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YEAST GLUTATHIONE REDUCTASE

STUDIES OF THE KINETICS AND STABILITY OF THE ENZYME AS A FUNCTION OF pH AND SALT CONCENTRATION

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Summary

1. The pH dependencies of the apparent Michaelis constant for oxidized glutathione and the apparent turnover number of yeast glutathione reductase (EC 1.6.4.2) have been determined at a fixed concentration of 0.1 mM NADPH in the range pH 4.5—8.0. Between pH 5.5 and 7.6, both of these parameters are relatively constant. The principal effect of low pH on the kinetics of the enzyme-catalyzed reaction is the observation of a pH-dependent substrate inhibition by oxidized glutathione at pH $\leqslant 7$, which is shown to correlate with the binding of oxidized glutathione to the oxidized form of the enzyme.

2. The catalytic activity of yeast glutathione reductase at pH 5.5 is affected by the sodium acetate buffer concentration. The stability of the oxidized and reduced forms of the enzyme at pH 5.5 and 25 °C in the absence of bovine serum albumin was studied as a function of sodium acetate concentration. The results show that activation of the catalytic activity of the enzyme at low sodium acetate concentration correlates with an effect of sodium acetate on a reduced form of the enzyme. In contrast, inhibition of the catalytic activity of the enzyme at high sodium acetate concentration correlates with an effect of sodium acetate on the oxidized form of the enzyme.

Introduction

The flavoenzyme glutathione reductase from yeast (NAD(P)H: oxidized glutathione oxidoreductase, EC 1.6.4.2) catalyzes the NADPH-dependent reduction of the disulfide bond of oxidized glutathione. The enzyme is a dimer

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with one FAD per monomer [1]. Both the enzyme-bound FAD and a protein disulfide have been implicated [2] as electron acceptors in the catalytic mechanism. Catalysis involves electron transfer from NADPH to the oxidized form of the enzyme to produce a 2-electron reduced form of the enzyme, followed by electron transfer from the reduced enzyme to GSSG [2,3]. Icén [4] reported that the kinetics of the reverse reaction catalyzed by the yeast enzyme required a sequential [5] mechanism. More recently, branching mechanisms composed on interconnected loops of sequential and ping pong mechanisms have been proposed for the yeast enzyme by Mannervik [6] and Moroff and Brandt [7].

In this paper we report studies of the pH dependence of the catalytic activity and stability of yeast glutathione reductase in the range pH 4.5—8.0. A pH-dependent substrate inhibition by GSSG, which has not been previously reported for the yeast enzyme, is observed at pH $\leqslant$ 7.0. The substrate inhibition is shown to correlate with the binding of GSSG to the oxidized form of the enzyme. At pH 5.5, the catalytic activity of the enzyme depends upon the buffer concentration and is inhibited by NaCl in a manner similar to that observed at pH 7.6 [7]. The substrate inhibition is interpreted as supporting the existence of separate binding sites for GSSG and pyridine nucleotide on both the oxidized and reduced forms of the enzyme. In addition, the relationship between the effect of buffer concentration on the catalytic activity of the enzyme and the stability of the oxidized and reduced forms of the enzyme at pH 5.5 has been studied.

Materials and Methods

Yeast glutathione reductase, obtained from Sigma Chemical Co., was purified further as described previously [7] to a specific activity of 262—297 units/mg protein when assayed at pH 7.6 and 25 °C according to Massey and Williams [2]. Stock enzyme solutions were stored at 4 °C in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. Bovine serum albumin was obtained from Pentex Laboratories. NADPH and GSSG were obtained from Calbiochem; NADPH was further purified as described previously [7]. All inorganic salts were of reagent grade.

