The Presence of Bound Cyanide in the Naturally Inactivated Form of Nitrate Reductase of Chlorella vulgaris

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SUMMARY

In the presence of NADH and cyanide, NADH-nitrate oxidoreductase (EC 1.6.6.1) from Chlorella vulgaris is converted to an inactive form which is readily reactivated by ferricyanide. Experiments with H14CN indicated that the inactivation process is associated with the firm binding to the protein of 0.066 nmole of cyanide per unit of enzyme inactivated. This cyanide binding was linearly proportional to the amount of inactivation. No firm cyanide binding and no inactivation occurred in the absence of NADH or an equivalent reductant. The bound 14C was released when the enzyme was reactivated by ferricyanide. After the cells had been treated with ammonia for several hours prior to disruption, the crude cell extracts contained the nitrate reductase primarily in the inactive form. Critical examination of the methods of cell disruption suggested that the inactivation of the enzyme had truly occurred in vivo. After 300-fold purification, activation of this in vivo inactivated enzyme resulted in the release of 0.066 nmole of HCN per unit of enzyme activated. We conclude that the inactivation of nitrate reductase in vivo involves the formation of a firmly bound complex of reduced enzyme and cyanide.

The reaction between reduced enzyme and HCN may be written:

\[ E_R + HCN \xrightarrow{k_4} E_R-HCN \]

The measured value for \( k_a \) was 1.25 \( \times \) 10^6 M\(^{-1}\) min\(^{-1}\), for \( k_d \) 4.5 \( \times \) 10^-4 min\(^{-1}\), giving a dissociation constant, \( K_D = 3.6 \times 10^{-10} \) M.

In sonicated extracts of Chlorella vulgaris, grown with nitrate as the sole nitrogen source, NADH-nitrate reductase (EC 1.6.6.1) was found initially in an inactive form, which was slowly activated in vitro in the presence of nitrate and phosphate (1, 2), and rapidly activated by ferricyanide (3). A reversible inactivation of crude preparations of the ferricyanide-activated enzyme could be achieved by the addition of either NADH or NADPH (4). The activity of the associated NADH-cytochrome c reductase was not affected by such treatments. Thus, control of the activity of nitrate reductase appeared to be exercised by simple oxidation-reduction processes associated with the nitrate-reducing moiety of the enzyme.

Simultaneously and independently, Losada et al. made rather similar studies with Chlorella fusca (5-7). With this organism, special prior treatment of the cells was required to obtain extracts containing reversible inactive enzyme. Among these treatments was an incubation with ammonia (5), a procedure which may have been suggested by the results of earlier studies of Syrett and Morris (8) on the effect of ammonia on nitrate assimilation by Chlorella.

Subsequent purification of the nitrate reductase from C. vulgaris revealed that reduced pyridine nucleotides alone did not inactivate the enzyme. A second component, by far the most effective of which was cyanide, was required to achieve reversible inactivation (9). Kinetic studies showed that this inactivation reaction involved a stoichiometric reaction of cyanide with the enzyme.

MATERIALS AND METHODS

Growth of Chlorella—The cells were grown in continuous white light on a mineral salts medium, with nitrate as the only source of nitrogen, in a stream of 5% (v/v) CO\(_2\) in air, at 20-22\(^\circ\)C, as previously described (1, 12). For the present experiments, round bottom flasks containing 500 ml of medium were inoculated with 0.4 ml of cells per flask. After 24 hours, the yield, on the average, was about 2 ml of packed cells per flask. The cells were washed with 1/3 volume of distilled water and suspended in water (250 \( \mu \)l of cells per ml) prior to disruption.

