Biochimica et Biophysica Acta, 309 (1973) 32–43

BBA 66890

REVERSIBLE INACTIVATION OF THE NITRATE REDUCTASE OF *CHLORELLA VULGARIS* BEIJERINCK

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(Received December 14th, 1972)

SUMMARY

The ferricyanide-activated NADH:nitrate oxidoreductase (EC 1.6.6.1) of *Chlorella vulgaris* Beijerinck, in extracts freed of low molecular weight components, undergoes a reversible inactivation on addition of an ultrafiltrate containing cellular components in the molecular weight range 1000–10 000. This inactivation reaction, which goes virtually to completion, accounts for the fact that the enzyme in the original cell extracts is present almost entirely in the inactive form. NADH or NADPH mimic the effect of the unknown components of the ultrafiltrate. In all cases, the inactivation occurs more readily at pH 7.6 than at pH 6.7, and is prevented by nitrate. The inactivated enzyme can be reactivated with ferricyanide. A partial, reversible inactivation also occurs in the absence of any additions, when extracts freed of low molecular weight components, are brought to a pH of 8.8. The diaphorase component of the nitrate reductase is not inactivated to a substantial degree by any of these procedures.

Partially purified, activated nitrate reductase is only slowly and partially inactivated by added NADH or NADPH. The ultrafiltrate alone has no effect on the partially purified, activated enzyme, but enhances the inactivating effect of the reduced pyridine nucleotides. The inactivated enzyme can be reactivated with ferricyanide.

INTRODUCTION

The NADH:nitrate oxidoreductase (EC 1.6.6.1) of the Berlin strain of *Chlorella vulgaris* Beijerinck[1] is obtained in crude extracts largely in an inactive form[2,3], which can be activated by oxygen in the unfractionated extracts, or by ferricyanide[4]. The recent studies of Losada and his associates on the inactivation and activation of the nitrate reductase of *Chlorella fusca*[5,6] and of *Chlamydomonas reinhardti*[7,8], show that, in spite of differences, there are also strong similarities between the inactivation and activation of these latter enzymes and of the nitrate reductase of *C. vulgaris* Beijerinck. A major difference is that extracts obtained from cells harvested during active

autotrophic growth with nitrate as a nitrogen source, contain the enzyme primarily in the inactive form in the case of *C. vulgaris*, whereas similarly prepared extracts from the other two species contain the enzyme in the active form. The Spanish group have shown, however, that in the latter case, the active enzyme can be inactivated by added NADH or NADPH, and that the inactive enzyme can be activated by ferricyanide.

The present study is concerned primarily with the reversible inactivation of the activated enzyme of *C. vulgaris*, a process as yet rather ill-defined. We will present evidence that the cell extracts of the Berlin strain contain components which can cause a rapid and reversible inactivation of the ferri cyanide-activated enzyme. We will also show that NADH and NADPH can likewise cause a reversible inactivation of the unpurified activated enzyme preparation, but that one or more as yet unidentified components are also required to obtain complete and rapid inactivation of the partially purified enzyme.

METHODS

Preparation of cell extract SE

The cells of the Berlin strain of *C. vulgaris* Beijerinck were grown autotrophically in continuous light with nitrate as a source of nitrogen, harvested during active growth, and broken by sonication as previously described[2,3]. After storage at −20 °C for at least 24 h, the broken cell suspension, representing about 200 μl cells per ml, was thawed and clarified by centrifugation for 20 min at 28 000 × *g*, to give preparation SE.

Preparation and activation of $SE_{G\text{-}50}$

SE and $SE_{G\text{-}50}$ were prepared fresh each day from frozen extract. $SE_{G\text{-}50}$ was prepared from SE, at 4 °C. 6 ml SE were run through a column (1.4 cm × 24 cm) of Sephadex G-50 Medium, which had been equilibrated with 1 mM phosphate buffer, pH 7.6. The pale green colour was used as a guide for beginning sample collection. About 6.2 ml were collected, to give $SE_{G\text{-}50}$. To activate $SE_{G\text{-}50}$, 1 ml 0.5 M phosphate buffer, pH 6.7, and 2 ml 5.0 mM $K_3Fe(CN)_6$ were mixed with 6 ml $SE_{G\text{-}50}$. After 15 min at room temperature, the reagents were removed in the cold on another Sephadex column, in the manner just described.