Enzyme activity was measured at 25 °C by observing the initial, steady-state rate of oxidation of NADPH at 340 nm. Unless specified otherwise, kinetic experiments at pH 4.5, 5.0, and 5.5 were performed in 0.1 M sodium acetate buffer; from pH 6.0 to 8.0, 0.06 M sodium phosphate buffer was used. All assay mixtures contained 1.4 mg/ml bovine serum albumin and 0.5 mM EDTA. When purified NADPH was used, the assay mixture also contained 0.02 M NaCl from the purification procedure. The reaction was initiated by adding enzyme to the thermally equilibrated assay mixture. Stock solutions of NADPH were maintained at about pH 7.6 in 0.01 M NH_4HCO_3 containing 0.2 M NaCl, the buffer used in purifying the NADPH [7], or in 1 mM sodium phosphate (pH 8) until addition to the various assay mixtures. Thus the NADPH was exposed to acidic conditions for only a short time for thermal equilibration, generally less than 3 min, prior to addition of enzyme. Control experiments, in which 0.1 mM NADPH was incubated in the absence of enzyme under assay conditions at pH 5.5 and below, showed that acid-catalyzed

modification of NADPH amounted to less than 1 μM per min at pH 5.5, and less than 2 μM per min at pH 5.0 and 4.5. It is therefore assumed that the resulting low concentrations of the acid modification product of NADPH will have a negligible effect on the measured initial velocities.

Heat inactivation experiments were carried out at 55 °C in either 0.165 M sodium acetate, pH 5.5, or 0.06 M sodium phosphate, pH 7.6. All incubation solutions contained 3 mM EDTA, but bovine serum albumin was omitted. GSSG was included as indicated. At the indicated times a 50-μl sample of the incubation mixture was removed and assayed immediately at pH 7.6 and 25 °C using the standard assay conditions of Massey and Williams [2]. Zero time was taken to be the time at which the incubation mixture was immersed in the 55 °C water bath. At each pH, 100% activity represents the activity of a sample of that incubation mixture determined immediately after addition of stock glutathione reductase to the incubation mixture at 25 °C but prior to immersion in the 55 °C water bath.

Results

The initial, steady-state rate of NADPH-dependent reduction of GSSG catalyzed by glutathione reductase was determined as a function of GSSG concentration at 0.1 mM NADPH at pH values between 4.5 and 8.0. At pH $\leqslant$ 7, GSSG substrate inhibition was observed, with the onset of this inhibition occurring at lower GSSG concentration at successively lower pH values. This effect is illustrated in Fig. 1, where data obtained at pH 4.5, 5.5, and 6.0 are shown. No evidence of substrate inhibition by GSSG at concentrations up to 4 mM was observed at pH 7.6 or 8.0 (data not shown). The pH dependence of the apparent Michaelis constant for GSSG and the apparent turnover number of yeast glutathione reductase at 0.1 mM NADPH is summarized in Table I. It

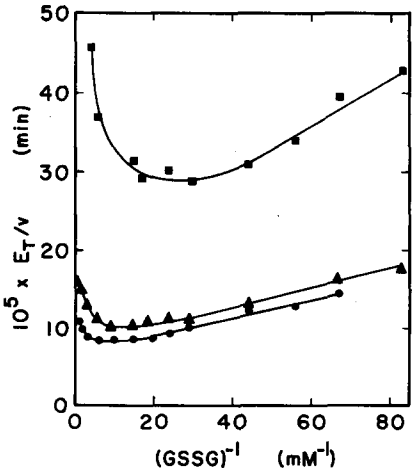


Fig. 1. Lineweaver-Burk plots of enzyme activity at 25 °C with GSSG as the variable substrate at various pH values. Purified NADPH was used at a concentration of 0.1 mM. On the ordinate legend, E_T is the μM concentration of enzyme-bound FAD in the assay mixture and v is the initial velocity in $\mu M \cdot min^{-1}$. Symbols: ●, pH 6.0; ▲, pH 5.5; ■, pH 4.5.

