

BBA 66986

## STUDIES ON THE KINETICS AND MECHANISM OF REDUCTION OF FLAVODOXIN FROM *PEPTOSTREPTOCOCCUS ELSDENII* BY SODIUM DITHIONITE

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(Received March 12th, 1973)

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### SUMMARY

1 The reactions of the oxidised and semiquinone forms of *Peptostreptococcus elsdenii* flavodoxin with sodium dithionite have been studied by stopped flow spectrophotometry. At low ionic strength where comproportionation of flavodoxin is slow compared with the overall rate of reduction, the reactions with an excess of dithionite follow first-order kinetics. Semiquinone is not detected during the reduction of oxidised flavodoxin, possibly because the semiquinone is reduced very much faster than the oxidised protein.

2 The rates of reduction are proportional to the square root of the dithionite concentration. This observation suggests that the reducing species is not dithionite itself, but a dissociation product. It is proposed that the reducing species is  $\text{SO}_2^-$ . The results support a mechanism in which the reduction of oxidised flavodoxin occurs by two successive one-electron transfers, and is limited by the rate of reduction to the semiquinone level.

3. The reaction of oxidized flavodoxin and fully reduced flavodoxin to form semiquinone is very sensitive to ionic strength and the salt composition of the medium. Increasing ionic strength causes a marked increase in rate. The rate constant at zero ionic strength, calculated by extrapolation, was  $100 \text{ M}^{-1} \cdot \text{min}^{-1}$  at 25 °C.

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### INTRODUCTION

The flavodoxins are a group of flavoproteins which serve as electron carriers between other oxidation–reduction proteins (see references in ref. 1). Although they do not normally react directly with small oxidizable substrates such as the reduced pyridine nucleotides, they are reduced by the non-specific reducing agent sodium dithionite. Earlier we reported titration experiments which showed that 1 mole of flavodoxin from *Peptostreptococcus elsdenii* is reduced by 1 mole of sodium dithionite, and that at half reduction the neutral form of FMN semiquinone is formed

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almost quantitatively<sup>2</sup>. Since it was also shown that the semiquinone can be generated from mixtures of oxidized and reduced protein<sup>2</sup>, we tentatively concluded that the semiquinone observed in titrations is formed by such a comproportionation reaction. There were indications that dithionite reduction of the semiquinone is more rapid than reduction of the oxidized protein, but titration experiments of this kind gave little further information about the overall reaction mechanism. Thus these experiments did not show whether fully reduced flavodoxin is produced directly from the oxidized protein by a single two-electron transfer from dithionite, or alternatively, whether two sequential one-electron transfers occur with the flavin semiquinone as an intermediate.

More recently, this question has been discussed in connection with a related flavodoxin (phytoflavin) from *Anacystis nidulans* (see discussion following ref. 3). In this case it was proposed that dithionite is a two-electron donor to the oxidized protein, but again direct experimental evidence was lacking.

We have now investigated this reaction with *P. elsdenii* flavodoxin by measuring the kinetics of reduction in a stopped flow spectrophotometer. This paper describes the kinetics of the reaction of dithionite with oxidized flavodoxin and flavodoxin semiquinone, and also a previously unreported effect of ionic strength on the comproportionation between oxidized and fully reduced flavodoxin.

#### METHODS AND MATERIALS

Flavodoxin was prepared from *P. elsdenii* as described elsewhere<sup>4</sup>. Anaerobic titrations were performed under purified nitrogen in the apparatus of Foust *et al*<sup>5</sup>. Solutions of sodium dithionite (Eastman Kodak) were standardized by titration into lumiflavin-*N*(3)-acetic acid, and with the use of a difference extinction coefficient of  $11\,300\text{ M}^{-1}\cdot\text{cm}^{-1}$  for oxidized *minus* reduced flavin (the difference extinction coefficient given previously for this compound<sup>5</sup> is now known to be incorrect). Extinction coefficients for sodium dithionite were determined from the same titrations by continuing the incremental addition of dithionite after the flavin had been completely reduced, and by measuring the subsequent increases in absorption at 315 nm (the absorption maximum of sodium dithionite).