Preparation of Ammonium-treated Cells—The contents of six 500-ml flasks (10 to 12 ml of cells) were harvested by centrifugation, washed with 2 liters of nitrate-free culture medium (20 mM MgSO\(_4\),...
Purification of inactive form of nitrate reductase

| Fraction            | Volume | Total Protein | Ammonium | Before Activation | After Activation | Specific Activity | Yield   |
|---------------------|--------|---------------|----------|------------------|-----------------|-------------------|---------|
| Crude extract...... | 280    | 5600          | unit     | 14               | 244             | 0.04              | 100     |
| Protamine sulfate   | 55     | 10            | mU/mg    | 10               | 215             | 0.84              | 88      |
| Bio-Gel A-1.5m      | 88     | 56            | mU/mg    | 13               | 215             | 3.1               | 70      |
| Zonal rotor c...    | 88     | 12            | mU/mg    | 19               | 160             | 13.6              | 66      |

RESULTS

H^14CN-binding Associated with Inactivation in Vitro—When purified nitrate reductase was incubated with a large molar excess of NADH and H^14CN, the enzyme was rapidly inactivated, although the activity of the associated NADH-cytochrome c reductase remained constant. Subsequent gel filtration of the incubation mixture revealed that radioactivity was incorporated into the fractions which co-eluted with the NADH-cytochrome c reductase activity (Fig. 1A). When the incubation was carried out in the absence of NADH, there was a slight loss of activity (3.8%) and practically no incorporation of radioactivity into the protein fraction (Fig. 1B).

In the experiment of Fig. 1A, 56.5 units of cytochrome c reductase, 8.7 units of inactive nitrate reductase, and 6.86 \times 10^4 dpm of H^14CN were recovered in the protein fractions. From this data and a measured specific radioactivity of 1.2 \times 10^6 dpm per \mu mole, we calculate that 1 unit of inactive enzyme binds 0.066 nmole of HCN. The same stoichiometry holds when the enzyme is only partially inactivated, as shown in Fig. 2. Intermediate levels of inactivation were achieved by incubation of the enzyme with limiting concentrations of NADH in the presence of excess H^14CN, or with limiting concentrations of H^14CN in the presence of excess NADH.

The binding of the H^14CN in the inactive enzyme complex is very firm. When 0.1 nmole of the inactive complex was incubated for 2 hours at 20°C in the presence of a 500-fold molar excess of unlabeled HCN and NADH, only about 10% of the radioactivity exchanged out of the inactive enzyme (Fig. 3A).

In contrast to the slow release of H^14CN from the inactive enzyme complex, all of the \(^{14}C\) was released within a few minutes upon activation of the enzyme by tert-butylamine (Fig. 3B).

Estimation of Dissociation Constant, K_{D}, of E_{R-CN} Complex—
Fig. 1. A, binding of H\textsuperscript{14}CN by nitrate reductase in the presence of NADH. NADH-nitrate reductase (11.4 units) containing 74 units of NADH-cytochrome c reductase was incubated in 0.655 ml of 0.075 M phosphate buffer (pH 7.6), 0.23 mM NADH, and 16 \mu M H\textsuperscript{14}CN for 5 min at 20°. The solution (0.5 ml) was then applied to a Sephadex G-25 column (0.9 X 20 cm) equilibrated with 0.025 M phosphate buffer (pH 6.8), 0.1 mM dithioerythritol, and 0.15 mM EDTA at 4°. Fractions (1.1 ml) were collected. \(\bullet\)---\(\bullet\), disintegrations per min of 1% per 0.1 ml; \(O--O\), NADH-cytochrome c reductase, units per ml; \(\times--\times\), NADH-nitrate reductase, units per ml. B, no binding of H\textsuperscript{14}CN by nitrate reductase in the absence of NADH. Conditions the same as in A except no NADH was present. Symbols as in A.

Fig. 2. Relationship between the binding of H\textsuperscript{14}CN by nitrate reductase and the extent of inactivation. \(O\), conditions identical to those in Fig. 1A except that limiting quantities of NADH were added so as to produce intermediary states of inactivation (9). \(\Delta\), conditions were as above except that intermediate states of inactivation were achieved by incubating various amounts of enzyme (2.3, 5.7, 11.4, and 22.8 units of NADH-nitrate reductase per 0.655 ml) for 40 min at 20° in the presence of 0.5 mM NADH and a limiting concentration of H\textsuperscript{14}CN (0.65 \mu M). At 100% inactivation, 8.7 units of inactive nitrate reductase bound 0.86 \times 10\textsuperscript{4} dpm of H\textsuperscript{14}CN.

The reaction between HCN and reduced enzyme is represented in Equation 1.

\[ E_R + HCN \xrightleftharpoons[k_d]{k_a} E_R\textsuperscript{-}\text{HCN} \]  

(1)

The dissociation constant, \(K_D\), for this reaction was estimated as follows.

