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HYSTERETIC BEHAVIOUR AND GSSG SUBSTRATE INHIBITION SHOWN BY GLUTATHIONE REDUCTASE FROM *PHYCOMYCES BLAKESLEEANUS*

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Phycomyces blakesleeanus glutathione reductase shows hysteretic behaviour under experimental conditions, when GSSG substrate inhibition is observed. The progress curves for the reaction show an acceleration phase. The degree of hysteresis varied inversely as the enzyme concentration. It increased when GSSG or NADPH concentration increased, whereas the addition of GSH or $NADP^+$ to the initial reaction mixture prevented it from occurring. In addition, hysteresis was dependent on pH, ionic strength and temperature, decreasing as any of these parameters increased. The parallel effects of pH and ionic strength on the GSSG substrate inhibition and hysteretic behaviour suggest a relationship between these two mechanisms. From the overall results reported in this paper, we propose that the hysteretic behaviour shown by *Phycomyces* glutathione reductase could be due to a process of time-dependent accumulation of reaction products rather than to a slow conformational change.

KEY WORDS: Hysteresis, GSSG inhibition, glutathione reductase, *Phycomyces*.

INTRODUCTION

Glutathione reductase (NAD(P)H: oxidized-glutathione oxidoreductase, EC 1.6.4.2) catalyzes the NAD(P)H-dependent reduction of the oxidized form of glutathione (GSSG) to GSH, this reaction being essential for the maintenance of glutathione levels[1–3]. Earlier we have described the purification, characterization and the kinetic mechanism of mycelial glutathione reductase from the fungus *Phycomyces blakesleeanus*[4,5]. During the course of our studies on the kinetic mechanism followed by the *Phycomyces* enzyme we have found that the rate of GSSG reduction increases during the time course of the reaction. This effect was especially noticeable at acidic pH values at which substrate inhibition by GSSG was also observed. The slow transient kinetics can be described in terms of the hysteretic concept[6]. Hysteretic enzymes are those which respond slowly, usually in terms of reaction velocity (or other parameters) to a rapid change in ligand concentration[7–9]. Enzymes showing hysteretic behaviour are a diverse group, but such a slow kinetic response would seem likely to have a physiological function controlling the metabolite flux through different metabolic pathways[9]. The present paper shows the results obtained concerning the study of the apparent hysteretic response in the reaction progress curves from *Phycomyces*

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glutathione reductase. The influence of enzyme concentration, pH, temperature and ligands on the slow kinetic response were examined. The implications of GSSG substrate inhibition on glutathione reductase hysteretic behaviour are discussed. On the basis of the studies reported here, we propose a mechanism whereby hysteresis results from products activation rather than on time-dependent conformational changes.

MATERIALS AND METHODS

Materials

Biochemicals were obtained from Boehringer Mannheim, Manheim, FRG. Sephadex G-100 and G-200 and molecular weight markers for gel filtration chromatography were from Pharmacia Fine Chemicals, Uppsala, Sweden. Agar No. 3 and yeast extract (Lab Lemco Powder) were from Oxoid Ltd, London, U.K. Bactocasitone from Difco, Detroit, MI, U.S.A. All other reagents were purchased from standard commercial suppliers and were of the best grade available.

Organism Cultivation and Preparation of Purified Glutathione Reductase

Phycomyces blakesleeanus wild-type strain NRRL 1555(–) was used (see De Arriaga *et al.*[10] for liquid minimal medium and general method of cultivation). The preparation of purified mycelial glutathione reductase was described in a previous paper[4].

Protein Determination

Protein concentration was determined by the method of Lowry *et al.*[11] or spectrally using[4] $A_{280}^{0.1\%} = 1.16$ at pH 7.5.

Enzyme Assay

The kinetics of GSSG reduction were monitored at 340 nm by measuring the change in absorbance associated with oxidation of NADPH. Assays were performed at 30°C in a Shimadzu recording spectrophotometer model UV-260. The reaction substrates (GSSG and NADPH) at indicated concentrations in each experiment were in a final reaction volume of 1 ml. Assays were performed routinely in 50 mM sodium phosphate buffer or 25 mM sodium citrate buffer containing 1 mM EDTA, at the pH value indicated for each experiment.

