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ACTIVE AND INACTIVE NITRATE REDUCTASE

EFFECTS OF MILD TREATMENT WITH DENATURING AGENTS OF PROTEIN

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Summary

Nitrate reductase (NAD(P)H:nitrate oxidoreductase, EC 1.6.6.2) of the unicellular alga *Cyanidium caldarium* can exist in two interconvertible forms; one catalytically active and one inactive. The inactive nitrate reductase can be activated by mild treatment with denaturing agents of protein. By treatment with urea or mersalyl, activation of both the NADPH and benzyl viologen activities can be realized under mild conditions, whereas by treatment with heat, the activation of benzyl viologen activity is concomitant with loss of the NADPH activity. On the other hand, both activities are activated and destroyed concomitantly by ethylene glycol. In the present of FAD, either activation of benzyl viologen activity or loss of NADPH activity upon heating occur only at higher temperatures.

The existence of a controlling region in the nitrate reductase molecule is postulated.

Introduction

The assimilatory nitrate reductase (NAD(P)H:nitrate oxidoreductase, EC 1.6.6.2) of the red alga *Cyanidium caldarium* is extracted in a catalytically-active form from cells grown with a growth rate-limiting nitrogen source [1], and in an inactive form from cells which have had access to ammonia [2—4]. Also in other unicellular algae, nitrate reductase may become inactive or may occur mainly as an inactive enzyme [5,6].

In previous papers [7,8], it was reported that in vitro protein denaturants

can destabilize the inactive to favour the active form of nitrate reductase. It was also demonstrated that inactive nitrate reductase of *C. caldarium* when heated reattains its original activity with benzyl viologen but concomitantly loses activity with NADPH. It was proposed, on the basis of Arrhenius activation energies, that a single process of protein denaturation, occurring at the same level of the nitrate reductase molecule, was responsible for both phenomena [7].

In recent years Zumft et al. (in *Chlorella*) [9] and Barea et al. (in *Chlamydomonas*) [10] showed that FAD protects the NADH nitrate reductase against heat denaturation.

Here it will be shown that in *C. caldarium*, FAD not only protects the NADPH activity against heat denaturation, but also results in an increase in temperature where heat activation of the benzyl viologen activity occurs. Studies on the effects of urea, mersalyl and ethylene glycol on the inactive enzyme will also be reported.

Materials and Methods

C. caldarium was grown autotrophically in light at 42°C and at pH 1.9 as reported earlier [2]. 0.025 M glutamic acid was used as the sole nitrogen source. Cultures were bubbled with air enriched with 5% CO_2. Cell-free extracts were prepared with the aid of a French-pressure cell, as previously [2]. The active nitrate reductase was obtained from glutamate-grown cells as shown previously [1]; these cells possess only the active enzyme. The inactive nitrate reductase was obtained from cells to which ammonia was added 1 h before harvesting. The presence of ammonia brings about a conversion of the enzyme from the active into the inactive form. Nitrate reductase was assayed by a colorimetric estimation of the nitrite produced [2].

The experiments were carried out with crude extracts, since all attempts to purify nitrate reductase of *C. caldarium* have, so far, been unsuccessful, due to extreme lability of the enzyme during purification. In particular, the inactive enzyme loses its inactive state from the first step, whatever the purification procedure.

Results

Effect of heat on active and inactive nitrate reductase in the presence or absence of FAD

Fig. 1 shows that in the absence of FAD, the NADPH nitrate reductase of *C. caldarium* was destroyed in 10 min by incubation at 50°C, whereas in the presence of FAD it was destroyed in 10 min only at 60°C.

Fig. 2 shows that heating, at 52°C for 30 min, an inactive nitrate reductase brought about a 4-fold increase of benzyl viologen activity. By contrast, heating in the presence of FAD at a higher temperature, 57°C, was almost without effect, and it was at 62°C in the presence of FAD that benzyl viologen nitrate reductase underwent a time-dependent increase in activity.