TABLE I

pH DEPENDENCE OF THE APPARENT MICHAELIS CONSTANT (K_{GSSG}(app)) AND APPARENT TURNOVER NUMBER (TN(app)) OF YEAST GLUTATHIONE REDUCTASE

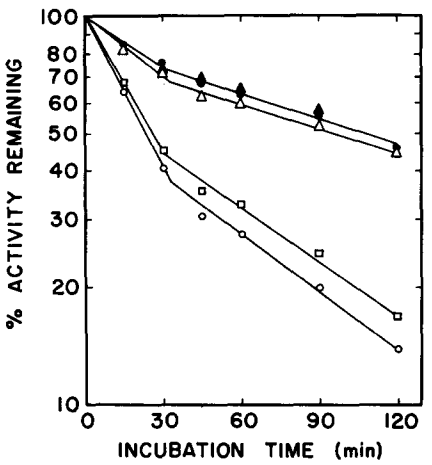
General assay conditions are described under Materials and Methods. K_{GSSG}(app) and TN(app) were determined at 0.1 mM NADPH by extrapolating the linear portion of Lineweaver-Burk plots at low GSSG concentration where substrate inhibition is minimal. Corrections were made for the spontaneous rate of decomposition of NADPH at pH 4.5, 5.0, and 5.5. Purified NADPH was used.

pH	K_{GSSG}(app) (μM)	TN(app) (min^{-1})
4.5	30	7400
5.0	15	8700
5.5	18	13900
6.0	18	15600
6.5	40	17000
7.0	50	17500
7.6	64	14900
8.0	110	13800

can be seen that the apparent turnover number varies only 20% between pH 5.5 and 8.0.

The concentration of NADPH used in the measurements reported in Table I is close to saturating at pH 7.6, where reported values for the Michaelis constant for NADPH range from 3 to 13 μM [1,2,7]. Evaluation of the limiting Michaelis constants for NADPH at the acidic pH values is complicated by the substrate inhibition by GSSG. However, the apparent Michaelis constant for NADPH was determined in the range pH 5.5—7.6 at a fixed concentration of 60 μM GSSG, a concentration below the onset of significant substrate inhibition. Values of K_{NADPH} (app) obtained were 5 μM at pH 7.6 and 7.0, 4 μM at pH 6.5, and 3 μM at pH 6.0. At pH 5.5 when the NADPH concentration was varied from 10 to 100 μM at 60 μM GSSG, only a very slight dependence of the initial velocity on NADPH concentration was observed, from which we estimate the value for K_{NADPH} (app) is 1 μM or less. Based on these data, we conclude that the NADPH concentration used in the measurements reported in Table I is close to saturating over the pH range 5.5—7.6.

Substrate inhibition can result from the binding of substrate, at high concentration, to an enzyme form other than the form with which that substrate normally interacts in the catalytic cycle, producing an inactive (or less active) abortive complex [8]. Since GSSG would normally be expected to interact with the 2-electron reduced form of the enzyme in the catalytic cycle, the GSSG substrate inhibition might result from formation of a complex between the oxidized enzyme and GSSG. Fig. 2 shows the results of experiments in which the loss of enzyme activity which occurs upon incubation of the oxidized form of the enzyme at 55°C was determined as a function of time at both pH 5.5 and 7.6. The oxidized enzyme is clearly less stable at pH 5.5 that at pH 7.6. If the incubation at 55°C is performed in the presence of 2 mM GSSG, a concentration at which substrate inhibition is observed at pH 5.5 but not at pH 7.6, substantial protection against inactivation is observed at pH 5.5. No protection by 2 mM GSSG against inactivation is detectable at pH 7.6.



