ at 340
nm was used for NADH and NADPH (15). Turnover numbers were calculated by dividing the observed velocity by the concentra-
tion of enzyme-bound FAD.
(\(V_{\text{max}}/K_m\) (NAD\(^+\)). \(V_{\text{max}}/K_m\) is the apparent first order rate constant for hydrolysis of NAD\(^+\) and is equal to 120 \(\mu M\) per min divided by 500 \(\mu M\) – 0.24 min\(^{-1}\). This corresponds to a half-time of 2.9 min, so that during the 10 min following addition of the NADH, more than 88% of the NAD\(^+\) produced on reduction should be hydrolyzed.

It can be seen that Curves 2 and 3 are virtually identical, the small differences between them probably being due to experimental error or possibly incomplete reduction in the case of Curves 2. Curves 2 and 3 in Fig. 2 are quantitatively identical between 425 and 650 nm, within experimental error, to the spectrum (Fig. 5 in Reference 2) reported by Massey and Williams for yeast glutathione reductase reduced anaerobically by a 526-fold molar excess of NADPH. Curve 4 in Fig. 2 is the spectrum obtained on anaerobic reduction of the enzyme-bound FAD of glutathione reductase by a 34-fold molar excess of NADPH. It is clear that this spectrum of the NADPH-reduced enzyme is different from that of the NADH-reduced enzyme. Curve 4 is virtually identical at wave lengths greater than 500 nm to the spectrum (Fig. 11 in Reference 2) reported by Massey and Williams for anaerobic reduction of yeast glutathione reductase by an 11.9-fold molar excess of NADPH; between 400 and 500 nm Curve 4 exhibits lower absorption at all wave lengths than that reported by Massey and Williams. The difference between the NADH-reduced and NADPH-reduced enzyme species is considered in detail in an accompanying paper (9).

The stoichiometry of the interaction between NADH and glutathione reductase was also studied. Fig. 3A shows the results of an anaerobic titration of glutathione reductase with NADH in which the spectral changes at 462 nm and 530 nm were followed. It can be seen that the spectral species characterized by the long wave length absorption at 530 nm and partial bleaching at 462 nm is produced on addition of one NADH, i.e., 2 electrons, to each enzyme-bound FAD. It therefore corresponds to the stoichiometry reported by Massey and Williams (2) for the species with \(\epsilon_{500} \sim 0.36 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}\) produced by NADPH reduction of glutathione reductase. In Fig. 3B are plotted the results obtained on anaerobic titration of glutathione reductase with NADH as followed at 356, 358, and 360 nm. The observation that the absorbivity of the enzyme decreases slowly out to about 1 mole of NADH per mole of enzyme-bound FAD at 360 nm, increases slowly at 356 and 358 nm, and remains constant at 358 nm demonstrates that 358 nm is an isosbestic point between the oxidized and the 2-electron reduced enzyme. Beyond 1 mole of NADH per mole enzyme-bound FAD the absorbivity increases at each wave length in direct proportion to added NADH due to the accumulation of unreacted NADH after formation of the 2-electron reduced enzyme is complete.