Analysis of Kinetic Data

Enzyme assay curves exhibiting a lag in catalytic activity were analysed on the basis of a unimolecular change in enzyme activity, the rate of change in velocity being described by the equation[9]

$$\ln (v_{ss} - v_t) = \ln (v_{ss} - v_i) - kt \quad (1)$$

where v_{ss} is the steady-state velocity, v_i is the initial velocity, v_t is the instantaneous velocity of the reaction at time t, and k represents the apparent first-order rate

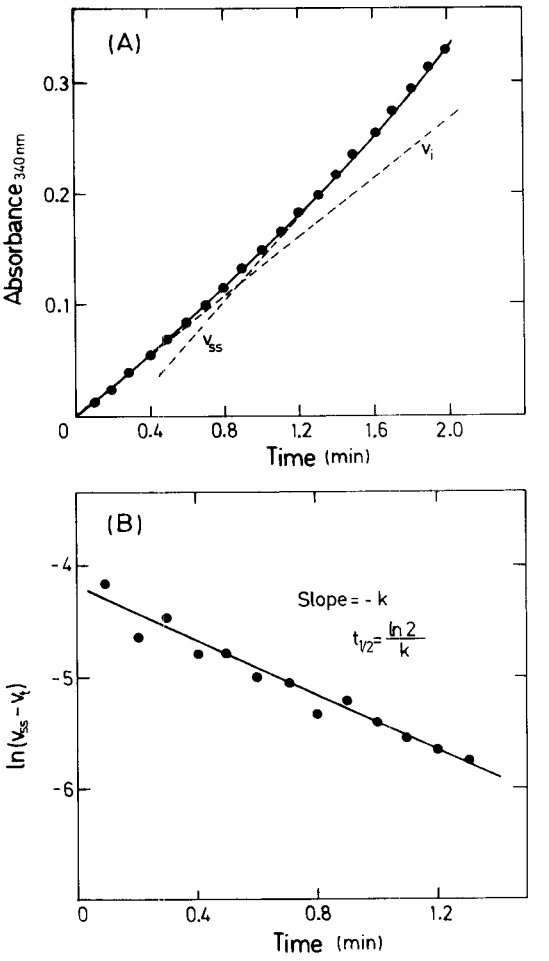

FIGURE 1 Hysteretic behaviour of *Phycomyces blakesleeanus* glutathione reductase. A) Progress curve of GSSG reduction at 1 mM GSSG and 100 μM NADPH in 25 mM sodium citrate/citric acid buffer pH 5.5 containing 1 mM EDTA. All reactants were pre-incubated at 30°C for 2 min and then the assay was started by adding the enzyme (1.6 μg/ml reaction mixture). B) Exponential decay of the transient. Details are given in Materials and Methods. v_i, initial velocity, v_{ss}, steady-state velocity and v_t, instantaneous velocity at time t.

constant for relaxation of the enzyme to its final constant velocity. The product-time coordinates of the progress curves were manually digitized at 6 s intervals, and the tangents to the curves at these constant time intervals represent v_t at time t. The validity of this analysis was tested by plotting ln $(v_{ss} - v_t)$ *vs.* time, which should be linear to ensure that the process is truly first order (Figure 1). The apparent k value was calculated routinely from the linear portions of the progress curves. The extrapolation of the linear portion of the curve representing v_{ss} to $t = 0$ yields an apparent k value equal to $-(v_{ss} - v_i)$/ordinate intercept. Both methods gave similar k values.

The kinetic parameters, K_m and V_{max}, were calculated by the Direct Linear plot[12] using the Enzpack computer program [13] after graphically checking that the double-reciprocal plots of reaction rate *versus* substrate concentration were linear. Allosteric GSSG substrate inhibition kinetics were analyzed according to Kurganov[14] from a ln $[(V_{max} - v)/v]$ *vs.* ln [substrate inhibitor] plot, V_{max} being the generally accepted value of V_{max} for the ascending branch of the velocity curve, i.e., the maximum value of the velocity for the hyperbolic branch, and v the velocity at any specified inhibitory substrate concentration. The maximal slopes (h) of the above Hill plots were estimated by fitting the experimental inhibition data to straight lines by the least-squares method; in addition, the substrate inhibition constant values $[GSSG]_{0.5}$, were determined as the abscissa intercept when the ordinate was zero.

RESULTS AND DISCUSSION

Phycomyces glutathione reductase shows substrate inhibition by GSSG to be pH-dependent, which is marked at acidic pH values but not detectable at pH 7.5, even with a GSSG concentration of 9 mM[5,15]. In addition, glutathione reductase underwent a slow change in activity dependent on GSSG concentration under assay conditions where substrate inhibition by GSSG was noticeable. The progress curve for the reaction occurs as a lag type of transient (hysteretic response) in which the rate of reaction was initially low and later increased to a higher steady-state (Figure 1.A). When the data from Figure 1.A were replotted in Figure 1.B, it showed that the apparent slow transition followed first-order kinetics with an apparent k value of 1.2 min^{-1} and corresponding half-time ($t_{1/2}$) of 0.57 min (where $t_{1/2} = \ln 2/k$), indicating, thus, that the transition can be considered as a unimolecular process. At low GSSG concentrations, at which substrate inhibition was not apparent, hysteretic effects were also not detected.