Fig. 3 shows that upon heating in the absence of FAD for a fixed period of time (5 min) at various temperatures, a significant increase of benzyl viologen

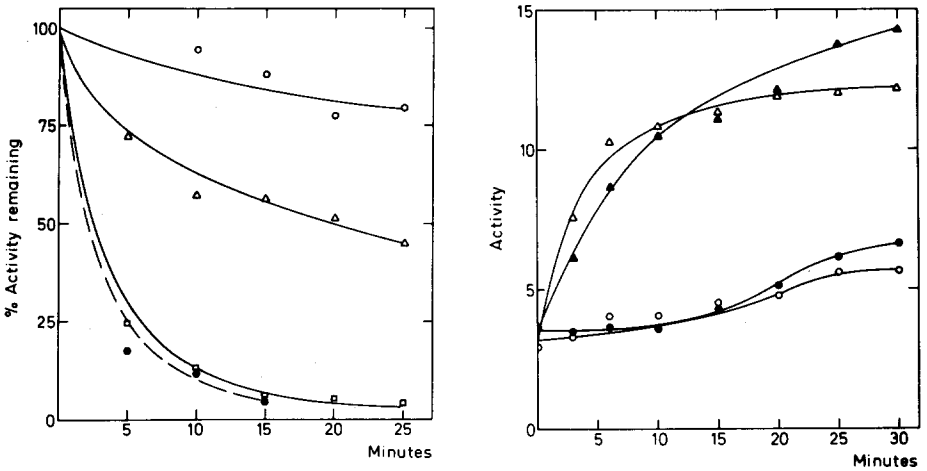

Fig. 1. Time course of thermal denaturation of NADPH nitrate reductase at 50° C (○———○), 55° C (△———△) and 60° C (□———□) in the presence of FAD, and at 50° C (●- - - - -●) in the absence of FAD (the control). Microtubes containing in a final volume of 0.2 ml, 50 µl of active nitrate reductase, 3 mM phosphate buffer (pH 7.5) and 0.02 mM FAD (except in the control), were kept at the reported temperatures, successively removed and chilled in ice, and then assayed at 30° C for residual activity. The assay mixture contained (in a final volume of 2 ml) 0.08 M phosphate buffer (pH 7.5)/25 mM KNO_3/0.25 mM $NADPH_2$. Activity at 100%; 113 nmol nitrite produced/min per ml extract.

Fig. 2. Time course of thermal activation of benzyl viologen nitrate reductase at 47° C (●———●) and 52° C (▲———▲) in the absence of FAD, and at 57° C (○———○) and 62° C (△———△) in the presence of FAD. Microtubes containing (in a final volume of 0.2 ml) 25 µl of inactive nitrate reductase, 3 mM phosphate buffer (pH 7.5) and 0.02 mM FAD (only the tubes at 57° C and 62° C), were kept at the above reported temperatures, successively removed and chilled, and then assayed at 30° C for activity. Assay procedure as in Fig. 1, except that $NADPH_2$ was replaced by 2.5 mM dithionite and 0.06 mM benzyl viologen.

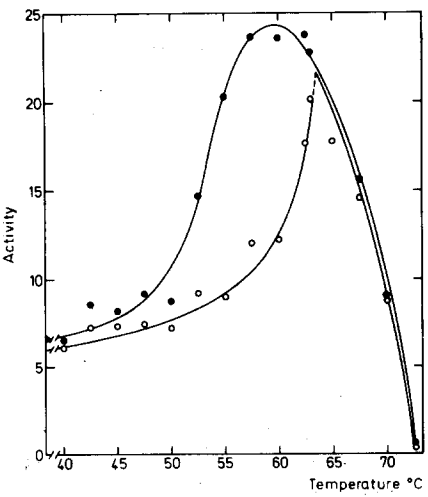

Fig. 3. Temperature-dependent activation of benzyl viologen nitrate reductase in the presence or absence of FAD. Microtubes containing, in a final volume of 0.2 ml, 25 µl of inactive nitrate reductase and 3 mM phosphate buffer, pH 7.5, without (●———●) or with (○———○) 0.02 mM FAD, were incubated for 5 min at indicated temperatures, then chilled and assayed at 30° C for benzyl viologen nitrate reductase. Assay procedure and activity as in Fig. 2.

nitrate reductase activity occurred in the range 45—57°C, whereas in the presence of FAD, the increase in activity occurred in the range 57—62°C. FAD, however, was without effect on the thermal denaturation of the benzyl viologen activity, which, above 60°C, was destroyed similarly either in the presence or in the absence of FAD (Fig. 3).