Preparation of ultrafiltrate Fraction X

About 50 ml of fresh SE were filtered through a PM 30 Amicon Diaflo membrane under N_2 at 4 °C. The first 40 ml of filtrate were collected. Then 20 ml of water were added to the retained protein solution, and another 20 ml of filtrate were collected. The combined filtrates were passed through a PM 10 membrane in a similar fashion, and then concentrated on a UM 2 membrane to a volume of about 2.5 ml. This is Fraction X.

For some experiments, Fraction X was further fractionated on a Sephadex G-25 column (1.4 cm × 24 cm) in water. The PM 30 filtrate from 150 ml SE was passed through PM 10 and concentrated on UM 2 to about 3 ml. This was added to the Sephadex column, and 2.5-ml fractions were collected. The 13th and 14th fractions

(designated Fraction X_{13} and X_{14}, *etc.*) contained most of the activity, which was estimated as described under Results.

Preparation of partially purified nitrate reductase

Nitrate reductase was purified as previously described[3] through the protamine sulphate step. The final protamine sulphate precipitate containing the nitrate reductase activity was extracted overnight at 4 °C with a solution containing 0.1 M phosphate buffer, pH 7.2, 0.1 mM dithioerythritol, 0.1 mM EDTA and 0.01 mM chloramphenicol. After centrifugation for 15 min at 30 000 × *g* (4 °C), the clarified extract was concentrated to a volume of 3.0 ml in an Amicon Model 12 ultrafiltration cell equipped with an XM 100A membrane. The concentrated extract was applied to a Bio-Gel A1.5m column (2 cm × 24 cm, $V_0 = 29$ ml) equilibrated with 0.05 M sodium–potassium phosphate buffer, pH 7.2, 0.1 mM dithioerythritol, 0.1 mM EDTA and 0.01 mM chloramphenicol at 4 °C. Fractions of 6.5 ml were collected. The peak of nitrate reductase and cytochrome *c* reductase activities (eluted concurrently, $V_e = 42$ ml) was found in Fraction 7. From an initial 700 units of nitrate reductase (after ferricyanide activation) with a specific activity of 0.11 unit/mg protein in the SE extract, 85 units with a specific activity of 10.3 units/mg protein were recovered in this peak fraction, which had an $A_{280\ nm}/A_{412\ nm}$ absorbance ratio of 5.1.

1 ml of the peak fraction was activated by incubation for 20 min at 0 °C in 0.25 M phosphate, pH 6.7, 0.1 mM $K_3Fe(CN)_6$. Specific activity after activation was 10.3 units of nitrate reductase per mg protein. After activation, the solution was passed through a Sephadex G-50 column (0.9 cm × 12 cm), equilibrated with 0.05 M phosphate, pH 7.6, 0.1 mM dithioerythritol, 0.1 mM EDTA and 0.01 mM chloramphenicol, to remove the ferricyanide. The removal of ferricyanide under these conditions caused no significant loss of activity.

Assay of nitrate reductase and protein determinations

Nitrate reductase was assayed by measuring the rate of oxidation of NADH photometrically at 20 °C as previously described[2]. One unit of nitrate reductase is that amount which catalyzes the oxidation of 1 μmole of NADH per min by nitrate, under the conditions of the assay.

Protein was determined by a modification of the method of Lowry *et al.* (see ref. 9) or by the biuret method[10], as described in the Biochemica Test Combination of Boehringer Mannheim GmbH. The protein content of partially purified enzyme was also estimated from measurements at 280 and 260 nm[10].

Phosphate buffers were prepared from Na_2HPO_4 and KH_2PO_4, Tris buffers from Tris and HCl.