Most hysteretic mechanisms involve, at least, two different states of the enzyme with different physical and kinetic properties, where the slow transition to the second state occurs on a time scale observable in the enzymatic assay. The slow phase of the hysteretic transition may be a conformational change (isomerization), a dissociation-

TABLE I

Pre-incubation experiments of *Phycomyces* glutatione reductase with the reaction components. The reactants listed below were pre-incubated for 2 min at 30°C in 25 mM sodium citrate buffer, containing 1 mM EDTA, pH 5.5 and pH 6.5, respectively, the reaction being started by the addition of the omitted reactant. The pre-incubation mixtures were carried out in a constant volume of 0.98 ml. The GSSG concentration used was 25 mM at pH 5.5 and 7 mM at pH 6.5. In both cases, the NADPH concentration was 0.1 mM and the enzyme concentration 1.6 μg/ml of reaction mixture.

Premixed reactants	Reaction initiated by addition of	v_i/v_{ss}*		$t_{1/2}$ (min)	
		pH 5.5	pH 6.5	pH 5.5	pH 6.5
GSSG, NADPH	enzyme	0.49	0.57	0.50	0.39
GSSG, enzyme	NADPH	0.50	0.57	0.40	0.39
NADPH, enzyme	GSSG	-**	0.44	-	1.42
enzyme	NADPH, GSSG	0.49	0.57	0.40	0.31

*A value lower than 1.0 indicates hysteresis (lag type of transient), whereas a value of 1.0 indicates no hysteresis.

**Enzyme inactivation.

association reaction of the enzyme, or a direct displacement of a bound ligand by a second ligand[7–9]. To determine whether the hysteretic response requires the presence of either GSSG or NADPH, or both ligands, a series of pre-incubation experiments were performed where the enzyme was pre-incubated with various combinations of reactants, the reaction being initiated by addition of the omitted reactant. These conditions are given in Table I. As can be seen, in all cases except in the pre-incubation

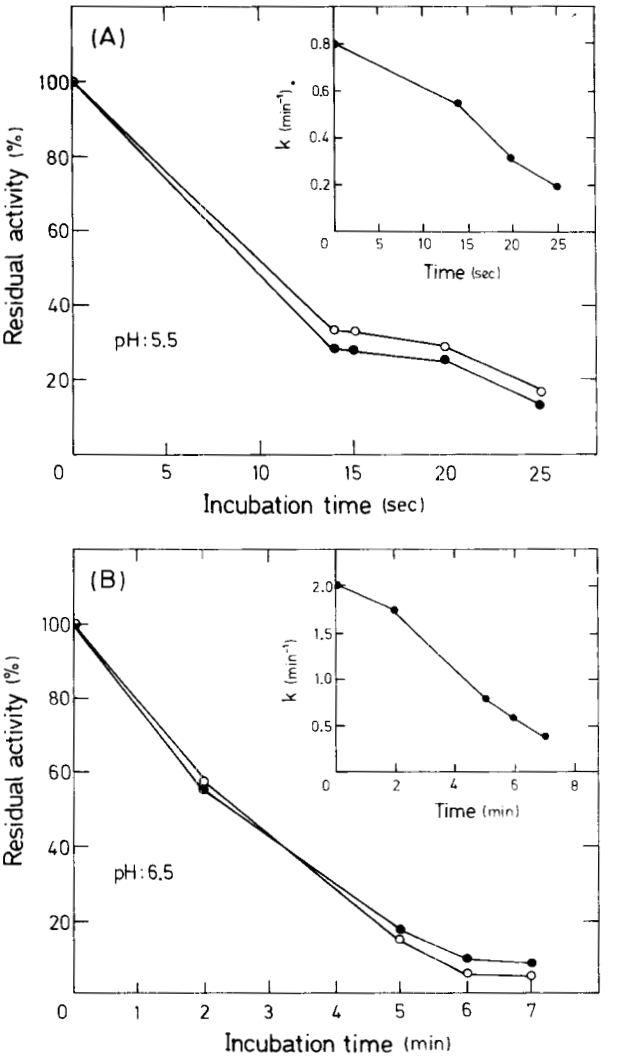

FIGURE 2 Inactivation by NADPH and hysteretic behaviour of *Phycomyces blakesleeanus* glutathione reductase. The reaction mixtures were incubated at 30°C containing 1.6 μg of enzyme and 0.1 mM NADPH in 25 mM sodium citrate buffer, in a final volume of 0.9 ml. In both cases, the reaction was started by adding 2.5 mM GSSG. A) pH 5.5 and B) pH 6.5. v_i (○) and v_{ss} (●) are expressed in % activity taking the activity at zero time incubation as 100%. *Insets*: Dependence of the apparent hysteretic rate constant (k) on the inactivation process.