The kinetics of reduction of the enzyme-bound FAD of glutathione reductase by NADH in the absence of GSSG were also determined using stopped flow techniques. The reaction was conveniently followed by observing the decrease in absorbance at 462 nm on mixing NADH with glutathione reductase. Experimental conditions were chosen such that the concentration of enzyme-bound FAD was much less than the total NADH concentration. This results in pseudo-first order conditions, simplifying analysis of the kinetic data, since it can be assumed that the NADH concentration remains constant throughout the reduction process. A tracing of a photograph of a typical oscilloscope trace showing the increase in percentage of transmittance at 462 nm with time on mixing NADH with enzyme is shown in Fig. 4A, and a plot of log (per cent unreacted) against time for the data taken from the photograph is shown in Fig. 4B. The total absorbance change for the reaction observed in the photograph (\(\Delta A_{\text{kinetic}} = 0.0089\)) corresponds well with that calculated from the spectra in Fig. 2 for a 2 cm light path and 1.7 \(\mu M\) enzyme-bound FAD (\(\Delta A_{\text{spectra}} = 0.0095\)). It is apparent that the reaction follows first order kinetics for at least 90% of the total reaction. For the experiment shown in Fig. 4A, \(k_{\text{obs}} = 0.99 \text{ sec}^{-1}\). The values of \(k_{\text{obs}}\) obtained at various concentrations of NADH are plotted against NADH concentration in Fig. 1 (solid). The values of \(k_{\text{obs}}\) are directly proportional to NADH concentration out to 148 \(\mu M\). Not only was similar behavior observed with the steady state rate data for the NADH-GSSG activity of glutathione reductase, but as seen in Fig. 1 the slopes of the two lines are very similar. The stopped flow data fit the equation \(k_{\text{obs}} = k_{\text{tr}} (\text{NADH})\), where \(k_{\text{tr}}\) is the apparent bimolecular rate constant for reduction obtained from the stopped flow data. Evaluation of \(k_{\text{tr}}\) from the slope of the line in Fig. 1 gives a value of \(k_{\text{tr}} = 1.8 \times 10^4 \text{ M}^{-1} \text{sec}^{-1}\).

Observation of the reduction process at 358 nm, an isosbestic point between oxidized and 2-electron reduced enzyme, on mixing NADH with glutathione reductase in the stopped flow apparatus gave identical values of \(k_{\text{obs}}\) to those obtained at 462 nm. At 358 nm the only change in absorbance should be due to oxidation of NADH to NAD\(^+\). The number of moles of NADH oxidized
yeast glutathione reductase by NADH. This trace was obtained from the oscilloscope trace. A molar final concentrations after mixing of 1.7 M phosphate buffer, pH 7.6, containing 3.0 mM EDTA at 25°C to give the order rate constant for this experiment, calculated as described. An 800-mv signal corresponds to 100% T. An RC noise filter with a time constant of 3 msec was used. The horizontal trace results from a second triggering of the oscilloscope to establish the t = ∞ transmittance. B, plot of the data taken from the trace in Fig. 4. A, tracing of a photograph of a typical stopped flow oscilloscope trace for the reduction of the enzyme-bound FAD of yeast glutathione reductase by NADH. This trace was obtained on mixing aerobic solutions of enzyme and NADH in 0.06 M sodium phosphate buffer, pH 7.6, containing 3.0 mM EDTA at 25°C to give final concentrations after mixing of 1.7 μM enzyme-bound FAD and 50 μM NADH. Changes in transmittance at 462 nm were followed. An 800-mv signal corresponds to 100% T. An RC noise filter with a time constant of 3 msec was used. The horizontal trace results from a second triggering of the oscilloscope to establish the t = ∞ transmittance. B, plot of the data taken from the trace in Fig. 4. A as log (% Unreacted) versus time. The observed, pseudo-first order rate constant for this experiment, calculated as described under "Experimental Procedure," is 0.99 sec⁻¹.

per mole of enzyme-bound FAD during the reduction process can be calculated from the difference between the initial and final absorbance evaluated from the oscilloscope trace. A molar absorptivity of 4.5 × 10⁴ M⁻¹ cm⁻¹ at 359 nm for NADH was calculated from spectra of the NADH used in these experiments, assuming a molar absorptivity of 6.22 × 10⁴ M⁻¹ cm⁻¹ at 340 nm (15). The absorbance change in the oscilloscope trace was 0.0136 for a 2-cm light path. This corresponds to the oxidation of 1.5 μM NADH on interaction with 1.7 μM enzyme-bound FAD, indicating that 0.88 moles of NADH are oxidized per mole of enzyme-bound FAD during the reaction. Thus the kinetically determined stoichiometry agrees with the statically determined stoichiometry shown in Fig. 3A, confirming the 2-electron nature of the process.