RESULTS

Inactivation of nitrate reductase by ultrafiltrate Fraction X

SE and $SE_{G\text{-}50}$, prepared as described under Methods, both contain nitrate reductase mainly in the inactive form. The behavior of the two preparations is different in the following respects: (a) The striking effect of oxygen on the activation, and the inhibition of the O_2 effect by CO, as previously described for SE (ref. 4), cannot be demonstrated with $SE_{G\text{-}50}$. (b) The activity of nitrate reductase in $SE_{G\text{-}50}$ increases

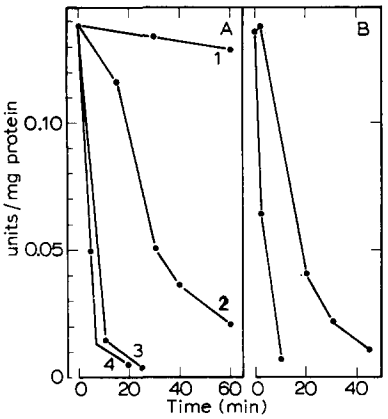

Fig. 1. Inactivation of activated nitrate reductase in SE_{G-50} by the ultrafiltrate, Fraction X. (A) Effect of different amounts of Fraction X. The reaction mixtures all contained 1.0 ml activated SE_{G-50} containing 5.7 mg protein in a final volume of 1.5 ml 0.033 M phosphate buffer, pH 7.6, with the following amounts of Fraction X: 1, none; 2, 0.01 ml; 3, 0.05 ml; 4, 0.10 ml. Incubation was at 20 °C. Small aliquots were removed for assay of nitrate reductase at the times indicated. (B) Effect of pH. The reaction mixtures contained 1.0 ml activated SE_{G-50} containing 6.2 mg protein, and 0.4 ml Fraction X_{13} in 1.5 ml 0.033 M phosphate buffer, pH 6.7 (upper curve) or pH 7.6 (lower curve). Procedure as for (A). There was no appreciable change of activity in the absence of added Fraction X.

slowly on storage, whereas the activity of nitrate reductase in SE decreases.

This latter difference can be explained by the fact that the low molecular weight fraction removed from SE by Sephadex treatment, promotes the inactivation of the activated enzyme. Thus, Fig. 1 shows how the activated nitrate reductase in SE_{G-50} is inactivated after addition of the ultrafiltrate, Fraction X. For the experiment of Fig. 1A, different amounts of Fraction X were added to a given amount of SE_{G-50} in phosphate buffer, pH 7.6. Curve 1 shows the small change in activity in the absence of added ultrafiltrate. Curves 2, 3 and 4 show the decline in activity after addition of 0.01, 0.05 and 0.10 ml Fraction X, respectively, per ml of SE_{G-50}. The active components of Fraction X have been concentrated about 20-fold. The amount of X added to the reaction mixture of Curve 3 was sufficient therefore, to restore the ratio, Fraction X/protein, of the original SE preparation. The experiment of Fig. 1B shows that the inactivation of activated enzyme proceeds more rapidly at pH 7.6 (lower curve) than at pH 6.7 (upper curve).

The inactivation of nitrate reductase after addition of Fraction X, proceeds virtually to completion, if a sufficient amount of Fraction X is employed. In the present enzyme assays, no blanks were subtracted from the measured rates. These blank values (rate of oxidation of NADH in the absence of added nitrate) give values of about 0.002 unit/mg protein. As the enzyme activity measurements approach this level, the measurements become very inaccurate.

The inactive enzyme formed after addition of Fraction X has a stability comparable to that of the inactive enzyme initially present in SE. It can also be reactivated by ferricyanide to the prior high activity level of the activated SE_{G-50} used for the experiment.

It has not been possible, as yet, to devise a reliable method for the quantitative

assay of the active components of Fraction X, partly because the magnitude of response to a given amount of Fraction X varies from one SE_{G-50} preparation to the next, partly because of the complex kinetics of the inactivation reaction. Thus, a lag period in the inactivation by relatively small amounts of Fraction X (Fig. 1A) is characteristic. Note also the 2-min lag period in the upper curve of Fig. 1B. The procedure shown in the experiment of Fig. 1A was therefore used only to compare the activity of different ultrafiltrate preparations, by determining what quantity of each was necessary to give the same response with the same preparation of activated SE_{G-50}.