experiments of the enzyme with NADPH, similar extents of hysteresis were observed. These experiments rule out the possibility of the enzyme dilution in the reaction mixture contributing to hysteretic behaviour. On the other hand, the hysteresis was independent of the pH value at which the stock enzyme solution was maintained. In addition, these premixing studies suggest either that a slow conformational change is not responsible for the hysteretic behaviour, or that such a conformational change occurs only in the presence of all of the substrates for reaction, i.e., in the active ternary complex.

Phycomyces glutathione reductase is inactivated by incubation with NADPH[4]. At pH 5.5, the pre-incubation of the enzyme with NADPH produces a total inactivation of catalytic activity, but at pH 6.5 the partial inactivation (about 50%) was associated with an increased lag with a $t_{1/2}$ of 1.42 min, suggesting a possible relationship between both phenomena. Thus, we carried out a series of experiments to analyze the effect of NADPH inactivation of the enzyme on hysteretic behaviour. Figure 2 shows the NADPH inactivation pattern followed by v_i and v_{ss}, as well as the dependence of the hysteretic rate constant (k) on the inactivation process at two pH values (5.5 and 6.5). In both cases v_i and v_{ss} showed a similar inactivation pattern but the inactivation rate constant value at pH 6.5 was ten times lower than at pH 5.5. Hysteretic behaviour was appreciable at zero time incubation, resulting in an increase in the lag simultaneous to inactivation. This increase in the lag was associated with a decrease in the k value from 0.82 min^{-1} at zero time to 0.21 min^{-1} after 25 s incubation at pH 5.5 and from 2.1 min^{-1} at zero time to 0.40 min^{-1} after 7 min incubation at pH 6.5. However, in a similar experiment at pH 7.5, no hysteresis was observed at zero time incubation, or even after 50 min NADPH incubation when the glutathione reductase activity was only 3% of that shown at zero time incubation. Under these last conditions, the

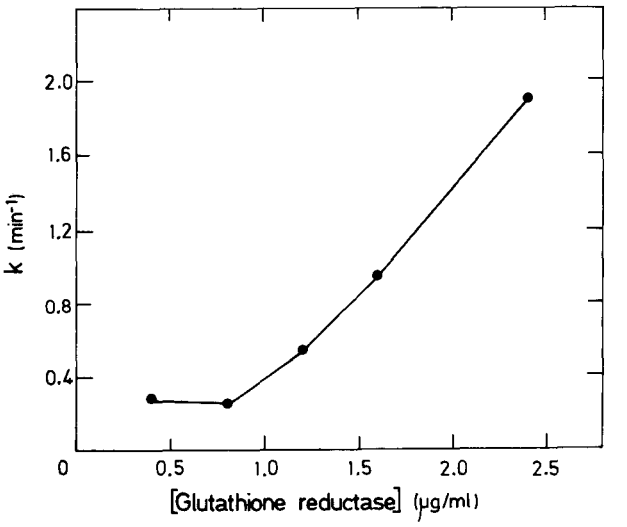

FIGURE 3 Effect of enzyme concentration on hysteresis shown by *Phycomyces* glutathione reductase. Variation of the apparent hysteretic rate constant (k) as a function of enzyme concentration. All assays were carried out at 30°C, in 25 mM sodium phosphate buffer pH 6.5 with 5 mM GSSG and 30 μM NADPH.

inactivation rate constant value was 100 times lower than at pH 5.5. Alternatively, when a series of similar experiments were performed by starting the reaction by addition of 0.1 mM GSSG instead of 2.5 mM GSSG to the above experiment, hysteretic behaviour was only detected after 13 s NADPH incubation at pH 5.5 (the k value decreasing from 1.55 min^{-1} to 0.65 min^{-1} at 45 s incubation), whereas both v_i and v_{ss} showed a similar inactivation pattern between then (data not shown). In addition, the inactivation rate constant values were identical to those obtained in the above experiment. These results indicated that, although both phenomena are pH-dependent (decreasing as the pH value increases), a slow conformational change produced during the inactivation process cannot be responsible for the hysteretic behaviour. In addition, it is also evident that the hysteretic behaviour is GSSG-dependent but the NADPH inactivation is not.