As mentioned in the Experimental Procedure section, these stopped flow kinetic experiments were done aerobically. It was established that the rate of reoxidation of the NADH-reduced enzyme by dissolved oxygen was much slower than the reduction process, thereby causing no interference. When glutathione reductase (5.0 μM enzyme-bound FAD) is reduced by 1.0 μM NADH in an air-saturated solution, the initial relatively rapid reduction is followed by a very slow reoxidation as evidenced by a gradual increase in absorbance at 462 nm and corresponding decrease at 530 nm. The rate is too slow to measure accurately, but an upper limit for the pseudo-first order rate constant for the reduction of these conditions can be set at 0.0004 sec⁻¹. This corresponds to a half-life of 29 min for the 2-electron reduced enzyme under conditions of air saturation. The lowest kobs measured for enzyme-bound FAD reduction by NADH was 0.64 sec⁻¹. Thus, the rate of aerobic reoxidation of the NADH-reduced enzyme is much too slow to have any effect on determination of the rate of enzyme-bound FAD reduction by NADH.

DISCUSSION

The data presented in this paper demonstrate that NADH is capable of functioning, albeit poorly in comparison with NADPH, as a substrate for yeast glutathione reductase. Four observations are especially relevant. First, the enzyme-bound FAD of glutathione reductase is reduced by NADH to produce the species with spectral properties virtually indistinguishable from those reported by Massey and Williams (2) for glutathione reductase reduced by a large excess of GSH, the normal substrate for the reverse reaction. That the spectral changes observed result from reduction of the enzyme-bound FAD is postulated on the basis of kinetic studies at 358 nm of the reaction of NADH with glutathione reductase. As described above, the kinetically observed spectral change at this isosbestic point between free oxidized enzyme and NADH-reduced enzyme corresponds to that expected for oxidation of 0.88 mole of NADH per mole of enzyme-bound FAD. This near integral value is most simply interpreted to represent actual oxidation of NADH, and hence, reduction of the enzyme-bound FAD, although it cannot be rigorously excluded that NADH simply binds in such a way that environmental effects drastically alter its spectral properties. For the purposes of this discussion, the process will be hereafter considered to involve reduction.

The second observation is the demonstration of the 2-electron stoichiometry of the reduction, as shown in Fig. 3 by the requirement for 1.0 to 1.2 moles of NADH per mole of enzyme-bound FAD whether the reduction is observed at 358, 462, or 530 nm. This is identical with the stoichiometry reported by Massey and Williams (2) for reduction of yeast glutathione reductase by NADPH, the physiological substrate. The identical stoichiometry and qualitative similarity in shape of the spectra of the NADH- and NADPH-reduced enzymes suggests that somewhat similar processes occur on interaction with NADH and NADPH, despite the low kinetic activity of the former.

Third, the stopped flow kinetic data on the reduction of the enzyme-bound FAD of glutathione reductase by NADH exhibit simple first order kinetics and an absorbance change at 462 nm which agrees with that predicted from the statically determined spectra in Fig. 2. It is noteworthy that the largest kobs measured at 25°C for reduction of the enzyme by NADH, 2.7 sec⁻¹, is much smaller than the rate of enzyme-bound FAD reduction by NADPH as reported by Massey. Massey et al. (17) report that formation of the 2-electron intermediate "is extremely fast, and not rate-limiting," indicating that the rate constant for reduction by NADPH must be at least 250 sec⁻¹, the maximum turnover number for the NADPH-GSSG activity at 25°C. Massey has also reported (5) that the limiting rate constant for reduction by NADPH at 5°C is 83 sec⁻¹. It is evident that the extra phosphate attached to NADPH has a pronounced effect on its interaction with oxidized enzyme and the kinetics of reduction relative to NADH.
Finally, the agreement between $k_{a1}$ for reduction of the enzyme-bound FAD of glutathione reductase by NADH and $k_{a2}$ evaluated from the steady state NADH-GSSG activity of the enzyme argues strongly that the steady state NADH-GSSG activity of the enzyme directly involves the enzyme-bound FAD and the 2-electron reduced enzyme. The agreement between the stopped flow data and the steady state data in Fig. 1 can be explained by postulating that the rate-limiting step in the NADH-GSSG activity of yeast glutathione reductase is the reduction of the enzyme-bound FAD by NADH. If this is the case, i.e. reoxidation of the 2-electron reduced enzyme by GSSG is much faster than production of that species by NADH, the apparent insensitivity of the steady state turnover number to GSSG concentration can also be explained. The enzyme can turn over in the steady state no faster than the 2-electron reduced enzyme species can be produced, and this is limited by the kinetics of reduction by NADH. Consequently, increasing the rate at which the 2-electron reduced enzyme is reoxidized by increasing the GSSG concentration can have no effect on the over-all rate.