In the absence of FAD, the maximum extent of increase occurred at 57°C and it was up to 4-fold, whereas in the presence of FAD the maximum extent of increase occurred at 62°C and it was only 3-fold. This means that in the presence of FAD full activation occurred at temperatures above 62°C, but this could not be observed since denaturation occurring at these temperatures caused a lower activity to be measured than that which could have been present.

In contrast with the benzyl viologen activity, the low NADPH activity detectable before the heat treatment was completely lost after the treatment. In the presence of FAD, however, a slight activation of NADPH activity always occurred at temperatures just below those which denature it. Activation of benzyl viologen nitrate reductase of *C. caldarium* by heat is probably similar to the activation of the methyl viologen system in *Neurospora crassa* described by Garrett and Nason [11].

Effects of urea or mersalyl on inactive nitrate reductase

Fig. 4 shows that treatment of inactive nitrate reductase with urea (1—2.5 M) brought about a 4-fold increase of both benzyl viologen and NADPH activities. At urea concentrations above 3 M, there occurred a loss of NADPH

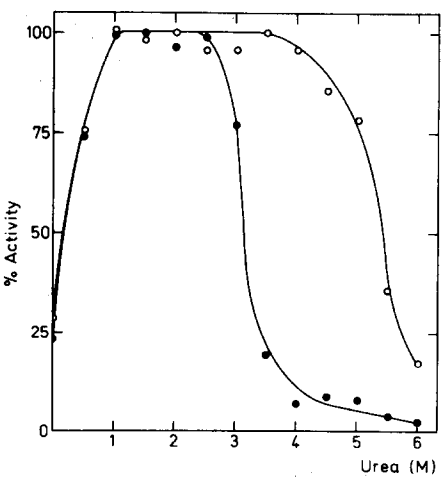

Fig. 4. Effect of urea on the benzyl viologen (○———○) and NADPH (●———●) activities of an inactive nitrate reductase. Aliquots of inactive enzyme were incubated at 0° C with the reported concentration of urea in 3 mM phosphate buffer pH 7.5. For control under the same conditions urea was replaced with water. After 1 h, samples of 50 and 100 µl from urea treated and control tubes were withdrawn and tested for benzyl viologen and NADPH activities respectively. Assay procedure as in Figs. 1 and 2. It is to note that when samples incubated with urea are translated in the assay mixture, urea undergoes a 20 or 40-fold dilution reaching concentrations which do not affect nitrate reductase activity during the assay. Activities at 100% for benzyl viologen and NADPH, 290 and 52 nmol nitrite produced per min per ml extract respectively.

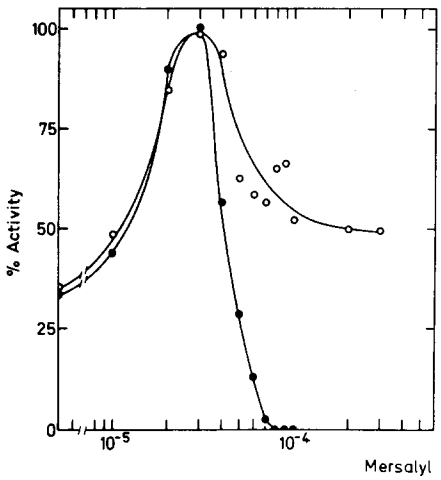

Fig. 5. Effect of mersalyl on the benzyl viologen (○———○) and NADPH (●———●) activities of an inactive nitrate reductase. Activities were tested in assay systems containing 50 µl of inactive nitrate reductase and the reported concentrations of mersalyl in addition to the usual components of the assay mixture. Assay procedure as in Figs. 1 and 2. Activities at 100% for benzyl viologen and NADPH, 320 and 66 nmoles nitrite produced per min per ml extract respectively.

activity and at urea concentrations above 5 M loss of benzyl viologen activity. Similar results were obtained in the presence of FAD.