Recently, Arscott *et al.*[16] have described that the NADPH inactivation of *E. coli* glutathione reductase is a process inversely dependent on the enzyme concentration, involving an equilibrium between an inactive-monomeric-two-electron-reduced form and an active-dimeric-two-electron-reduced form. These studies lend weight to our explanation that the apparent relationship between NADPH inactivation and the hysteretic behaviour of *Phycomyces* glutathione reductase could be due to a decrease in the active enzyme concentration. By varying the enzyme concentration over a 10-fold range under otherwise identical conditions, it is apparent that the hysteresis phenomenon decreases (Figure 3) as the enzyme concentration increases (k value increasing from 0.30 min^{-1} to 1.92 min^{-1} when the enzyme concentration was raised from 0.4 μg/ml to 2.4 μg/ml respectively). Non-hysteretic behaviour was detected using a 4 μg/ml enzyme concentration. Enzyme-concentration-dependent hysteresis is often associated with reversible enzyme polymerization with the various polymers having different kinetic properties[6,8,9]. However, no evidence of polymerization was

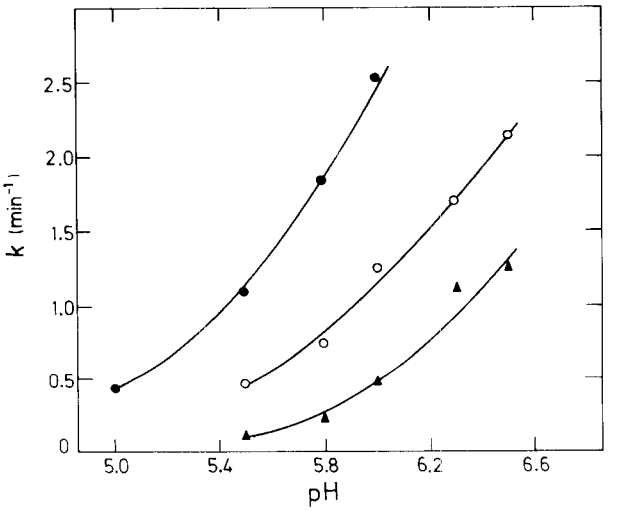

FIGURE 4 Effect of pH on hysteresis behaviour shown by *Phycomyces* glutathione reductase. The experiments were performed at 30°C in 25 mM sodium citrate buffer pH 5.0–6.5 or 50 mM sodium phosphate buffer pH 6.5, using 100 μM NADPH and 1.6 μg of glutathione reductase/ml of reaction mixture. 2 mM GSSG (●), 5 mM GSSG (○) and 7 mM GSSG (▲).

detected for *E. coli* dihydrofolate reductase[17] and bovine glutamate dehydrogenase[18], as was the case for *Phycomyces* glutatione reductase. Gel filtration chromatography showed that the enzyme elution volume did not change in columns equilibrated with 25 mM sodium citrate buffer pH 5.5 or 50 mM sodium phosphate buffer pH 6.5, in the absence or in the presence of 5 mM GSSG, even when the enzyme concentration applied to the column ranged from 20 nM to 800 nM. Therefore, there was no evidence of enzyme aggregation under conditions which showed hysteresis in the assay.