Inactivation of nitrate reductase by NADH or NADPH

Rapid and reversible inactivation of activated nitrate reductase in SE_{G-50} also occurs after addition of NADH or NADPH, as shown in Fig. 2. Fig. 2A shows the

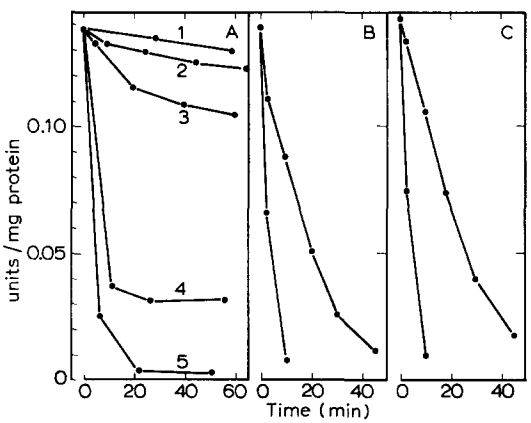

Fig. 2. Inactivation of nitrate reductase by reduced pyridine nucleotides. (A) Different amounts of NADPH. The reaction mixtures contained 1.0 ml activated SE_{G-50} with 5.7 mg protein in a final volume of 1.5 ml 0.033 M phosphate, pH 7.6, with the following concentrations of NADPH: 1, none; 2, 0.004 mM; 3, 0.02 mM; 4, 0.04 mM; 5, 0.2 mM. Procedure as in the legend to Fig. 1. (B) Effect of pH on inactivation by NADPH. The reaction mixtures contained 1.0 ml activated SE_{G-50}, containing 6.2 mg protein, in 1.5 ml 0.033 M phosphate, with 0.4 mM NADPH. Upper curve, pH 6.7; lower curve, pH 7.6. Procedure as in the legend to Fig. 1. (C) Effect of pH on inactivation by NADH. As in B, but 0.4 mM NADH instead of NADPH.

inactivation with different amounts of added NADPH. Results with NADH were similar to those with NADPH except that NADH was more effective at concentrations below 0.04 mM. Comparison of the inactivation seen at concentrations of 0.2 mM led to variable results. With some SE_{G-50} preparations, NADPH was definitely more effective than NADH. With others, there was no clear difference. The inactivation by reduced pyridine nucleotides was more rapid in phosphate buffer of pH 7.6 than in phosphate of pH 6.7. Fig. 2B shows the results with NADPH, Fig. 2C, the results with NADH (*cf.* Fig. 1B). In every case, the inactive product could be reactivated with ferricyanide.

Effect of oxidized pyridine nucleotides on the inactivation by reduced pyridine nucleotides