As shown in Fig. 3A, there is a slow but measurable exchange reaction between H\textsuperscript{14}CN of the \(E_R\text{-}^{14}CN\) complex and unlabeled HCN. This reaction reflects the rate of dissociation of the \(E_R\text{-}^{14}CN\) complex since unlabeled HCN is present in great excess. In an experiment similar in design to that shown in Fig. 3A, \(E_R\text{-}^{14}CN\) was incubated at 20° with an excess of unlabeled HCN, and the loss in \textsuperscript{14}C from the \(E_R\text{-}^{14}CN\) complex was determined as described in the legend for Fig. 4. As shown in Fig. 4, the dissociation of the \(E_R\text{-}^{14}CN\) complex is a first order reaction. From the slope of this plot, a value of 4.50 \times 10\textsuperscript{-4} min\textsuperscript{-1} was calculated for the first order reaction constant, \(k_a\), for the reaction of HCN with the reduced enzyme. The second order rate constant, \(k_a\), for the reaction of HCN with the reduced enzyme is 1.25 \times 10\textsuperscript{6} M\textsuperscript{-1} min\textsuperscript{-1}. This value was calculated from the data given in Fig. 3 of Ref. 9, on the assumption that one enzyme unit equals 0.066 nmole of enzyme. From these two values a dissociation constant, \(K_D\), was calculated as 3.6 \times 10\textsuperscript{-11} M. At the end of the experiment shown in Fig. 4, there was a 70% loss in NADH-nitrate reductase activity but only a 12% loss in reduced methyl viologen-nitrate reductase activity, which indicates that the nitrate-reducing moiety remains largely catalytically active under these conditions. It has previously been shown that regulation occurs on the nitrate-reducing moiety (9). However, because it is not known what effect, if any, irreversible loss of enzyme activity may have on the rate of dissociation of the \(E_R\text{-}CN\) complex, the values for \(k_d\) and \(K_D\) as determined above should be regarded as approximate values.
inactive enzyme, and addition of nitrate to the medium in which the cells are disrupted has less effect on the proportion of the enzyme present in the inactive form (Table II, Experiment 2). In two other similar experiments, there was no significant effect on the other hand, the extracts contain more active enzyme than the average, in Ribi extracts of normal cells. To obtain Ribi extracts containing most of the enzyme was inactive. We found, however, that when nitrate is added to the cell suspension immediately prior to cell disruption, then its presence should prevent any inactivation which might occur during or after the disruption process.

In earlier studies from this laboratory, water-washed cells were disrupted by sonic and consistently yielded extracts in which most of the enzyme was inactive. We found, however, that when nitrate is added to the cell suspension immediately prior to sonic, the extracts contain a considerable proportion of the enzyme in the active form (Table II, Experiment 1). When the extracts are disrupted with a Ribi cell fractionator, on the other hand, the extracts contain more active enzyme than inactive enzyme, and addition of nitrate to the medium in which the cells are disrupted has less effect on the proportion of the enzyme present in the inactive form (Table II, Experiment 2). In two other similar experiments, there was no significant effect of added nitrate. About 30% of the enzyme was inactive, on the average, in Ribi extracts of normal cells. To obtain Ribi extracts containing most of the nitrate reductase in the inactive form, we used an ammonia treatment similar to that which Losada et al. employed to obtain inactive nitrate reductase from Chlorella fusca (5) and Chlamydomonas reinhardii (15, 16). Experiment 3 of Table II shows that addition of nitrate prior to cell disruption had no effect on the activation state of the enzyme from such cells.

Because nitrate added prior to cell disruption did not decrease the amount of inactive enzyme found in the Ribi extracts of ammonium-treated cells, we conclude that enzyme inactivation had, in fact, occurred in vivo. This conclusion is supported also by the fact that these Ribi extracts do not cause rapid inactivation of added, active enzyme, as do the sonicated extracts (Fig. 5).