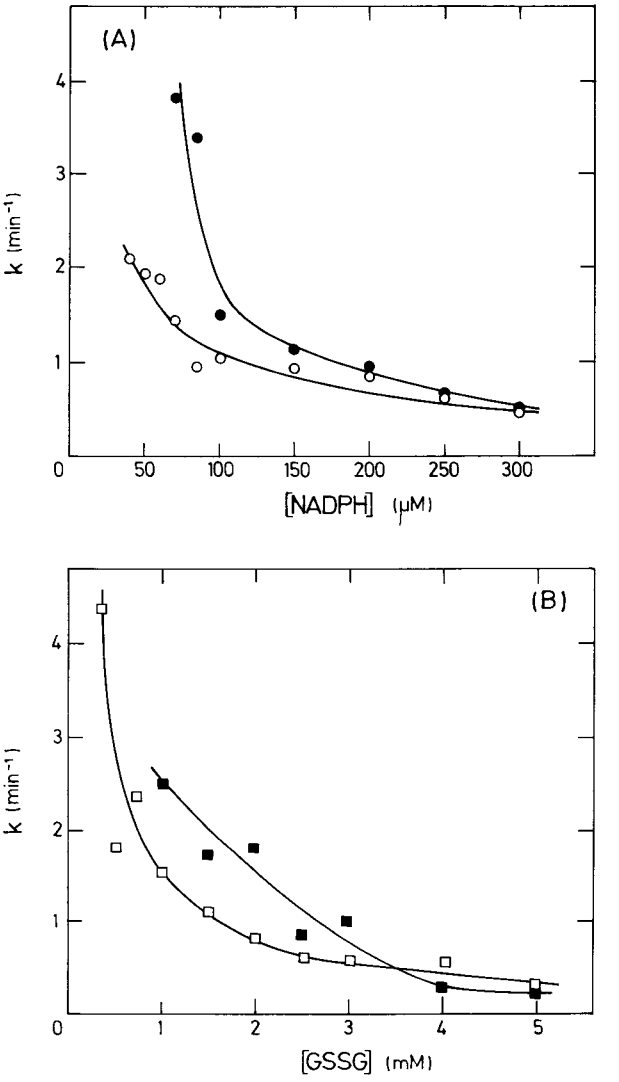

FIGURE 5 Dependence of hysteretic behaviour shown by *Phycomyces* glutathione reductase on substrate concentration. The assays were carried out at 30°C in 25 mM sodium citrate buffer pH 5.5 (I = 0.09 M) using 1.6 μg of enzyme/ml of reaction mixture. A) At variable NADPH concentration and two fixed GSSG concentrations: (●) 1 mM, (○) 2 mM. B) At variable GSSG concentration and two fixed NADPH concentrations; (■) 50 μM, (□) 100 μM.

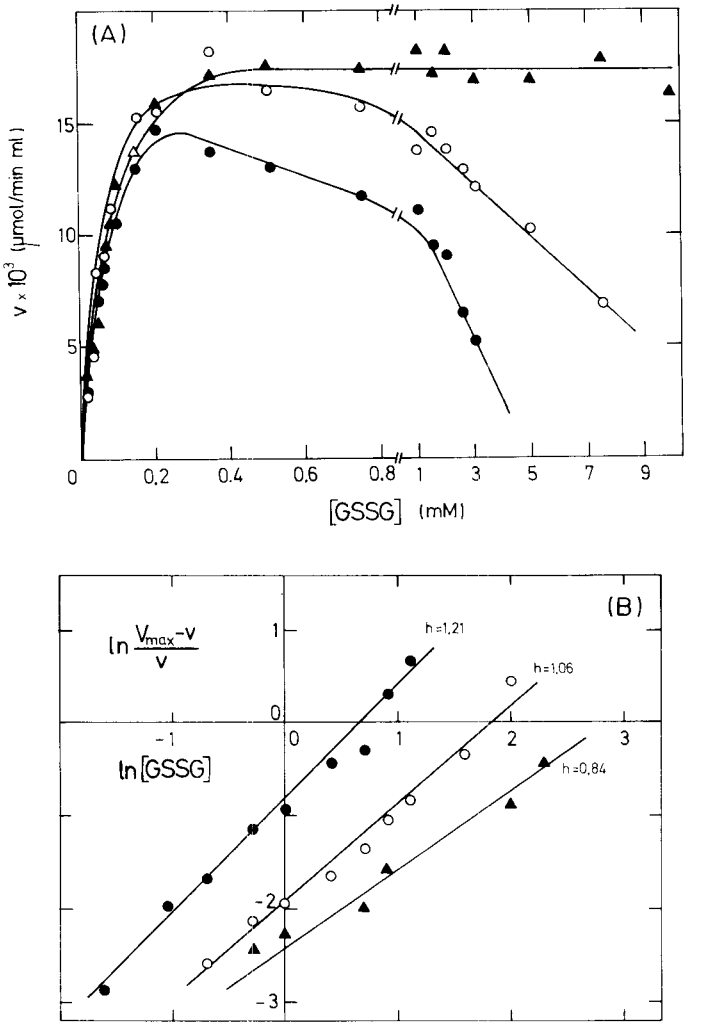

FIGURE 6 Effect of pH on the GSSG substrate inhibition shown by *Phycomyces* glutathione reductase. All assays were performed at 30°C at constant ionic strength (I = 0.12 M) in sodium phosphate buffer at the pH values indicated and a constant NADPH concentration (100 μM) using 0.8 μg of enzyme/ml of reaction mixture: (●), pH 6.0; (○), pH 6.5 and (▲), pH 7.0. A) Dependence of v_{ss} with pH. B) Hill plots of the data according to Kurganov[14].

The degree of hysteresis was affected by pH and the ionic strength (I) of the buffer used. As can be seen in Figure 4, the apparent k value increases substantially with increasing pH, indicating thus that hysteresis is less prominent at basic pH values. On the other hand, it was observable that as the pH value increased, a higher GSSG concentration was required to obtain a similar k value, although the pattern of the hysteresis dependence on pH was unchanged when the GSSG concentration varied. A similar pattern of the hysteresis dependence on the ionic strength was obtained. At 2 mM GSSG, the apparent k value increased from 0.44 min^{-1} to 1.26 min^{-1} when the ionic strength was raised from 0.045 M to 0.36 M in sodium citrate buffer pH 5.5.