Added NAD^+ or $NADP^+$ cause no inactivation reaction. They both inhibit the

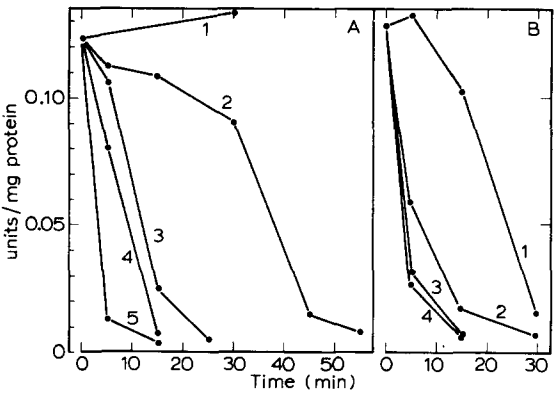

Fig. 3. Effect of oxidized pyridine nucleotides on inactivation by reduced pyridine nucleotides. (A) Effect of adding $NADP^+$ to NADPH. Reaction mixtures contained 1.0 ml $SE_{G\text{-}50}$ with 5.13 mg protein in 1.5 ml 0.033 M phosphate buffer, pH 7.6, with the following additions: 1, none; 2, 0.2 mM NADPH and 1.0 mM $NADP^+$; 3, 0.2 mM NADPH and 0.4 mM $NADP^+$; 4, 0.2 mM NADPH and 0.2 mM $NADP^+$; 5, 0.2 mM NADPH. Procedure as in the legend to Fig. 1. (B) Effect of different combinations. Reaction mixtures contained 1.0 ml $SE_{G\text{-}50}$ with 5.8 mg protein in 1.5 ml 0.033 M phosphate buffer, pH 7.6. 1, 1.0 mM $NADP^+$ and 0.2 mM NADPH; 2, 1.0 mM $NADP^+$ and 0.2 mM NADH; 3, 1.0 mM NAD^+ and 0.2 mM NADH or 0.2 mM NADPH; 4, 0.2 mM NADH or 0.2 mM NADPH. Procedure as in the legend to Fig. 1.

inactivation by the reduced pyridine nucleotides, to a decidedly different extent, $NADP^+$ being the more effective. This is shown in Fig. 3. In Fig. 3A, Curve 1 shows the change in activity with no added pyridine nucleotides, Curve 5 shows the inactivation by 0.2 mM NADPH, and Curves 4, 3 and 2 show the inactivation by 0.2 mM NADPH with 0.2, 0.4 and 1.0 mM $NADP^+$, respectively. A similar experiment with NADH showed decidedly less inhibition of inactivation by added NAD^+. Thus 0.2 mM NAD^+ had no clearly detectable effect on the inactivation by 0.2 mM NADH. It was necessary to use 2.0 mM NAD^+ to obtain a clearly discernable effect, which was not as great as that seen in Curve 4.

The experiment of Fig. 3B shows again how $NADP^+$ effectively retards the inactivation of nitrate reductase by NADPH (Curve 1), and shows further that $NADP^+$ is not as effective in retarding inactivation by NADH (Curve 2). Curve 2 of Fig. 3A and Curve 1 of Fig. 3B represent identical measurements, but with two different preparations of activated $SE_{G\text{-}50}$. Some of the differences between the two pyridine nucleotides might possibly be explained in terms of differences in binding constants. It is not so easy to account for the lag period induced in the inactivation reaction by $NADP^+$, but it is interesting to note that a similar lag period is often observed with the ultrafiltrate Fraction X (Fig. 1). The results of Fig. 3 show, in general, that a high ratio of reduced to oxidized pyridine nucleotide is not essential for the inactivation reaction.

Inactivation at high pH

The inactivation of nitrate reductase does not proceed at a maximum rate at the pH of 7.6 employed in most of our experiments. Though the rate of inactivation increases with an increase in pH from 7.6 to 8.8, we have used the pH of 7.6 in order to avoid confusion with a different type of inactivation, which becomes more pro-

nounced as the pH is raised. Fig. 4 shows some of the results of incubating activated SE_{G-50} in Tris buffer, pH 8.8. The lower curve shows the results of activity measurements when the rates were calculated from the amount of absorbance change noted in the 1st min (approximately) after addition of an aliquot to the enzyme assay system. The upper curve shows the results of activity measurements when the rates were calculated from the amount of absorbance change which occurred in the 5th min after commencement of the assay measurements. The activity, increasing in the assay system, was almost doubled in 5 min. One can generally observe an activation of inactive enzyme in the assay system, but the change is usually too slow to be clearly perceptible within 5 min.

When the incubation of activated enzyme at pH 8.8 is continued for several hours, there is a gradual formation of a more stable inactive enzyme, which requires ferricyanide for rapid reactivation, but considerable irreversible inactivation may also occur. In other words, nitrate reductase in activated SE_{G-50} undergoes a kind of auto-inactivation at high pH, but the procedure is not recommended for enzyme inactivation because of attendant irreversible loss of activity.