Presence of Bound Cyanide in In Vivo-inactivated Enzyme—The procedure developed for the purification of activated nitrate reductase can be applied equally well to the purification of the

**Table II**

| Experiment | Method of cell disruption | Ammonia treatment | NaNO₃ (10 mM) | Presence of enzyme (before activation) | Inactive enzyme |
|------------|---------------------------|-------------------|--------------|----------------------------------------|-----------------|
| 1          | Sonication                | –                 | +            | 0.21                                    | 1.90            |
| 2          | Ribi cell fractionator    | –                 | +            | 2.35                                    | 3.96            |
| 3          | Ribi cell fractionator    | +                 | +            | 3.12                                    | 3.87            |

Fig. 4. Rate of dissociation of the nitrate reductase-[¹⁴C]-cyanide complex. Enzyme-[¹⁴C]-cyanide complex (0.3 μM) in 0.08 m phosphate buffer (pH 7.6), 0.25 mM HCN, 0.05 mM NADH, 0.1 mM dithioerythritol, and 0.1 mM EDTA was incubated in a well-stoppered tube at 20°. At the indicated times, 0.5-ml aliquots were applied to a Sephadex G-25 column (1 X 18 cm) equilibrated with the incubation buffer. The decrease in bound H¹⁴CN was determined and expressed as the log of the percentage of initially bound H¹⁴CN. The initial total enzyme units per ml of incubation mixture (measured after activation of an aliquot with 0.4 mM K₃Fe(CN)₆) were 4.1 units of NADH-nitrate reductase and 7.6 units of reduced methyl viologen-nitrate reductase. After the 1500-min incubation period, the activities had decreased to 1.5 units and 0.7 units, respectively. There was no significant nitrate reductase activity before activation. Methyl viologen-nitrate reductase was assayed as described previously (9).

Methods of Cell Disruption—Previous studies have shown that nitrate prevents the inactivation of the enzyme by reduced pyridine nucleotides and HCN. Unlike ferricyanide, nitrate does not cause a rapid activation of the cyanide-inactivated enzyme. The slow activation caused by nitrate is insignificant on the time scale under consideration. It was, therefore, reasoned that if nitrate was added to the cell suspension immediately prior to cell disruption, then its presence should prevent any inactivation which might occur during or after the disruption process.

Fig. 5. Change in nitrate reductase activity with time after addition of purified, active enzyme to crude cell extracts. Extract (0.5 ml) plus 0.1 ml of a solution containing 1.2 units of purified active enzyme were incubated at 20°, and aliquots were assayed for nitrate reductase at the times indicated, as described under “Materials and Methods.” Ordinate shows activity expressed as the percentage of initial activity in the incubation mixture. The extracts were prepared with and without 10 mM NaNO₃ as described in the legend for Table II, and under “Materials and Methods.” ○, sonicated extract; □, sonicated extract (with nitrate); ■, Ribi extract; ×, Ribi extract (with nitrate). Results with Ribi extracts of ammonium-treated cells were essentially like those with Ribi extracts of normal cells.
zyme inactivation is several orders of magnitude smaller than one may infer that the concentration of HCN required for enzyme. From the firm binding of cyanide to the reduced enzyme, the results have been expressed as 0.066 nmole per unit of enzyme. Pending determination of molecular weight by other methods, the paper establishes the stoichiometry of the reaction. Pending a second order reaction between enzyme and HCN. The present study also shows that the inactive nitrate reductase present in Ribi extracts of ammonia-treated Chlorella releases the same stoichiometric amount of HCN upon activation as the inactive labeled cyanide complex formed in vitro. This establishes that the inactive enzyme present in the extracts is indeed the En-CN complex.

Preliminary studies had shown that the inactive nitrate reductase of sonicated extracts also releases HCN on activation. This extracts contain much more HCN than is necessary to inactivate the enzyme (11), and the possibility that the formation of HCN might be an artifact caused us some concern. The Ribi cell fractionation procedure gives, on the average, more nitrate reductase than the sonication process. Values above 20 units of total enzyme per ml of packed cells have been observed. Most important, with the Ribi procedure the activation state of the nitrate reductase is not strongly influenced by addition of nitrate prior to cell disruption, so that the activation state of the enzyme in the extracts most probably reflects its condition in vivo. Finally, the ammonia treatment was chosen to obtain in vivo-inactivated enzyme, because it is firmly established that ammonia plays a role as a regulator of nitrate reductase in intact algae (6, 8).