Fig. 5 shows that upon addition of mersalyl to an inactive nitrate reductase, at mercurial concentrations between 0.01—0.04 mM, there occurred a 4-fold increase of both NADPH and benzyl viologen activities. Higher concentrations of mersalyl inhibited the NADPH activity 100% and the benzyl viologen activity only 50%. FAD did not significantly protect the enzyme against activation or inhibition by mersalyl.

The selective destruction of NADPH activity with respect to the benzyl viologen activity observed at intermediate concentrations of urea or mersalyl is consistent with previous findings [12].

Effect of ethylene glycol on active and inactive nitrate reductase

Fig. 6 shows that when active nitrate reductase of *C. caldarium* was treated with ethylene glycol, both benzyl viologen and NADPH activities were lost. The 50% loss of activity, however, occurred at an ethylene glycol concentration of 25 vol.% for benzyl viologen activity and at 35 vol.% for NADPH activity. It is evident that NADPH protected the enzyme against the effect of ethylene glycol. FAD did not protect.

Fig. 6 also shows that when inactive nitrate reductase was treated with ethylene glycol, both NADPH and benzyl viologen activities were activated. The maximum extent of activation occurred at 25% glycol concentration for benzyl viologen activity and at 35% for NADPH activity. Higher concentrations of ethylene glycol were inhibitory, with a loss of activity comparable to that occurring in the active enzyme treated with similar glycol concentrations.

It should be noted that the maximum increase in activity produced by ethylene glycol was up to 2-fold, whereas the maximum increase produced by

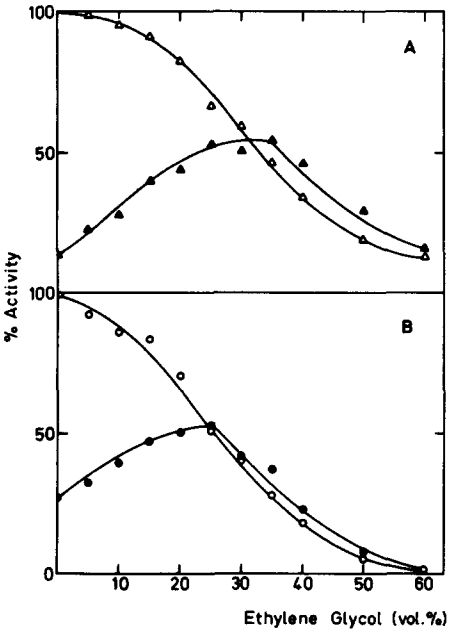

Fig. 6. A and B. Influence of ethylene glycol on NADPH (A) and benzyl viologen (B) activities of an inactive (full symbols) and of an active (open symbols) nitrate reductase. The enzyme activities were tested in systems containing the reported concentrations of ethylene glycol in addition to the usual components of the assay mixture. △———△, NADPH activity of active enzyme; ▲———▲, NADPH activity of inactive enzyme; ○———○, benzyl viologen activity of active enzyme; ●———●, benzyl viologen activity of inactive enzyme. Assay procedure as in Figs. 1 and 2. Active enzyme: activities at 100% for benzyl viologen and NADPH, 380 and 110 nmol nitrite produced per min per ml extract respectively. Inactive enzyme activities at 100% for benzyl viologen and NADPH, 290 and 52 nmol nitrite produced per min per ml extract respectively. The 100% activity of inactive enzyme corresponds to activity found in the same enzyme preparation activated by urea 2 M for 1 h.

2 M urea was up to 4-fold (tested with the same enzyme preparation). This means that upon treatment with ethylene glycol, concomitantly with activation, there were enzyme molecules which remained destroyed. On the other hand, the concentrations of ethylene glycol below 25—35% which produced an increased activity with the inactive enzyme, produced a partial loss of activity with the active enzyme (as referred above). A comparable loss of activity is certainly the reason why the inactive enzyme cannot attain full activity upon treatment with ethylene glycol.

FAD did not protect nitrate reductase against the effect of ethylene glycol.

Discussion

As referred in previous papers [1—4], the nitrate reductase of *C. caldarium* can modulate in vivo between the active and the inactive form. In particular, the enzyme which occurs in a catalytically-active form in cells which are grown in a growth rate-limiting nitrogen source, is transformed in a few minutes into an inactive enzyme if the cells are supplied with ammonia and it is transformed once more into the active form upon removal of ammonia. In nitrate-grown cells, nitrate reductase occurs partially in the inactive form. The interconver-

sion of the two forms of nitrate reductase probably represents the main mechanism regulating the assimilatory reduction of nitrate to ammonia [13].