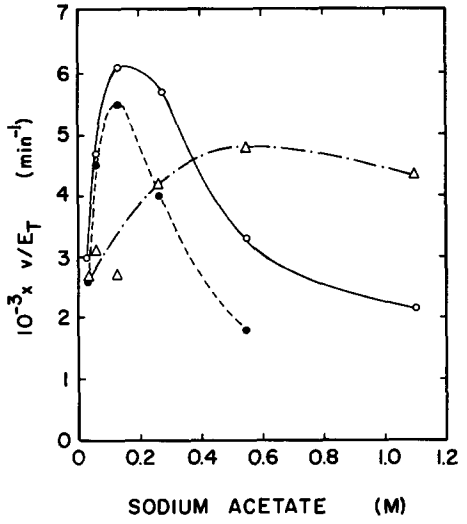


Fig. 2. Heat inactivation of yeast glutathione reductase at 55°C at pH 5.5 and 7.6. The data are plotted as log (percent activity remaining) vs time. No bovine serum albumin was present in the incubation mixture, and the 100% value for activity was determined as described under Materials and Methods. Open symbols, pH 5.5; the concentration of GSSG in the incubation mixture was: ○, no GSSG; □, 40 μM GSSG; △, 2 mM GSSG. Filled symbols, pH 7.6; the concentration of GSSG in the incubation mixture was: ●, no GSSG; ▲, 2 mM GSSG.

Fig. 3. The effect of sodium acetate buffer concentration on the catalytic activity of yeast glutathione reductase at pH 5.5 and 25°C. Initial velocity was measured at the indicated concentrations of sodium acetate, pH 5.5, in the presence of 1 mM EDTA, 1.4 mg/ml bovine serum albumin, 0.1 mM NADPH, and: ●, 20 μM GSSG; ○, 60 μM GSSG; △, 1.2 mM GSSG. Commercial NADPH was used without further purification to avoid added NaCl in the reaction mixture. On the ordinate legend, E_T is the μM concentration of enzyme-bound FAD in the assay mixture and v is the initial velocity in $\mu M \cdot min^{-1}$.

Furthermore, a low concentration of GSSG (40 μM) affords only minimal protection against inactivation at pH 5.5. These results are consistent with the observed substrate inhibition by GSSG at acidic pH being a consequence of formation of an abortive complex between the oxidized enzyme and GSSG.

At both pH 5.5 and 7.6, the inactivation processes shown in Fig. 2 are apparently biphasic in both the absence and presence of GSSG. Biphasic heat inactivation kinetics have also been reported [9] for the human erythrocyte glutathione reductase. While biphasic heat inactivation profiles could result from the presence of a mixture of two isozymes with different inherent susceptibility toward heat inactivation, we believe this is unlikely in the case of the yeast enzyme. Yeast glutathione reductase with a specific activity comparable to that used in this work has been found [3] to be at least 99% homogeneous on polyacrylamide disc gel electrophoresis. Furthermore, extrapolation of the slower phase of heat inactivation back to zero time indicates a different fraction of total activity at pH 5.5 and 7.6 in what would be the more stable isozyme. Other possible explanations for biphasic inactivation kinetics have been discussed by Seidel [10] and by Autor and Fridovich [11]. No further studies of this phenomenon have been carried out.

It has been previously reported [7] that the catalytic activity of yeast glutathione reductase at pH 7.6 is sensitive to the buffer concentration and to

the concentration of monovalent salts in the assay mixture. A similar effect is observed at pH 5.5. Fig. 3 shows the dependence of catalytic activity on the sodium acetate buffer concentration at pH 5.5 at each of three different concentrations of GSSG. At 20 and 60 μM GSSG, concentrations at which substrate inhibition by GSSG is minimal, increasing the sodium acetate concentration from 0.034 to 0.14 M results in an increase in enzyme activity. Increasing the acetate buffer concentration above 0.14 M results in inhibition. At 1.2 mM GSSG, a concentration which produces significant substrate inhibition, activation of enzyme activity is also seen on increasing the acetate buffer concentration from 0.034 M, but maximal activity is not achieved until the concentration reaches about 0.55 M. The results in Fig. 3 at 0.14 M sodium acetate, pH 5.5, are consistent with the substrate inhibition by GSSG shown in Fig. 1. Enzyme activity increases as the concentration of GSSG is increased from 20 to 60 μM, but then decreases substantially at 1.2 mM GSSG. However, at 0.55 M sodium acetate, pH 5.5, the data in Fig. 3 suggest that substrate inhibition by GSSG is prevented; catalytic activity increases as the concentration of GSSG is increased from 60 μM to 1.2 mM.