Stopped flow spectrophotometry was performed in the apparatus of Gibson and Milnes<sup>6</sup>. Solutions of sodium dithionite for use in stopped flow experiments were made by transferring samples from an anaerobic stock solution of the reagent (approximately 0.01 M in 0.1 M glycine-NaOH buffer, pH 8.5) into stopped flow tonometers which contained an appropriate amount of anaerobic buffer solution. After completion of a series of stopped flow experiments, a sample from the diluted solution was transferred anaerobically from the tonometer to the burette of the titration apparatus mentioned above<sup>5</sup>, and the dithionite determined by titration into lumiflavin-*N*(3)-acetic acid. The anaerobic transfer of dithionite was made through a short length of butyl rubber tubing using different pressures of nitrogen in the burette and tonometer.

#### RESULTS

##### *Properties of dithionite*

As reported by Dixon<sup>7</sup>, the reducing properties of anaerobic solutions of sodium dithionite are not appreciably changed during long term storage at pH

values above 7. In contrast to Dixon<sup>7</sup>, however, we find that dithionite is also moderately stable under mildly acidic conditions. For example, the concentration of a solution in 0.1 M sodium acetate, pH 6, fell from 2.7 to only 1.6 mM during 4 days, as determined by titration *versus* lumiflavin-*N*(3)-acetic acid. At pH 5.6 in citrate buffer, the rate of loss of dithionite was slightly higher (19% in 24 h), but this rate is considerably lower than those observed by Dixon<sup>7</sup> who reported a decay rate of about 10% /min at pH 6.

In order to quantitate the results of stopped flow experiments described later, we required a reliable method for the determination of dithionite. Initially we proposed to determine this compound from its absorption at 315 nm<sup>7</sup>. However we observed firstly, that the extinction coefficient of freshly prepared solutions of sodium dithionite in 0.1 M sodium pyrophosphate, pH 8.3, shows a considerable variation (6450 M<sup>-1</sup>·cm<sup>-1</sup> to 7980 M<sup>-1</sup>·cm<sup>-1</sup> for different solutions), and secondly, that the extinction coefficient of a stock solution decreases slowly during storage without undergoing any noticeable qualitative changes in its absorption spectrum. The rate and extent of this decrease were somewhat variable, but in a typical case, the determined extinction coefficient dropped from 7980 to 5600 M<sup>-1</sup>·cm<sup>-1</sup> during 8 days. There were no detectable changes in the reducing properties of the solution during this time. While we have no chemical explanation for these observations, they may help to explain the wide variation in reported values for the extinction coefficient of sodium dithionite<sup>7-10</sup>.

We have also observed an effect of concentration on the extinction coefficient of dithionite at low pH (Fig. 1). In these experiments a stock solution of dithionite at pH 8.3 was added in increments to anaerobic buffer at pH 5.6. After the first additions, there is a rapid first order decrease of absorption at 315 nm during several minutes, followed by a very much slower decrease over many hours. The rate and extent of the rapid change depend on the dithionite concentration. When further additions of dithionite are made to the same diluted solution, the rate and also the extent of the rapid decrease become progressively lower. At high concentrations (2·10<sup>-4</sup> M), only the slow changes occur, and the extinction changes parallel those seen during a similar experiment at pH 8.3 (Fig. 1). It is possible that the rapid changes