At least two different mechanisms exist whereby ammonium controls the utilization of nitrate. One of these is associated with the repression of nitrate reductase synthesis by ammonium, an effect which requires many hours to become fully evident (18, 19). The other mechanism is associated with the rapid cessation of nitrate assimilation upon the addition of ammonium to the culture medium. Nitrate utilization does not resume until nearly all the ammonium is assimilated. Syrett and Morris have described this effect in detail (8). They showed further that ammonium did not inhibit the reduction of nitrate to ammonium by carbon-starved cells, which assimilated little ammonium. They concluded that nitrate reduction was probably inhibited by products of ammonium assimilation. Thacker and Syrett (20) have more recently examined the control of nitrate reduction by ammonium in Chlamydomonas reinhardtii and come to similar conclusions. It is tempting to speculate that the product of ammonium assimilation which inhibits nitrate reduction is HCN. We have no evidence, however, that cyanide is synthesized in response to ammonium, except the fact that an increased quantity of cyanide is bound to nitrate reductase after the cells have been incubated with ammonium. Nor do we yet understand the relationships between ammonium assimilation and cyanide synthesis, if such exist. It seems relevant, however, that the products of ammonium assimilation, the amino acids, are the precursors of the cyanogenic glucosides and cyanide, in all of the cyanogenic fungal, bacterial, and higher plant systems that have been examined to date (21-23).

A rapid, in vitro activation of in vivo-inactivated nitrate reductase, independent of de novo enzyme synthesis, has yet to be demonstrated unequivocally. Furthermore, the rapid in vitro activation of the inactive enzyme has until now only been achieved with artificial oxidant. The activation with nitrate is slow. It is possible that some other substance is required in addition to nitrate. The possibility that the inactivation is irreversible in vivo has not been excluded. It might, for example, be a part of a mechanism involving the continual turnover of the enzyme.

We have touched on only a few of the problems raised by our

### Table III

| Purification stage | Nitrate reductase | HCN recovered |
|--------------------|-------------------|---------------|
|                    | After full activation with ferricyanide | Before activation | After partial slow activation | nmol | nmol/ unit activated |
| Protamine sulfate... | 17 | 4.9 | 8.8 | 0.26 | 0.067 |
| Protamine sulfate... | 68 | 0.81 | 11.0 | 43 | 2.16 | 0.067 |
| Zonal rotor centrifugation... | 54 | 13.6 | 9.6 | 20.0 | 0.685 | 0.066 |

inactive enzyme. Starting with a Ribi extract of ammonium-treated cells, two preparations were carried through a protamine sulfate precipitation, and one preparation was further purified by gel filtration and density gradient centrifugation in a zonal rotor to give a product estimated to be about 30% pure (see under "Materials and Methods"). Aliquots of these preparations were partially activated by overnight incubation with added nitrate and phosphate buffer in the main compartment of Warburg vessels containing base in the center well. HCN (pKₐ = 9.15) is completely undissociated at neutral pH and will readily distill into the alkali trap. The use of ferricyanide as activator was avoided, since it is a potential source of cyanide. The enzyme preparation was assayed before and after the activation process, and the contents of the center well were analyzed for cyanide. The results are summarized in Table III. In all three experiments, the amount of cyanide obtained per unit of enzyme activated was virtually the same, 0.066 to 0.067 nmole per unit activated, in agreement with the previous measurements of the amount of H⁺CN bound during enzyme inactivation in vitro.

### Discussion

The reaction of HCN with the reduced form of nitrate reductase to give an inactive enzyme complex which can be reactivated by ferricyanide proceeds as shown in Equation 1. This was first surmised from Solomonson's demonstration that the inactivation of the reduced enzyme has the kinetics of a second order reaction between enzyme and HCN. The present paper establishes the stoichiometry of the reaction. Pending an accurate determination of molecular weight by other methods, the results have been expressed as 0.066 nmole per unit of enzyme. From the firm binding of cyanide to the reduced enzyme one may infer that the concentration of HCN required for enzyme inactivation is several orders of magnitude smaller than that which would inhibit other vital processes such as respiration. At such low concentrations, HCN is probably a rather specific inhibitor for nitrate reductase.
results. Experiments are in progress to distinguish between the many models to which the current findings can be fitted.

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