The activation and inhibition of the catalytic activity of yeast glutathione reductase as a function of sodium acetate buffer concentration at pH 5.5 are qualitatively similar to the effects seen on varying the sodium phosphate buffer concentration at pH 7.6 [7]. Monovalent anions were also found [7] to be inhibitors of yeast glutathione reductase at pH 7.6, exhibiting non-competitive inhibition with respect to GSSG. Fig. 4 shows the inhibition pattern observed for NaCl in the presence of 0.1 M sodium acetate, pH 5.5. Extrapolation of the linear portions of the curves at low GSSG concentration to the ordinate shows that NaCl inhibition is characterized by an effect on both the slope and the ordinate intercept, and can thus be classified as non-competitive [12]. However, the extrapolated lines do not appear to intersect at a common point. Qualitatively the effects of buffer concentration and monovalent salts on the

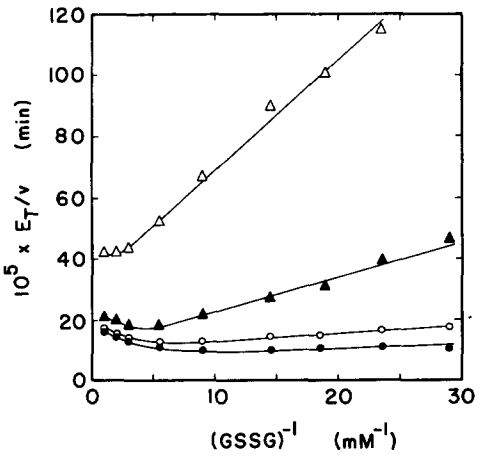


Fig. 4. Lineweaver-Burk plot of inhibition of glutathione reductase by NaCl at pH 5.5 and 25°C with GSSG as the variable substrate. Purified NADPH was used at a concentration of 0.1 mM. NaCl concentration: ●, 0.02 M; ○, 0.12 M; ▲, 0.27 M; △, 0.52 M.

catalytic activity of yeast glutathione reductase at pH 5.5 are similar to those reported [7] previously at pH 7.6.

It can also be noted from the data in Fig. 4 that high NaCl concentration diminishes substrate inhibition by GSSG, in that a higher concentration of GSSG is necessary to produce deviation from linearity in the Lineweaver-Burk plots at high NaCl concentration. This observation is consistent with the data in Fig. 3 showing that in 0.55 M sodium acetate, pH 5.5, substrate inhibition by GSSG appears to be prevented.

Glutathione reductase is unstable when incubated at low concentration at 25 °C in the absence of bovine serum albumin, as measured by assaying samples of the incubation mixtures under standard conditions at pH 7.6. The extent of this inactivation depends upon the pH of the incubation mixture, with a greater extent of inactivation being observed at more acidic pH. The time course of inactivation in the absence of serum albumin is complex. Typical results for incubation of 33 nM enzyme in 0.034 M sodium acetate, pH 5.5, in the absence and presence of NADPH are shown in Fig. 5 (open circles). An immediate loss of activity is detected upon dilution of the stock enzyme solution into the pH 5.5 buffer, followed by a further slow loss of enzyme activity over a 2-h period. The reason for the immediate loss of enzyme activity is not known, but it was not reversed by a subsequent 5-min incubation in 0.06 M sodium phosphate, pH 7.6, prior to assay.

It should be noted that these losses of enzyme activity can be largely prevented by including bovine serum albumin in the incubation mixture. In the presence of 1.4 mg/ml serum albumin, less than 10% of the enzyme activity is lost on incubation of the enzyme in the absence of NADPH for 1 h at pH values between 5.0 and 8.0; the same is true in the range pH 5.5—8.0 when NADPH is present. Thus, the enzyme is stable under the conditions employed