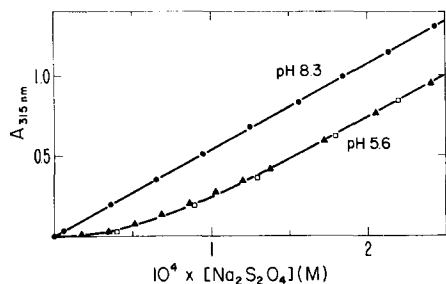


Fig. 1. Effect of pH and concentration on the absorbance of sodium dithionite. A stock solution of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (4.3·10<sup>-3</sup> M in 0.1 M sodium pyrophosphate, pH 8.3) was added in increments<sup>5</sup> to 3 ml anaerobic buffer at pH 8.3 (0.1 M sodium pyrophosphate) or pH 5.6 (0.1 M sodium citrate) at 20 °C. The absorbance at 315 nm in the buffer at pH 5.6 was measured after completion of the rapid decrease described in the text. Δ, □, data from separate titrations. The extinction coefficient of the stock solution of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> was 5370 M<sup>-1</sup>·cm<sup>-1</sup> as determined with lumiflavin-*N*(3)-acetic acid.

at low concentrations of dithionite correspond to the decay rate of 10% /min reported by Dixon<sup>7</sup> at pH 6.

While we do not understand these effects, they do indicate that the estimation of dithionite from absorption measurements at 315 nm is subject to uncertainty, and that it is preferable to determine the reducing equivalents in a solution by direct titration into a suitable oxidation-reduction compound similar to the one under investigation. All solutions of sodium dithionite used in this work were therefore standardized by titration into lumiflavin-*N*(3)-acetic acid.

#### *Effect of ionic strength on the comproportionation of flavodoxin*

It was reported previously that oxidized and fully reduced flavodoxin from *P. elsdenii* react together in a second-order reaction to give flavodoxin semiquinone<sup>2</sup>. In the course of the present experiments, it became clear that under certain conditions this comproportionation reaction is rapid and can become important in the overall reduction of oxidized flavodoxin by sodium dithionite. In seeking conditions to eliminate this complication, we discovered that the rate of comproportionation is drastically influenced by ionic strength (*I*). At low ionic strength (*I* 0.015) the reaction is slow and requires several hours for completion; an increase of ionic strength causes a marked increase in rate, such that at *I* 0.24, for example, the reaction is complete in less than 2 min. Second order rate constants for the reaction were determined in glycine-NaOH buffer, pH 8.5, with NaCl to give the required ionic strength (Fig. 2A). The rate at zero ionic strength, obtained by extrapolation of a plot of  $\log_{10}$  rate constant versus  $\sqrt{I}$  (Fig. 2B), was approximately  $100 \text{ M}^{-1} \cdot \text{min}^{-1}$ ; at *I* 0.16 the measured rate constant is  $1.4 \cdot 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ .

These observations are in accord with collision theory which suggests that when two ions of like charge interact, an increase of ionic strength of the solution will enhance the rate of reaction<sup>11</sup>. At pH 8.5, flavodoxin carries a net negative charge (isoelectric point < 5). The charge is probably similar in all three oxidation-reduction states of the molecule, although it should be noted that fully reduced flavodoxin has a slightly different charge due to an ionization at  $\text{p}K' = 5.8$  (ref. 2).

The rate of comproportionation at low ionic strength is not appreciably

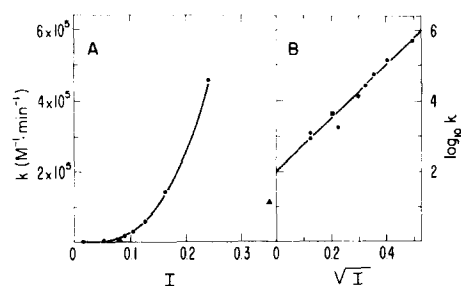


Fig. 2. Effect of ionic strength on the rate of formation of semiquinone from oxidized and fully reduced flavodoxin. Flavodoxin ( $0.04 \mu\text{mole}$ ) in 2.5 ml buffer, pH 8.5, was titrated to full reduction with an equal amount of sodium dithionite. Oxidized flavodoxin,  $0.04 \mu\text{moles}$  in 0.15 ml buffer, was then added from a side arm and the reaction followed at 580 nm. Second-order rate constants were determined<sup>2</sup> from the experimental curves. ●, glycine-NaOH buffer plus NaCl to give the indicated ionic strength; △, Sodium pyrophosphate buffer; ■, Tris-HCl buffer. (A) Plot of the observed second-order rate constant ( $k$ ) versus  $\sqrt{I}$ . Temperature, 25 °C.