The observation that both the steady state turnover number and the pseudo-first order rate constant for enzyme-bound FAD reduction by NADH are directly proportional to NADH concentration in the range studied and do not show a saturation effect requires some comment. For steady state data this behavior will in general be observed if the concentrations of the varied substrate used lie below the apparent Michaelis constant for that substrate. Reported Michaelis constants for NADH with glutathione reductase range from 193 $\mu$M for the human erythrocyte enzyme (7) to 300 $\mu$M for the enzyme from P. chrysogenum (8). For deviation of less than 10% from direct proportionality at 161 $\mu$M NADH a Michaelis constant of 1.4 mA would be required, considerably greater than 300 $\mu$M. It must be concluded that these enzyme preparations have a sufficiently large $K_m$ that at the highest NADH concentration used the deviation from direct proportionality is not detected.

The stopped flow data on enzyme-bound FAD reduction by NADH are relevant in explaining the apparent large $K_m$ for NADH in terms of individual rate constants. Direct proportionality of $k_{a2}$ for enzyme-bound FAD reduction to concentration of reducing agent has previously been observed by Gibson, Swoboda, and Massey (18) for the reduction of the enzyme-bound FAD of Aspergillus niger glucose oxidase by D-glucose, D-mannose, D-galactose, and D-xylene. Indeed, with the latter three substrates, the turnover number is also directly proportional to sugar concentration in the range studied and numerically equal to the rate constant for enzyme-bound FAD reduction. If the reaction for glutathione reductase is assumed to involve formation of an oxidized enzyme-NADH complex followed by electron transfer, and if the reaction is assumed to go virtually to completion, it can be represented in its simplest form by Equation 6, where $E$ and $F$ represent the oxidized and 2-electron reduced enzyme, respectively. As pointed out by Gibson et al. (18), the pseudo-first order rate constant for formation of $F$ will be directly proportional to the NADH concentration if $k_3 \gg k_{a1}, k_2$ (NADH). In effect, then, at the NADH concentrations used the rate limiting step in enzyme-bound FAD reduction and, hence, steady state NADH-GSSG activity, is the bimolecular combination of $E$ with NADH to give a productive complex. Although $k_{a1}$ obtained from the stopped flow data in Fig. 1 ($k_{a1} = k_1 - 1.8 \times 10^4$ $M^{-1} sec^{-1}$) is small compared with many bimolecular rate constants for interaction of small molecules with proteins (19), it is of similar magnitude to that reported (18) for reduction of glucose oxidase by D-glucose. Since $k_1$ is in the denominator of the expression for the Michaelis constant for NADH regardless of whether the ping pong mechanism postulated by Massey and Williams (2) or the ordered sequential mechanism postulated by Staal and Veeger (4) is correct, a small value for $k_1$ as is found here will result in a large $K_m$.

Massey and Williams (2) have reported that addition of 11.9 moles of NADPH per mole of enzyme-bound FAD causes complete (4-electron) reduction of yeast glutathione reductase only in the presence of $N. crassa$ NAD$^+$ glycohydrolase. The criteria for 4-electron reduction are the absence of 330 nm absorption and a change in absorptivity at 462 nm ($\Delta\varepsilon_{462}$ of $1.03 \times 10^4$ $M^{-1} cm^{-1}$). (In the absence of NAD$^+$ glycohydrolase the $\Delta\varepsilon_{462}$ observed in this work on addition of a 34-fold molar excess of NADPH is 0.38 $\times 10^4$ $M^{-1} cm^{-1}$ (Fig. 2).) Since NAD$^+$ glycohydrolase hydrolyzes NADP$^+$ but not NADPH (16), Massey and coworkers (2, 20) have suggested that NADP$^+$ binds to the 2-electron reduced enzyme and protects it against further reduction by the excess NADPH. As they have pointed out (20), a very tight complex between NADP$^+$ and the 2-electron reduced enzyme would be required to account for the slow rate of production of the 4-electron reduced enzyme in the presence of excess NADPH and NAD$^+$ glycohydrolase. This is especially so since under the anaerobic conditions of the reduction the concentration of NADP$^+$ produced on formation of the 2-electron reduced enzyme can be no greater than the enzyme-bound FAD concentration (about 5 $\mu$M in Fig. 2).

