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EFFECT OF HEAT TREATMENT ON THE ACTIVITY *IN VITRO* OF NITRATE REDUCTASE FROM *CYANIDIUM CALDARIUM*

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SUMMARY

Nitrate reductase from the unicellular alga *Cyanidium caldarium* is present partly in a fully active form and partly in a latent form.

The latent nitrate reductase becomes expressed at 0°C: the reactivation of enzyme is dependent on the ionic strength of the buffer. It is expressed also upon heat treatment above 42°C; however, this heat-activated nitrate reductase can utilise only reduced benzyl viologen and not NADPH as electron donor for nitrate reduction. The ΔH_a for conversion from latent into active benzyl viologen nitrate reductase is 69.5 kcal.

When fully active nitrate reductase is heated at temperatures above 42°C, a loss of the NADPH-dependent activity occurs; the ΔH_a for this process is 68.5 kcal. Inactivation of the benzyl viologen-dependent activity occurs at temperatures above 64°C with an ΔH_a of 114 kcal.

The Arrhenius plot exhibits a break at 24°C; above this temperature the energies of activation calculated are: benzyl viologen, 6.15 kcal; NADPH, 7.55 kcal.

INTRODUCTION

Studies on nitrate reductase from various photosynthetic organisms showed that it utilises as electron donors the reduced forms of either pyridine nucleotide (preferentially NADH) viologens and flavins. It is well established both in higher plants[1,2] and in unicellular algae[3,4] that the activity with pyridine nucleotide is much more sensitive to mild heat inactivation and to *p*-chloromercuribenzoate (PCMB) than is that with benzyl viologen.

More recently[5] it was found that extracts from cells of the unicellular alga *Cyanidium caldarium*, an acidophilic and thermophilic organism, contain two different forms of enzyme when grown on nitrate: a fully active enzyme (functional with benzyl viologen and NADPH), and, in a small amount, a latent enzyme measurable *in vitro* only after activation. Further, when cells with fully induced nitrate reductase are supplied with ammonium ions, the active enzyme is transformed into the inactive species. In addition, when this inactive nitrate reductase is heated above 42°C, the benzyl viologen-dependent activity undergoes an irreversible time- and temperature-

Abbreviation: PCMB, *p*-chloromercuribenzoate.

dependent activation, resulting in about a quadrupling of the initial activity. In contrast, the pyridine nucleotide activity of the fully active enzyme is destroyed under these conditions.

This report describes the kinetics and energetics of thermal inactivation of the fully expressed nitrate reductase. The kinetics and energetics of thermal activation of the benzyl viologen activity of the latent enzyme was also investigated and is discussed.

MATERIALS AND METHODS

The unicellular alga *Cyanidium caldarium*, obtained from Prof. T. D. Brock, Indiana University, U.S.A., was grown autotrophically at 45°C and at pH 1.5 as described previously[5]. Cell-free extracts were prepared in 0.01 M phosphate buffer (pH 7.5), with a French press and centrifugation at 27000 × *g* for 20 min. The crude extract was centrifuged at 270000 × *g* for 2 h in a Spinco centrifuge Model L 3-50; all the activity was in the supernatant solution. The enzyme was precipitated with ammonium sulphate at 35 % of saturation, resuspended in 0.01 M phosphate buffer (pH 7.5), and dialysed against the same buffer for 6 h (3 changes, 1.5 l each change). The studies reported in this article were performed with this type of preparation (except as otherwise noted).

Nitrate reductase activity was assayed colorimetrically by measuring the nitrite formed. Full details are given in a previous paper[5].

The fully active nitrate reductase was obtained from nitrate grown cells. If any latent enzyme was present in the crude extract, this was totally activated during the $(NH_4)_2SO_4$ precipitation and thus all the enzyme was present in the fully active form.