The fact that nitrate reductase can be extracted in two well-defined forms, active or inactive, depending on the nutritional conditions of the cell, and the fact that in a crude extract each form can maintain its orginal state, together suggest that nitrate reductase may undergo regulatory mechanisms comparable to those operating with other interconvertible enzymes [14]. However, so far it has been impossible to verify the molecular basis of the nitrate reductase interconversion since all attempts to reproduce in vitro the inactivation of nitrate reductase have been unsuccessful. Furthermore, difficulties encountered in obtaining pure Cyanidium nitrate reductase have prevented comparative studies of the two forms of the enzyme. For the moment the only useful information about regulatory properties of the nitrate reductase molecule of *C. caldarium* can be drawn from the action of denaturants on active and inactive enzyme.

The results described in the present paper support the contention that there exists a region of the nitrate reductase molecule which is not concerned with the catalytic activity, but is concerned with the maintainance of the inactive state of the enzyme. This part may represent a control region which, in response to some presently unknown biological signal, can promote and maintain the inactive state of nitrate reductase.

Urea and mersalyl under sufficiently mild conditions, promote the activation of both benzyl viologen and NADPH activities of the inactive enzyme without apparent loss of activity, and it is only at higher concentrations that these denaturants promote a loss of NADPH activity first and of benzyl viologen activity after. Thus it seems that urea and mersalyl under mild conditions act on a control region of the nitrate reductase producing activation, and only at higher concentrations do they act progressively on that part of the nitrate reductase which bears the active site for NADPH, producing loss of NADPH activity and, ultimately, on the part of nitrate reductase which bears the active sites for benzyl viologen and nitrate, producing loss of the benzyl viologen activity.

Ethylene glycol also promotes activation of both benzyl viologen and NADPH activities of inactive nitrate reductase, and this is also compatible with the contention that there exists a controlling region of the enzyme which under the effect of the denaturant remains affected producing activation. However, simultaneously with activation, ethylene glycol produces also a partial loss of both benzyl viologen and NADPH activities. Characteristically, the loss of benzyl viologen activity and the loss of NADPH activity are coincident. It is apparent that ethylene glycol, different with respect to the other denaturants tested here which act progessively on the controlling part, on the NADPH activity and, ultimately, on the benzyl viologen activity, acts simultaneously on the controlling part and on the catalytic part. With respect to the latter action, it seems that ethylene glycol acts on a catalytic part with which either NADPH activity and benzyl viologen activity are equally concerned. Perhaps it acts at level of the part of nitrate reductase which bears the active site for nitrate.

Heat differs from the other denaturants tested in that it produces an activation of only the benzyl viologen activity and, concomitantly, a loss of the NADPH activity. FAD, which protects the NADPH activity against heat

denaturation, also allows for the activation of benzyl viologen activity to occur at a higher temperature. It seems that it is an effect of heat on that part of nitrate reductase which bears the active site for NADPH that brings about inactivation of NADPH activity on the one hand, and activation of benzyl viologen activity on the other. This means that the integrity of the entire nitrate reductase molecule is required for the maintainance of the inactive state of the nitrate reductase, and not of only its controlling part.

The effect of heat on the inactive nitrate reductase best supports the view that the other denaturants produce an activation through an action on parts of the enzyme different from those involved in the catalytic process. Thus, in the case of heat, where activation is due apparently to denaturation of an enzyme part involved in the catalytic reduction of nitrate by NADPH, activation cannot be seen with respect to the NADPH activity, but can with respect to the generally less heat-sensitive benzyl viologen activity.

Examples already exist of the activation of enzymes by treatment with protein denaturant agents; among interconvertible enzymes a phosphorilase *b* from rabbit muscle in the presence of hydrophobic solvents is stabilized in some partially active conformations [15].

The effects of denaturants on nitrate reductase may recall the desensitization of allosteric enzymes in the sense that, like allosteric enzymes, loss of enzyme integrity brings about a loss of its regulatory properties [16].

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