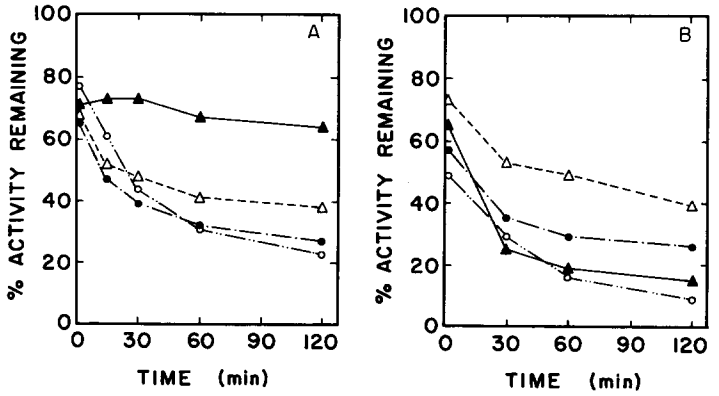


Fig. 5. The effect of sodium acetate buffer concentration on the stability of glutathione reductase in the absence of bovine serum albumin at pH 5.5 and 25 °C. Enzyme (33 nM) was incubated in sodium acetate buffer of the indicated concentration containing 1 mM EDTA in the: A, absence of NADPH; B, presence of 0.1 mM NADPH. At the end of the 2-h period, it was determined that excess NADPH was still present. At the indicated times, a 50-μl sample was removed and assayed under standard conditions at pH 7.6. The control value (100% activity) represents the activity of a sample of enzyme treated identically except for being incubated in the presence of 0.06 M sodium phosphate, pH 7.6, in the absence of NADPH. Concentration of sodium acetate: ○, 0.034 M; ●, 0.14 M; △, 0.28 M; ▲, 1.1 M.

for the kinetic experiments in Table I and Figs 1, 3 and 4, since these rate measurements were made in the presence of serum albumin.

It was previously suggested [7] that the activation of the catalytic activity of yeast glutathione reductase at pH 7.6 by low concentrations of phosphate buffer or monovalent salts was due to anion binding to the 2-electron reduced form of the enzyme, while inhibition of the catalytic activity at higher buffer or salt concentration resulted from anion binding to the oxidized form of the enzyme. The instability of glutathione reductase at pH 5.5 and 25°C in the absence of serum albumin provided a basis for testing this hypothesis.

Glutathione reductase was incubated in the absence of serum albumin at 25°C in four different concentrations of sodium acetate, pH 5.5, in both the absence and presence of NADPH. Fig. 5 shows the results of these experiments. In the absence of NADPH (Fig. 5A), it is assumed that the stability of the oxidized form of the enzyme is being measured. In the presence of NADPH (Fig. 5B), the stability of a reduced form of the enzyme is being measured. However, because the enzyme concentration is too low to detect spectrophotometrically under these conditions, it is not possible to directly determine the oxidation-reduction state of the enzyme-bound FAD in the incubation mixture. Subsequent to the immediate loss of enzyme activity observed for the zero-time point, it can be seen in Fig. 5 that both the oxidized and reduced forms of the enzyme undergo a substantial further loss of activity during a 2-h period in 0.034 M sodium acetate. Increasing the sodium acetate buffer concentration from 0.034 to 0.28 M significantly increases the stability of the reduced form of glutathione reductase, while only a small increase in the stability of the oxidized form of the enzyme is seen. This low buffer concentration range correlates reasonably well with the range of sodium acetate buffer concentration in which activation of the catalytic activity of the enzyme at pH 5.5 occurs (Fig. 3). The oxidized form of the enzyme is stabilized against the time-dependent loss of activity in the presence of 1.1 M sodium acetate, a concentration at which inhibition of the catalytic activity of the enzyme is evident in Fig. 3. However, the presence of 1.1 M sodium acetate also affects the stability of the reduced form of the enzyme, reversing the stabilizing effect of 0.28 M sodium acetate.