The inactive enzyme was obtained from nitrate grown cells. These were withdrawn, washed once and again resuspended in nitrate containing medium. After 2 h, NH_4^+ (0.01 M) was added (without removal of nitrate) to inactivate the enzyme, and the incubation continued for an additional 2 h. More than 75 % of the enzyme obtained from these cells was in the inactive form. The extract, after high speed centrifugation, was not treated with $(NH_4)_2SO_4$ since this treatment itself activates the latent enzyme.

RESULTS

Effect of the buffer concentration on latent nitrate reductase

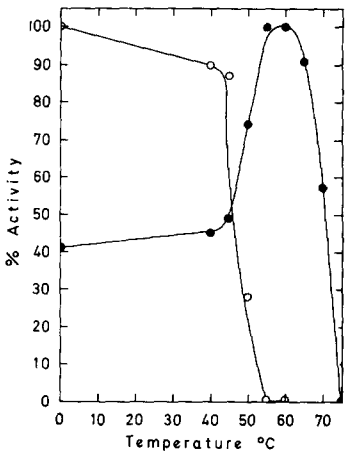
The latent enzyme lacks the ability to reduce nitrate *in vitro* both with NADPH and benzyl viologen. However, the viologen activity may be activated about 4 fold by exposure of the enzyme to a temperature above 42°C (see below). The latent nitrate reductase could also be reactivated (both benzyl viologen and NADPH activities in this case) simply by keeping the extract at 0°C for some hours. This reactivation depends on the ionic strength of the buffer. It is rapid in 0.07 M buffer and slow in 0.025 M buffer (Table I). This spontaneous reactivation was not observed for at least 1 day in presence of 0.01 M buffer (not shown). We have not investigated this point further.

TABLE I

EFFECT OF BUFFER CONCENTRATION ON THE RATE OF LATENT NITRATE REDUCTASE ACTIVATION AT 0 °C

Separate aliquots of extract containing latent nitrate reductase were incubated in the presence of varying concentration of phosphate buffer (pH 7.5) at 0°C. Activity was measured at the times indicated at 30°C as described previously[6].

Buffer (mM)	*Electron donor*	*Activity (nmoles nitrite formed per ml extract)*		
		0 h	*2 h*	*4 h*
25	Benzyl viologen	116	171	196
	NADPH	23	32	41
50	Benzyl viologen	116	245	343
	NADPH	23	40	55
75	Benzyl viologen	116	338	401
	NADPH	23	45	56



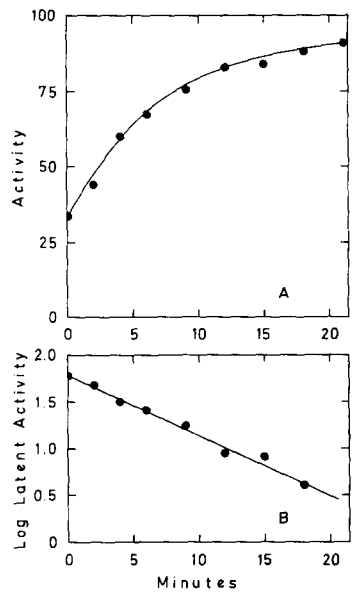


Fig. 1. Temperature dependence of activation of benzyl viologen and disappearence of NADPH activities. Aliquots were incubated at the indicated temperatures for 5 min, chilled, and then assayed at 30°C. ○—○, NADPH activity; ●—●, benzyl viologen activity.

Fig. 2. Kinetics of activation of latent benzyl viologen–nitrate reductase at 50°C. Microtubes containing the extract (0.05 ml) and water (1 ml) were immersed in a bath at 50°C, then successively withdrawn and chilled in ice. Enzymatic assays were made at 30°C. A. Increase of activity. B. Log of residual latency. Slope = 0.065 min^{-1}. Activity, nmoles of nitrite produced per min per system.

Effect of heat treatment on the nitrate reductase activity

When aliquots of crude extract of nitrate grown cells, containing about equal amounts of fully active and latent enzyme, were heated for 5 min at various temper-

atures (35–75°C) and then assayed at 30°C, a definite increase of benzyl viologen activity was observed for the temperature range of 40–55°C; in contrast, a corresponding and progressive loss of NADPH-dependent nitrate reductase occurred in the same temperature range. On the other hand, the inactivation of benzyl viologen activity occurs at temperatures above 64°C (Fig. 1).