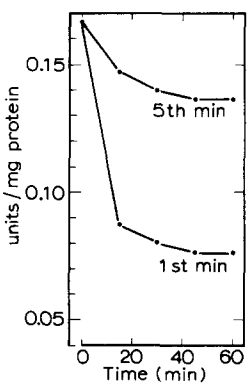

Fig. 4. Inactivation of nitrate reductase at pH 8.8. The activated SE_{G-50}, containing 5.7 mg protein per ml, was mixed with an equal volume of 0.1 M Tris buffer, pH 8.8, and incubated at 20 °C. Aliquots were added to the assay system at the times indicated. The rate of absorbance change during the first minute was difficult to measure accurately. The first reading was generally taken 10–15 s after addition of sample. Activity increase continued beyond the 5th min but at a diminishing rate. All activity was recovered after ferricyanide addition.

The effects of high pH are not specific for Tris buffer, but can also be observed with borate buffer. It does not appear that they are completely excluded in phosphate buffer of pH 7.6, but they are much diminished.

Prevention of inactivation by nitrate

The inactivation of nitrate reductase by NADPH can be completely prevented by nitrate. Thus, Curve 5 of Fig. 5 shows the course of the inactivation with 0.2 mM NADPH and Curve 1 shows the activity change with 0.2 mM NADPH and 13.3 mM KNO_3. The particular, activated SE_{G-50} preparation here employed had undergone a little spontaneous inactivation on storage, so that there was a small gain in activity with NADPH and nitrate. Curiously, this gain was somewhat greater than in the

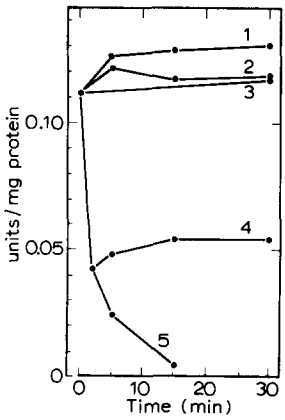

Fig. 5. Prevention of inactivation by nitrate. Reaction mixtures contained 1.0 ml activated SE_{G-50} containing 5.2 mg protein, in 1.5 ml 0.033 M phosphate, pH 7.6, with the following additions: 1, 0.2 mM NADPH and 13.3 mM KNO_3; 2, 13.3 mM KNO_3; 3, none; 4, 0.2 mM NADPH, 13.3 mM KNO_3, after 2 min; 5, 0.2 mM NADPH. Incubation was at 20 °C. Aliquots were assayed at the times indicated.

presence of nitrate alone (Curve 2). Curve 4 shows the course of the reaction when nitrate was added 2 min after commencement of the inactivation. Nitrite measurements, performed at the end of the incubation period, showed that about 0.1 μmole nitrite was formed in 30 min in the presence of NADPH and nitrate.

The nitrate was added in large excess in the experiment of Fig. 5. Complete inhibition of inactivation could be achieved in a similar experiment with 0.2 mM KNO_3. When 0.01 mM KNO_3 was added, a lag period was introduced in the inactivation reaction.

The inactivation caused by the ultrafiltrate, Fraction X, is also prevented by nitrate. It is possible, but not yet established, that the lag periods in the inactivation observed with Fraction X may reflect the time required to reduce a small amount of nitrate. Nitrate has little or no effect on the auto-inactivation in Tris buffer at pH 8.8.

Because of the rapid reduction of nitrate by NADH in the presence of enzyme an experiment of the type shown in Fig. 5 is not practical with NADH. It has already been observed[3], however, in the assay system, that NADH without nitrate can cause a rapid decline in activity, whereas the reaction rate in the presence of nitrate is relatively stable. The inactivation of enzyme by NADH in the absence of nitrate as observed in the assay system[3] can be reversed by ferricyanide.