affected by addition of FMN (0.1 mole per mole of flavodoxin), showing that the observed increases in rate at high ionic strength are not due to dissociation of flavin from flavodoxin. Furthermore, the amount of semiquinone observed after comproportionation is independent of ionic strength; if significant amounts of the holo-protein dissociated, a corresponding reduction in the semiquinone concentration would be expected. In addition, since the yields of semiquinone were the same in all of these experiments, we can conclude that the semiquinone formation constant ( $K = [FlH^{\cdot-}]^2/[Fl_{ox}][Fl_{red}]$ ) is not appreciably affected by ionic strength, and therefore that the rate of dismutation of the semiquinone changes to correspond with the observed changes in the comproportionation rate.

There is some evidence that the comproportionation rate of this flavodoxin is also subject to specific ion effects. Thus the rate in pyrophosphate buffer is lower than in glycine-NaCl mixtures of similar ionic strength (Fig. 2A).

#### *Reduction of flavodoxin by dithionite*

At pH values above 6.7, *P. elsdenii* flavodoxin is reduced by 1 and 2 equivalents of dithionite to the semiquinone and fully reduced forms, respectively<sup>2</sup>. At lower pH values, reduction beyond the semiquinone is more difficult, and at pH 5.2 for example, approximately 12% of the flavin remains as the semiquinone in the presence of a 12-fold molar excess of dithionite. When 1 reducing equivalent of dithionite is added to the oxidized protein at pH 7 or above and at high ionic strength, semiquinone forms rapidly before measurements can be made in a static spectrophotometer<sup>2</sup>. The changes are slower at low ionic strength (0.05), and it is clear that under these conditions half of the oxidized protein becomes fully reduced and all of the dithionite absorption at 315 nm disappears before the semiquinone begins to accumulate. This suggested that the semiquinone observed in static titration experiments<sup>2</sup> is formed by comproportionation and not by a direct one-electron reduction of the oxidized protein. However, it did not rule out the semiquinone as an intermediate in the overall reduction of flavodoxin by dithionite, and to assess this possibility we examined the kinetics of the reaction in a stopped flow spectrophotometer.

To ensure that comproportionation of oxidized and reduced flavodoxin was slow compared with the measured rates of reduction, the reactions of flavodoxin and dithionite were usually studied at low ionic strength ( $I$  0.06). Under these conditions, the reaction of oxidized flavodoxin with an excess of dithionite follows first-order kinetics (Fig. 3). The reactions of Fig. 3 were followed at 445 nm, an absorption maximum in the spectrum of the oxidized protein. Within the limits of detection of our instrument, there were no changes at 580 nm, a wavelength where only the semiquinone absorbs<sup>2</sup>; the absorbance at 580 nm was zero throughout the reaction. The reaction of flavodoxin semiquinone with excess dithionite also follows first order kinetics (Fig. 4), but the rate of this reaction is very much faster than the rate of reduction of the oxidized protein (approximately 450 time greater). It is clear from these large differences in rate that if flavin semiquinone is formed as an intermediate in the overall reduction of the oxidized protein, then it would be almost impossible to detect directly.