Discussion

The apparent Michaelis constant for GSSG and the apparent turnover number of yeast glutathione reductase measured at 0.1 mM NADPH show only a slight pH dependence in the range pH 5.5—7.6. In this range, the apparent turnover number varies only 20% and the apparent Michaelis constant for GSSG varies only about 3-fold. These results show that no catalytically important acidic functional groups are titrated in this region. Furthermore, the effects of sodium acetate buffer concentration on catalytic activity, as well as the inhibition of enzyme activity by NaCl at pH 5.5 are qualitatively similar to the effects of sodium phosphate buffer concentration on catalytic activity and the inhibition of enzyme activity by monovalent anions at pH 7.6 reported previously [7]. This qualitative similarity, coupled with the lack of significant pH dependence of the apparent Michaelis constant for GSSG and the apparent

turnover number in the range pH 5.5—7.6, suggests that the mechanism of the enzyme-catalyzed reaction is unchanged in this region. Therefore, it is likely that the mechanisms of anion activation and inhibition are the same at both pH 5.5 and 7.6.

One major effect of lowering the pH from 7.6 to 5.5, other than the observation of substrate inhibition by GSSG (which is discussed below), is the decreased stability of the enzyme on incubation at pH 5.5 in the absence of serum albumin. Comparison of Figs 3 and 5 reveals that the activating effect of low concentrations of sodium acetate buffer at pH 5.5 on the catalytic activity of yeast glutathione reductase correlates with a stabilization of a reduced form of the enzyme against inactivation at 25 °C. These same low concentrations of sodium acetate produce at most a slight stabilization of the oxidized form of the enzyme. These observations support the hypothesis [7] that the activation effect on catalytic activity seen at low buffer concentration is the result of anion binding to the 2-electron reduced form of the enzyme.

It is apparent from Fig. 5 that 1.1 M sodium acetate significantly affects the stability of both the oxidized and reduced forms of yeast glutathione reductase at pH 5.5. The oxidized form of the enzyme is markedly stabilized against the slow, time-dependent inactivation, while the reduced form is destabilized relative to 0.28 M sodium acetate. This destabilization of the reduced form of the enzyme in 1.1 M acetate cannot be the direct cause of the inhibition of catalytic activity shown in Fig. 3 at this buffer concentration. The kinetic assays in Fig. 3 were performed in the presence of serum albumin, under which conditions the enzyme is stable. The effect of 1.1 M sodium acetate on the stability of the two enzyme forms at pH 5.5 does not provide definitive support for inhibition of catalytic activity resulting from anion binding to the oxidized form of the enzyme. However, taken in conjunction with the observations that inhibition of catalytic activity by NaCl is non-competitive with respect to GSSG at both pH 5.5 (Fig. 4) and pH 7.6 [7], and that at pH 7.6 inhibition by monovalent anions is competitive with respect to NADPH [7], the overall results strongly suggest that the effect of high buffer or monovalent salt concentration on catalytic activity is due to anion binding to the oxidized form of the enzyme.

The principal change in the kinetics of yeast glutathione reductase at pH $\leqslant$ 7 is the observation of substrate inhibition by GSSG. Substrate inhibition by GSSG has been reported [9,13,14] for the human erythrocyte enzyme using either NADPH or NADH as the electron donor at pH 7.0 or 6.8. Substrate inhibition by GSSG of the yeast enzyme does not appear to have been previously reported. The substrate inhibition by GSSG apparently accounts for the previously reported [2] decrease in the turnover number of the yeast enzyme at pH 6.2, since the earlier data were determined at only a single GSSG concentration of 3.25 mM.

The substrate inhibition appears to result from the binding of GSSG to the oxidized form of the enzyme. This conclusion follows from the observations that a high concentration of GSSG (2 mM) stabilizes the oxidized form of the enzyme against inactivation at pH 5.5 and 55 °C, while a lower concentration of GSSG (40 μM) has only a slight effect. At pH 7.6 where substrate inhibition by GSSG is not observed, 2 mM GSSG has no effect on the stability

of the oxidized form of the enzyme. Harding [15] has recently reported that at pH 6.6 yeast glutathione reductase binds to a modified agarose in which GSSG is linked to the agarose through a 1,6-diaminohexane spacer chain. In the absence of NADPH, the yeast enzyme was retained on a column of the GSSG-modified agarose. Thus the oxidized form of the enzyme has affinity for GSSG. Elution of the enzyme from the column by 1 M NaCl correlates with the results shown in Fig. 4 which illustrate that high concentrations of NaCl, in addition to inhibiting the enzyme, diminish the substrate inhibition by GSSG.