TABLE II

Effect of pH and ionic strength (I) on the allosteric parameters obtained from GSSG substrate inhibition using the v_{ss} data obtained from *Phycomyces* glutathione reductase. The assays were performed at 30°C, a constant NADPH concentration (100 μM) and variable GSSG concentration in 50 mM sodium phosphate buffer (I = 0.12 M) for experiment A and in sodium citrate buffer pH 5.5 for experiment B.

Experiment A			Experiment B		
pH	$[GSSG]_{0.5}$* (mM)	h**	I (M)	$[GSSG]_{0.5}$* (mM)	h**
6.0	2.0	1.21	0.045	1.73	2.34
6.5	6.3	1.06	0.090	2.74	1.22
7.0	18.6	0.84	0.360	5.20	0.95

*$[GSSG]_{0.5}$ is the substrate concentration at which half of the maximum inhibition is obtained.
**h = Hill coefficient.

The apparent k value decreased, and thus, hysteretic behaviour increased, when the GSSG or NADPH concentration increased at fixed values of a constant substrate, pH and ionic strength. Figure 5 shows the results of a series of experiments carried out in 25 mM sodium citrate buffer pH 5.5 at fixed NADPH and fixed GSSG concentration, respectively. As can be seen, the hysteresis dependence was similar, it being evident that the apparent k appeared to tend to a constant value independent of the constant substrate concentration; however, at low varied substrate concentration, the apparent k value was dependent on the fixed substrate concentration. The analysis of the kinetic behaviour with respect to v_i and v_{ss} was similar in both cases: v_i and v_{ss} showed allosteric GSSG substrate inhibition[14] that was dependent on pH and the

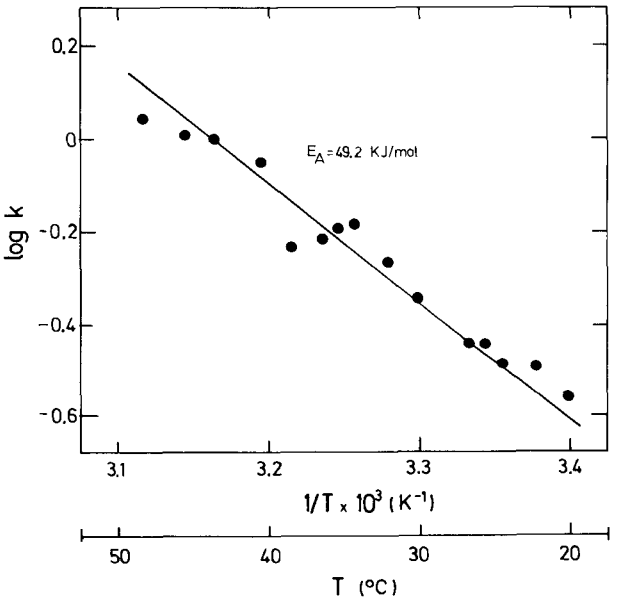

FIGURE 7 The Arrhenius plot of the dependence of hysteretic behaviour shown by *Phycomyces* glutathione reductase on temperature. The assays were carried out in 25 mM sodium citrate buffer pH 5.5, at 5 mM GSSGT and 100 μM NADPH, using 1.6 μg of enzyme/ml of reaction mixture. k is expressed in min^{-1}.

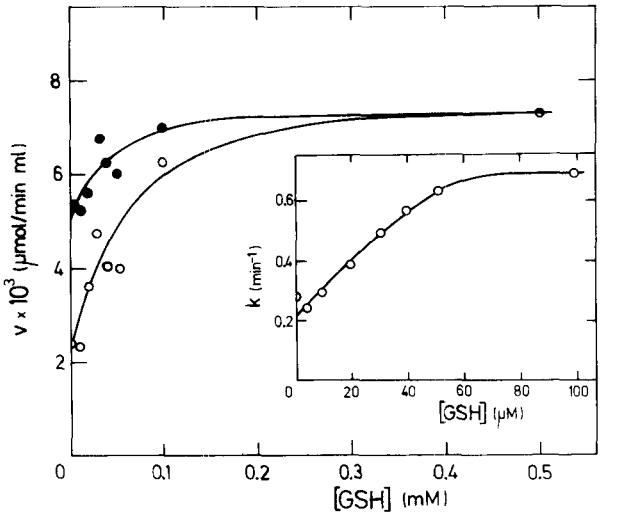

FIGURE 8 Effects of GSH concentration on the hysteretic behaviour shown by *Phycomyces* glutathione reductase. Plots of v_i (○) and v_{ss} (●) *versus* GSH concentration. The assays were performed at 30°C in 25 mM sodium citrate buffer pH 5.5, at constant GSSG (5 mM) and NADPH (100 µM) concentration, using 0.8 µg of enzyme/ml of reaction mixture. *Inset:* Variation of the hysteretic rate constant (k) with GSH concentration.