For a simple second-order reaction between flavodoxin and dithionite, the rate of reaction under pseudo-first-order conditions should depend directly on the concentration of dithionite. We find, however, that the observed rate constants are

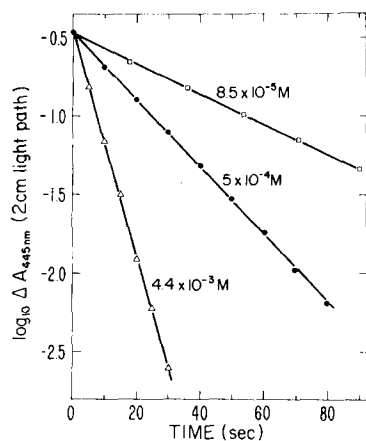


Fig. 3. Changes at 445 nm (2 cm light path) during the reaction of oxidized flavodoxin with sodium dithionite. Flavodoxin ( $4 \cdot 10^{-5}$  M) in 0.1 M glycine-NaOH buffer, pH 8.5, was mixed with sodium dithionite in the same buffer. The final concentration of dithionite is indicated on each curve. Temperature, 25 °C.

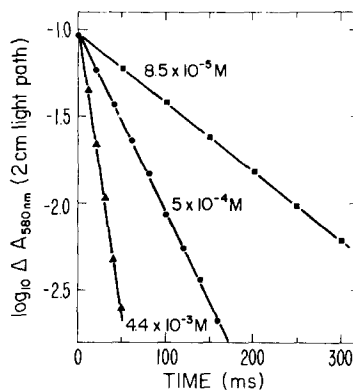


Fig. 4. Changes at 580 nm (2 cm light path) during the reaction of flavodoxin semiquinone with sodium dithionite. Flavodoxin semiquinone ( $2.6 \cdot 10^{-5}$  M), produced by photoirradiation in 0.1 M glycine-NaOH buffer, pH 8.5, and 0.001 M EDTA was mixed with sodium dithionite (in 0.1 M glycine-NaOH, pH 8.5) to give the final concentration indicated on each curve. Temperature, 25 °C.

not linear with dithionite over a 50-fold range of concentration. Instead, the rate constants increase with the square root of the dithionite concentration (Fig. 5). Such a dependency suggests that the reducing species is a dissociation product of dithionite, rather than dithionite itself.

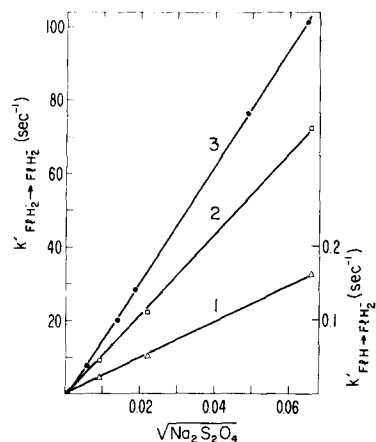


Fig. 5. Variation of the rate constant for the reduction of flavodoxin with the square root of the dithionite concentration. Curve 1, oxidized flavodoxin in glycine-NaOH, pH 8.5; Curve 2, flavodoxin semiquinone in glycine-NaOH, pH 8.5, Curve 3, flavodoxin semiquinone in 0.1 M sodium pyrophosphate, pH 8.5. The rate constants ( $k'$ ) are the observed pseudo first-order constants calculated from the data of Figs 3 and 4. The scale on the right hand axis is for Curve 1; the scale on the left for Curves 2 and 3.

The experiments described so far were done in glycine-NaOH buffer at an ionic strength of 0.06. Very similar results are obtained in Tris-HCl at the same pH and ionic strength. However, in buffer of higher ionic strength, the reduction of oxidized flavodoxin no longer follows pseudo-first-order kinetics and the reaction accelerates (Fig. 6). Again, semiquinone does not accumulate during the reaction. Nevertheless, the acceleration is probably due to the formation of semiquinone from oxidized and fully reduced flavodoxin and the subsequent rapid reduction of the semiquinone. Comproportionation is known to be fast under these conditions, and the rate of comproportionation would increase from zero to a maximum at 50% reduction when the concentrations of oxidized and fully reduced protein are equal. The rate of reduction of the semiquinone is somewhat enhanced at high ionic strength, but this reaction remains pseudo-first-order, and dependent on the square root of the dithionite concentration (Fig. 5, Curve 3).