The binding of GSSG to the oxidized form of the enzyme to produce substrate inhibition has interesting implications in relation to the mechanism of the enzyme-catalyzed reaction. Bulger and Brandt [16] have shown by spectrophotometric and kinetic studies at pH 7.6 that yeast glutathione reductase has a general pyridine nucleotide binding site on both the oxidized and 2-electron reduced forms of the enzyme. Staal and Veeger [14] have proposed an ordered sequential [5] mechanism for human erythrocyte glutathione reductase. Icen [4] has concluded that the mechanism of the reverse reaction catalyzed by yeast glutathione reductase is a sequential mechanism involving a ternary complex of enzyme with $NADP^+$ and GSH. Recently, Mannervik [6] has proposed a branching mechanism for the yeast enzyme which involves two interconnected loops corresponding to one ping pong and one sequential pathway. The common step interconnecting the two loops involves addition of NADPH to the oxidized form of the enzyme. A similar branching mechanism has been proposed by Moroff and Brandt [7]. The common feature of these proposals is that the enzyme must possess separate binding sites at the active site for both pyridine nucleotide and GSSG. Therefore, since NADPH can bind to both the oxidized and reduced forms of the enzyme [16], it would be expected that GSSG should also be able to bind to both the oxidized and 2-electron reduced forms of the enzyme.

The substrate inhibition by GSSG can be explained in terms of this hypothesis. It is known that the catalytic mechanism of yeast glutathione reductase involves electron transfer from NADPH to the enzyme-bound FAD [2,3]. The steady-state kinetic data are consistent with an ordered sequence of substrate binding, with NADPH binding first [4,6,14] for both the yeast and human erythrocyte enzymes. Thus, as proposed for both the yeast [7] and human erythrocyte [14] enzymes, the initial steps in the catalytic mechanism can be written as shown in Eqn 1, where E represents the oxidized form of the enzyme,

$$E + NADPH \rightleftharpoons E\text{-}NADPH \rightleftharpoons F\text{-}NADP^+ \quad (1)$$

and F is the 2-electron reduced form. The next step in the catalytic pathway would be the binding of GSSG to the $F\text{-}NADP^+$ complex to produce a ternary $F\text{-}NADP^+$-GSSG complex. Electron transfer from NADPH to the enzyme-bound FAD to produce $F\text{-}NADP^+$ may be accompanied by a conformational change in the enzyme, enhancing its affinity for GSSG. At sufficiently high concentrations of GSSG, however, GSSG may bind at its site on the oxidized enzyme, E, or to the E-NADPH complex. Within the resulting ternary E-NADPH-GSSG complex, electron transfer from NADPH to the enzyme-bound FAD may occur at a slower rate than in the binary E-NADPH complex of

Eqn 1, resulting in the observed substrate inhibition. Alternatively, the formation of a binary E-GSSG complex may inhibit the binding of NADPH. However, the structural dissimilarity of NADPH and GSSG, together with the marked specificity of yeast glutathione reductase for NADPH over NADH [3] and for GSSG over lipoic acid [2], would appear to require highly specific, separate substrate binding sites for pyridine nucleotide and disulfide substrate at the active site of the enzyme. Thus, it would be unlikely that the substrate inhibition by GSSG would be the result of the binding of GSSG at the pyridine nucleotide site.

We conclude that the more reasonable explanation for the substrate inhibition is that the binding of GSSG at its site on the oxidized form of the enzyme induces a conformational change in the enzyme which alters either the binding of NADPH to its site on the enzyme, or alters the rate of electron transfer from enzyme-bound NADPH to the enzyme-bound FAD. Finally, it should be noted that this binding of GSSG to the oxidized form of the enzyme must be pH dependent, since substrate inhibition was not observed at either pH 7.6 or 8.0, and became more pronounced at more acidic pH.

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