Inactivation of partially purified enzyme

The studies with activated SE_{G-50} suggest that the reduced pyridine nucleotides alone cause a rapid and complete inactivation of nitrate reductase. With partially purified enzyme, the reaction seemed more complex. Although both NADH and NADPH caused partial reversible inactivation, the process was sluggish, as shown in Fig. 6, where Curves 3 and 4 show the inactivation by NADH and NADPH, respectively. This inactivation was not appreciably increased by doubling the concentrations, or by combining NADH and NADPH (not shown). When Fraction X_{13} was added together with NADH or NADPH, however, there was a substantial increase

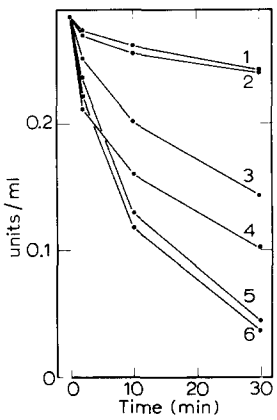

Fig. 6. Inactivation of partially purified, activated enzyme. Reaction mixtures contained 0.285 unit of activated, partially purified enzyme, prepared as described under Methods, per ml of solution containing 50 mM phosphate, pH 7.6, and 0.1 mM dithioerythritol, *plus* the following additions: 1, none; 2, 0.4 ml Fraction X_{13} per ml; 3, 0.3 mM NADH; 4, 0.3 mM NADPH; 5, 0.3 mM NADH and 0.4 ml Fraction X_{13} per ml; 6, 0.3 mM NADPH and 0.4 ml Fraction X_{13} per ml. Incubation was at 20 °C. Aliquots were assayed at the times indicated.

in the inactivation (Curves 5 and 6), though Fraction X_{13} alone (Curve 2) had no appreciable inactivating effect.

In the experiments with partially purified enzyme dithioerythritol was added as a stabilizing agent to ensure complete reactivation of the partially inactivated reaction mixtures by ferricyanide at the end of the incubation period. Dithioerythritol by itself did not cause an inactivation.

Effect of cyanide

It has been shown previously that when nitrate reductase is inactivated with NADH and HCN, the inactivation is faster than when the enzyme is incubated with NADH alone[3]. Nitrate reductase inactivated in the presence of HCN can be completely reactivated by ferricyanide. For example, 1.2 ml SE_{G-50} were added to a mixture of 0.4 ml 0.5 M phosphate, pH 7.1 and 0.2 ml 5 mM $K_3Fe(CN)_6$. After 40 min of incubation in an ice bath, a specific activity of 0.226 unit/mg protein had been reached. Then, 0.8 μmole NADH was added, followed after 10 min by sufficient 20 mM KCN to bring the final concentration to 0.4 mM HCN. Since the NADH was in excess, the ferricyanide was completely reduced prior to addition of cyanide. The sample was incubated in a stoppered tube in an ice bath for 90 min, after which time no activity could be detected. The reagents were removed on a column of Sephadex G-50 equilibrated with 1 mM Tris buffer, pH 8.0. The specific activity after gel filtration was less than 0.002 unit/mg protein. The preparation was reactivated with ferricyanide to give a specific activity of 0.245 unit/mg protein.

Effect of other reagents

With activated SE_{G-50}, there was no inactivation caused by added reduced glutathione, cysteine, dithioerythritol or ascorbic acid. With dithionite, a partial, reversible inhibition could be obtained, but only with a substantial loss in total

recoverable activity. Addition of flavin or of benzylviologen with dithionite did not improve the effectiveness of this reagent. Borohydride was likewise ineffective as an inactivating agent.

The activating effect of ferricyanide is not specific. Both dichlorophenolindophenol and ammonium persulphate cause activation of inactive enzyme, but these reagents are, for practical purposes, less useful than ferricyanide. We have not observed activation of inactive nitrate reductase with cytochrome *c*, but this may be due to the difficulty of adding the reagent in sufficiently high concentration.

The diaphorase activity associated with nitrate reductase (measured with cytochrome *c*) is not substantially inactivated by reduced pyridine nucleotides, or by the ultrafiltrate Fraction X, or by the treatment at pH 8.8 (described in the legend for Fig. 4).

DISCUSSION

The Berlin strain of *C. vulgaris* is not unique with respect to the properties of its nitrate reductase. At Kessler's suggestion, we have tested the nitrate reductase in extracts of *Chlorella pyrenoidosa* 211-8m from the Göttingen collection, using the procedures previously employed[1]. The enzyme in extracts of cells grown on nitrate was present largely in an inactive form which could be extensively activated, and it did not require added FAD. In these respects, the nitrate reductase of strain 211-8m resembles the nitrate reductase of the Berlin strain of *C. vulgaris*, and not the nitrate reductase of *C. fusca*. This is in agreement with the reclassification of *C. pyrenoidosa* 211-8m as *C. vulgaris* Beijerinck[11].