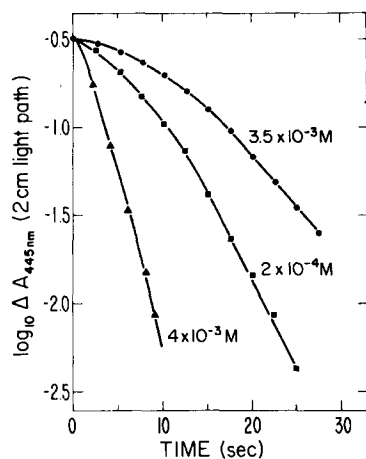


Fig. 6. Changes in absorbance at 445 nm (2 cm light path) during the reduction of oxidized flavodoxin with dithionite in pyrophosphate buffer. Oxidized flavodoxin ( $4 \cdot 10^{-5}$  M) in 0.1 M sodium pyrophosphate buffer, pH 8.5, was mixed with sodium dithionite in the same buffer. The final concentration of dithionite is indicated on each curve. Temperature, 25 °C.

## DISCUSSION

The kinetic pattern observed for the reduction of *P. elsdonii* flavodoxin can be explained if the reducing species is  $\text{SO}_2^-$  rather than dithionite. Solutions of sodium dithionite are known to contain  $\text{SO}_2^-$  (refs 12-16) according to Equilibrium 1



The complete reduction of oxidized flavodoxin may then occur in two sequential one-electron transfers according to equations 2 and 3.



Since the rate of Reaction 3 is fast compared with the overall reduction of oxidized flavodoxin, we conclude that in the latter reaction,  $k_3$  is limiting ( $k_4/k_3 = 450$ ) so that with an excess of dithionite, the semiquinone is not detected.

This proposed mechanism could explain the observed pseudo first-order reactions and their square root dependency on the dithionite concentrations, but only if Equilibrium 1 is maintained, and the concentration of  $\text{SO}_2^-$  does not change appreciably during the reaction. Lynn *et al.*<sup>15</sup> determined the equilibrium constant,  $K$  ( $K = k_2/k_1$ ) for Reaction 1 from measurements of the concentration of  $\text{SO}_2^-$  by electron spin resonance. Based on their value for  $K$  ( $1.6 \cdot 10^9 \text{ M}^{-1}$  at  $25^\circ \text{C}$  in  $0.1 \text{ M NaOH}$ ) and values published earlier for  $k_1/\sqrt{K}$  (refs 12 and 14) they obtained a value of  $40 \text{ s}^{-1}$  for  $k_1$ . With these data, a value of  $6.4 \cdot 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$  can be calculated for  $k_2$  ( $k_2 = Kk_1$ ). More recently, a lower value for  $k_2$  (approximately  $5.5 \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ ) has been determined from flash photolysis experiments<sup>16</sup>. Combination of this value with the equilibrium constant of Lynn *et al.*<sup>15</sup> gives a value of  $3.44 \text{ s}^{-1}$  for  $k_1$ .

For Equilibrium 1 to be maintained, the following condition must apply in the rapid reaction with flavodoxin semiquinone:

$$k_1[\text{S}_2\text{O}_4^{2-}] = k_2[\text{SO}_2^-]^2 > k_4[\text{FIH}_2^+][\text{SO}_2^-]$$

where  $k_4[\text{SO}_2^-]$  is the observed pseudo first order rate constant for the reduction of flavodoxin semiquinone, and is equivalent to  $k_4[\sqrt{\text{S}_2\text{O}_4^{2-}}/K]$ . Calculation shows that with the higher value for  $k_2$  obtained from the data of Lynn *et al.*<sup>15</sup>,  $k_2[\text{SO}_2^-]/k_4[\text{FIH}_2^+] \approx 10$  for the lowest dithionite concentration ( $3.5 \cdot 10^{-5} \text{ M}$ ) used in the present experiments. This ratio increases with increasing dithionite, such that at  $4 \cdot 10^{-3} \text{ M}$  dithionite it is approximately 80. However, with the value for  $k_2$  reported by Hayon *et al.*<sup>16</sup>, the ratio falls to 1 at the lowest dithionite concentration, a value insufficient to maintain Equilibrium 1.