ionic strength. The GSSG concentration at which substrate inhibition was detectable increased when the pH value or the ionic strength of buffer increased, the decrease in the degree of inhibition also being evident. Figure 6 shows the dependence of v_{ss} with respect to pH. The analysis by Hill plots of the allosteric GSSG substrate inhibition data with pH according to Kurganov[14] (Figure 6.B) showed that the $[GSSG]_{0.5}$ value shifted to higher values, and that the h value decreased when pH was raised from 6.0 to 7.0. Similar results were obtained from the study of the GSSG substrate inhibition dependence on ionic strength. The overall results are summarized in Table II. These results indicated a good correlation between loss of hysteresis and loss of allosteric substrate inhibition. According to Ainslie *et al.*[7], and Neet and Ainslie[9], the correlation of the changes in the kinetic transient with changes in the cooperativity observed as experimental conditions change is circumstantial evidence that the two processes are mechanistically related. In a kinetic mechanism of cooperativity, the progress curve transient and steady-state Hill coefficients are different combinations of the same rate constants and thus, a change in one necessitates a change in the other.

Hysteresis was also affected by temperature. The temperature dependence of k was explored over a temperature range from 20°C to 50°C. The apparent k value increased with increasing temperature (hysteresis decreased). An Arrhenius plot of the data showed an apparent activation energy of 49.2 KJ/mol (Figure 7). Catalytic activity (referred to v_i and v_{ss}) showed a similar value of apparent energy, indicating again that the changes leading to the hysteretic behaviour of the enzyme can be the same as those affecting its catalytic activity.

Finally, we examined the effects of the addition of the reaction products, $NADP^+$ and GSH, on hysteretic behaviour. Figure 8 shows the dependence of v_i, v_{ss} and k on GSH concentration. The following effects were seen; (a) at low concentrations, GSH

produced a marked activation effect on v_i (at 0.5 mM GSH, the v_i value was 325% with respect to that obtained in the absence of GSH), this effect on v_{ss} being much smaller, (b) the hysteretis effect diminished as the concentration of GSH increased; as can be seen (Figure 8, *inset*) the k value increased as the GSH concentration was raised, so at 0.5 mM GSH hysteretic behaviour was cancelled ($v_i = v_{ss}$). The apparent activation constant for GSH, obtained from a double reciprocal plot of the v_i data, was 9 μM; GSH concentration above 0.5 mM produced the characteristic product inhibition pattern[5]. Similar results were obtained in the presence of $NADP^+$.

All these results and the observation that the accumulation phase of the reaction (corresponding to v_{ss}) occurred after approximately the same concentrations of reaction products had been accumulated, led us to assume that the hysteretic phenomenon described here was clearly a time-dependent process, and could be due to a time-dependent process of accumulation of reaction products rather than to a slow conformational change. The dependence of hysteresis on the GSH or $NADP^+$ concentrations, especially, appears to support this hypothesis. The apparent activation constant values (9–10 μM) for GSH and $NADP^+$ were similar to the product concentrations accumulated at the onset of the acceleration phase of the reaction progress curves.

Since the hysteresis phenomenon is observed only when GSSG substrate inhibition is appreciable, the most simple explanation for our results could be consistent with a similar mechanism proposed for the hysteretic behaviour shown by bovine glutamate dehydrogenase[18] which supported a flip-flop mechanism[19]. Such models suggest that, in the steady state, half of the active sites are occupied by reactants and half by products. Under experimental conditions, where GSSG inhibition is noticeable, *Phycomyces* glutathione reductase is inhibited by GSSG binding to an inhibitory site[15]. The accumulation of reaction products and their binding to the active sites produce a conformational change that results in a decrease in the inhibitory effect produced by GSSG, and thus, an acceleration in the reaction progress curve. This model provides a rational explanation for the dependence of the apparent hysteretic behaviour on the enzyme concentration, since as the active enzyme concentration decreases, the time necessary for the accumulation of reaction products would increase as, therefore, does the time for appreciable acceleration of progress curve.

In conclusion, although the experimental data described in this paper may explain the hysteretic behaviour shown by *Phycomyces* glutathione reductase in terms of the model proposed above, we cannot fully rule out the possibility of the hysteretic phenomenon being produced by a slow conformational change between two different states of the active ternary complex, although this seems less likely to us.

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