Data were selected for presentation here in order to facilitate comparison of the control mechanism of the nitrate reductase of *C. vulgaris* with that of other species. Because of the similarity of our findings to those of the Spanish group, who have been working primarily with *C. fusca* and *C. reinhardti*, there is reason to anticipate that the phenomenon of activation of nitrate reductase by oxidation and inactivation by reduction, has a more widespread occurrence, whatever the differences in the individual characteristics of the enzymes from different sources may be. The present studies show why the nitrate reductase of *C. vulgaris* is present in crude extracts largely in an inactive form. An inactivation system is present and operating in these crude extracts. Such is apparently not the case for *C. fusca* extracts, unless the cells are given special prior treatment.

Perhaps ferricyanide will be found to be a better reagent than nickel salts for activating the nitrate reductase of sorghum[12] (*cf.* ref. 3). We anticipate this particularly because we have confirmed the report of Vega *et al.*[13] that nitrate reductase inactivated with NADH and HCN can be freed of reagents with Sephadex to give a preparation of inactive enzyme, which can be completely reactivated by ferricyanide treatment.

Similarities of the nitrate reductase of *C. vulgaris* to the latent nitrate reductase of *Cyanidium caldarium*[14,15] are also evident, although this clearly requires further study.

According to the simplest possible assumption, the nitrate reductase of *C. vulgaris* has only two relatively stable forms, one completely active, the other completely inactive. This assumption may require later revision. It is possible that the easily

reactivated product formed at pH 8.8 (Fig. 4) represents an intermediate step in the inactivation reaction, but this is not established.

The control mechanism here under study is clearly a short term process which can lead to large changes of activity in a few minutes. The nitrate reductase can be completely inactivated or completely activated without appreciable change in the associated NADH–cytochrome *c* reductase. This is in contrast to the suppression of enzyme synthesis which occurs when the cells are grown on ammonium[16]. The extracts of ammonium-grown cells contain little inactive or active nitrate reductase and a correspondingly small amount of NADH–cytochrome *c* reductase.

The current studies are still in progress. Ultrafiltrate Fraction X can reduce ferricyanide and dichlorophenolindophenol. A portion of this reducing power remains associated with activity during fractionation on Sephadex G-25 as described under Methods (Jetschmann, K., unpublished). Since the preparations are probably still very impure, no definitive conclusions regarding chemical structure can be drawn from these findings at present. The ultrafiltrate can, under other conditions, cause activation of inactive enzyme by O_2 (ref. 17). This oxidation is CO-sensitive, and provides an explanation for the effect of CO on the photoevolution of O_2 from nitrate, by illuminated *Chlorella* cells, as shown in the experiment of Fig. 1 in ref. 18.

The experiments with $SE_{G\text{-}50}$ preparations suggest an important role for the reduced pyridine nucleotides in the inactivation of nitrate reductase, but the experiments with partially purified enzyme (Fig. 6) also show that the inactivation can not be completely characterized in terms of a simple, direct redox reaction between enzyme and NADH or NADPH. Preliminary experiments (Solomonson, L. P., unpublished) have shown that ferrocyanide behaves similarly to ultrafiltrate Fraction X in its action on partially purified enzyme. That is, ferrocyanide alone has little inactivating effect, but when ferrocyanide is combined with NADPH, there is a marked stimulation of the inactivation. The inactivated product can be completely reactivated by ferricyanide. This leads to the suspicion that an active component of Fraction X may be a redox substance with some of the properties of the ferricyanide–ferrocyanide system.

ACKNOWLEDGEMENTS

We are grateful to Cornelia Bunge and Renate Borchers for competent technical assistance. We are grateful also to Professor Losada for sending us the manuscript of ref. 8 prior to publication.

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