It is clear that a final assessment of this proposed mechanism cannot be made without further studies on Equilibrium 1, and in particular under the conditions of pH and ionic strength used in the present work. Nevertheless, this mechanism seems as consistent as any with the kinetic results and the information presently available on dithionite chemistry.

From the preceding discussion it is evident that the formation of semiquinone on titration of a flavoprotein with dithionite is not necessarily indicative that the reducing species is  $\text{SO}_2^-$ . The possibility exists that semiquinone formation in such cases arises from a comproportionation reaction between oxidized and fully reduced species;  $\text{Fl}_{\text{ox}} + \text{Fl}_{\text{red}} \rightleftharpoons 2\text{FIH}^+$ . However, this situation is easily subject to experimental test. In the case of flavodoxin, as described in this paper, the comproportionation reaction proceeds readily, but with a rate markedly dependent on the ionic strength. Thus, even though the rate of reduction of the oxidized flavoprotein to the semiquinone is slower by two orders of magnitude than the subsequent reduction of the semiquinone to the fully reduced species, very substantial amounts of semiquinone are observed on addition of 1 electron equivalent of dithionite at high ionic strength, presumably because of the rapid comproportionation reaction under these conditions. With many other flavoproteins it may also be concluded that reduction by dithionite occurs in two discrete one-electron reduction steps, with  $\text{SO}_2^-$  probably being the reaction species. Thus, almost quantitative yields of semiquinone are obtained rapidly in the course of dithionite titration of such enzymes as glucose oxidase<sup>17,18</sup>,



D-amino acid oxidase<sup>17,18</sup>, L-amino acid oxidase<sup>19</sup>, yet under the same conditions equimolar mixtures of the oxidized and fully reduced enzymes form radical with half times of hours or days<sup>17-19</sup>. In these cases the comproportionation reactions are clearly unimportant; the finding of nearly quantitative yields of semiquinone on titration with 1 electron equivalent of dithionite indicates that the rate of reduction of the oxidized enzyme to the semiquinone form must be very much faster than the subsequent reduction of the semiquinone to fully reduced enzyme. In other cases (where comproportionation is also slow) such as old yellow enzyme<sup>20</sup>, much smaller yields of semiquinone are obtained at half reduction, indicating that the rates of reduction of both steps are similar. In other cases, such as *p*-hydroxybenzoate hydroxylase<sup>21</sup> or melilotate hydroxylase<sup>22</sup> essentially zero yields of semiquinone are found at half reduction. While such results could be indicative of a single two-electron reduction, they are equally well explained in terms of the two-step process, in which the rate of reduction of semiquinone is much faster than its rate of production from oxidized enzyme. In this case they would differ from flavodoxin only in lacking the comproportionation reaction.

Previously reported results with the *Azotobacter* flavoprotein, *Shethna* flavoprotein<sup>1</sup> have revealed yet another type of reactivity with dithionite. This protein is reduced to the semiquinone, but at neutral pH values, very little further reduction was found, even in the presence of considerable molar excesses of dithionite. These results may indicate that the oxidation-reduction potential of the couple  $\text{SO}_2^- - \text{SO}_2$  may not be as low as is usually considered.

#### ACKNOWLEDGEMENTS

We are grateful for helpful discussions with Drs D. Lambeth, G. Palmer and P. Hemmerich.

This work was supported by USPHS grant GM 11106.

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