In Fig. 2 it is seen that a 29-fold molar excess of NADH, as with NADPH in the absence of NAD$^+$ glycohydrolase, also does not produce complete (4-electron) reduction, despite the fact that no NADP$^+$ is present. Only the 2-electron reduced enzyme is formed, as evidenced by the 530 nm absorption and $\Delta\varepsilon_{530} = 0.26 \times 10^4$ $M^{-1} cm^{-1}$. The difference between the values of $\Delta\varepsilon_{462}$ for the NADH- and NADPH-reduced enzymes is considered in detail in the accompanying paper (9). However, the addition of a 29-fold molar excess of NADH causes no further spectral change, within experimental error, from that observed on addition of a 1.2-fold molar excess in the presence of NADP$^+$ glycohydrolase. It is conceivable that NADP$^+$ is functioning in a manner similar to that postulated (2, 20) by Massey and coworkers for NADP$^+$, but again, since the NAD$^+$ concentration can be no greater than the concentration of enzyme reduced, a very high affinity of the 2-electron reduced enzyme for NAD$^+$ would be required. If the large difference in $K_m$ between NADPH and NADH is reflected in the ability of NADP$^+$ and NAD$^+$ to bind to the 2-electron reduced enzyme, it seems unlikely that significant protection would be afforded by these concentrations of NAD$^+$ even if NADP$^+$ does function in this manner.

An alternative explanation which has been proposed by Sandri (21) is an inherent resistance of the 2-electron reduced enzyme to further reduction, i.e. a thermodynamic barrier. For example, if the reduction potential for the conversion of the 2-electron to the 4-electron species were lower than that of NADH or NADPH, significant reduction to the 4-electron state would be observed only if the product, NAD$^+$ or NADP$^+$, were removed...
from the reaction mixture, for example, by hydrolysis with NAD$^+$ glycolyldrase, thereby pulling the reaction to completion. In this case the slow rate of formation of the 4-electron reduced enzyme by excess NADPH in the presence of NAD$^+$ glycolyldrase reported by Massey and coworkers (2, 20) could be due to either an inherently slow reduction process or to tight binding of NADP$^+$ to the 4-electron reduced enzyme.

It has been pointed out above that the spectra in Fig. 2 of the NADH-reduced enzyme are identical within experimental error to the spectrum reported by Massey and Williams (2) for yeast glutathione reductase reduced by a 526-fold molar excess of GSH. The GSH-reduced enzyme clearly can have no oxidized or reduced pyridine nucleotide bound to it. The quantitative agreement between the spectra of the NADH- and the GSH-reduced enzyme is, then, most simply explained by concluding that the NADH-reduced enzyme also has no pyridine nucleotide bound to it. However, the spectral data do not rule out the existence of a complex of the NADH-reduced enzyme with either NADH or NADP$^+$ if the presence of these pyridine nucleotides bound to the 2-electron reduced enzyme does not significantly alter the spectral characteristics of the enzyme. All that can be unequivocally concluded is that if NADP$^+$ or NADH does bind, it does not affect the spectral properties of the 2-electron reduced enzyme.

Some evidence exists$^3$ which could be interpreted in terms of NADH binding to the 2-electron reduced enzyme. As described above, the NADH-reduced enzyme can be reoxidized slowly by dissolved oxygen. This suggests that the enzyme should catalyze the oxidation of NADH by oxygen. At 10 $\mu$M NADH and 0.94 $\mu$M enzyme-bound FAD the turnover number of NADH oxidation measured at 340 nm is 0.0005 sec$^{-1}$ while $k_{obs}$ for enzyme-bound FAD reduction is 0.17 sec$^{-1}$. Despite the fact that enzyme-bound FAD reduction is already much faster than the steady state NADH oxidation by oxygen, increasing the NADH concentration above 10 $\mu$M increases the rate of this steady state NADH-O$_2$ activity. Thus, at 177 $\mu$M NADH, the turnover number for the steady state NADH-O$_2$ activity is 0.0020 sec$^{-1}$. This increase in the rate of the NADH-O$_2$ activity can be accounted for in two possible ways. It could be due to a minor impurity in these glutathione reductase preparations which can also catalyze the oxidation of NADH by dissolved oxygen and which has a relatively high $K_m$ for NADH. If that is the case then the observed increase in NADH-O$_2$ activity at higher NADH concentrations is irrelevant. Alternatively it could be explained by postulating that NADH can bind to the 2-electron reduced enzyme and enhance its reactivity toward oxygen. Since high concentrations of NADH are required to produce this enhancement, the dissociation constant for the postulated complex between NADH and the 2-electron reduced enzyme must be large. A value of 60 $\mu$M has been estimated.$^3$ If the interpretation suggested here is correct, and if this NADH-O$_2$ activity is a property of glutathione reductase and not due to an impurity, Curve 3 in Fig. 2 represents a mixture of 2-electron reduced enzyme and its complex with NADH, since the NADH concentration is 146 $\mu$M. Since Curves 2 and 3 are the same, within experimental error, it must be concluded that if the above interpretation is correct and Curve 3 does represent the 2-electron reduced enzyme partially complexed with NADH, the binding of NADH does not significantly affect the spectral properties of the enzyme. Regardless, a dissociation constant of 60 $\mu$M permits production of the 2-electron reduced enzyme with a sufficiently small molar excess of NADH to assure a negligible association of the 2-electron reduced enzyme with excess NADH.

Concerning a possible complex of the NADH-reduced enzyme with NADP$^+$, there is little relevant data. Staal and Veeger have concluded (4) from their data that the oxidized and 2-electron reduced enzymes have little affinity for NADP$^+$ with the human erythrocyte enzyme. The latter conclusion is based on the lack of effect of 1 mM NADP$^+$ on the NADH-GSSG reaction. In the absence of data to the contrary, the NADH-reduced enzyme produced by a small molar excess of NADH (and therefore containing 5 $\mu$M or less NADP$^+$) will be considered to be the free, 2-electron reduced enzyme with no pyridine nucleotide bound to it. It will be denoted $F$.

It is concluded, then, from the data presented in this paper, that the enzyme-bound FAD of yeast glutathione reductase can be reduced by NADH to produce a stable, 2-electron reduced enzyme in the absence of NADP$^+$. The production of this intermediate in the absence of NADP$^+$ was desired for the reasons outlined in the introductory section. This 2-electron reduced enzyme is spectroscopically identical with that reported by Massey and Williams (2) to be formed on reduction by excess GSH and spectroscopically different from the species produced on reduction by excess NADPH. This 2-electron reduced enzyme is also stable to further reduction by large excesses of NADH. The rate of reduction of the enzyme-bound FAD of yeast glutathione reductase by NADH in the absence of GSSG to produce this 2-electron reduced species has been measured using stopped flow techniques. The rate is directly proportional to NADH concentration out to 148 $\mu$M and identical with the rate of the steady state NADH-GSSG reaction. It is concluded that the rate-limiting step in the steady state NADH-GSSG reaction is reduction of the enzyme-bound FAD by NADH. The bimolecular rate constant measured for the reduction is $1.8 \times 10^4$ M$^{-1}$ sec$^{-1}$.

REFERENCES


$^3$ J. E. Bulger and K. G. Brandt, unpublished results.


Yeast Glutathione Reductase: I. SPECTROPHOTOMETRIC AND KINETIC STUDIES OF ITS INTERACTION WITH REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE
James E. Bulger and Karl G. Brandt


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