Thermal activation of the benzyl viologen activity of the latent nitrate reductase: kinetic properties

The time course of activation of the benzyl viologen activity when latent enzyme is heated at 50°C is presented in Fig. 2. The process of heat activation is first order. Fig. 2A represents the time dependence; Fig. 2B the logarithm of the latent enzyme remaining. The first order rate constant derived from the slope shown in Fig. 2B is 0.065 min^{-1}. Fig. 3 is the plot of the logarithm of the first order rate constants obtained at 42, 44, 46, 48 and 50°C as a function of $1/T$, and from it a value for ΔH_a of 69.5 kcal was calculated.

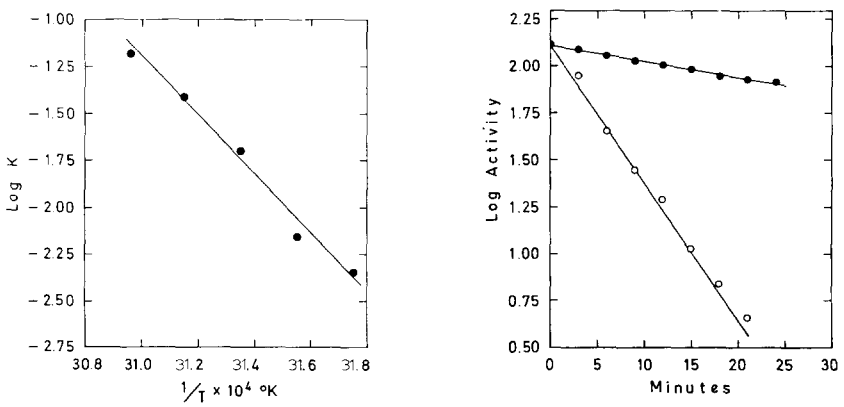


Fig. 3. Determination of the activation energy for the conversion of latent nitrate reductase into a manifest benzyl viologen–nitrate reductase. The log of the first order rate constants (computed in min^{-1}) at 42, 44, 46, 48 and 50°C are plotted as a function of $1/T$. The ΔH_a calculated from the slope is 69.5 kcal.

Fig. 4. Time course of thermal inactivation of NADPH–nitrate reductase activity at 42 (●—●) and 48°C (○—○). Test tubes containing 0.1 ml of fully active nitrate reductase and 1 ml of water were immersed in a bath at each temperature and removed successively as indicated. The residual activity was then assayed at 30°C. Activity, nmoles of nitrite produced per min per system.

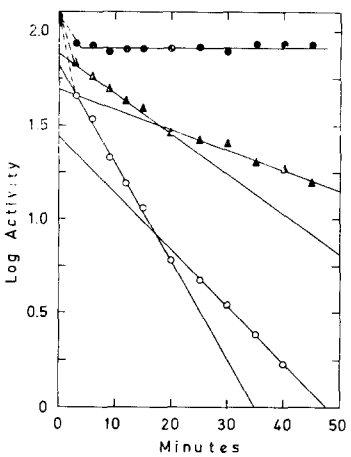
Kinetic properties of thermal inactivation of NADPH–nitrate reductase activity

Exposure of fully active nitrate reductase to a temperature above 42°C results in loss of the NADPH activity. The inactivation of the enzyme at 42 and 48°C is illustrated in Fig. 4 which shows the logarithm of residual activity as a function of time. It is apparent that the inactivation may be approximated by a first order rate constant, the value of which at 48°C is 0.074 min^{-1}.

The plot of the logarithm of first order rate constants obtained at 42, 44, 46, 48 and 50°C *versus* the reciprocal of absolute temperature indicated that the ΔH_a for the inactivation process is 68.5 kcal.

Thermal inactivation of fully expressed benzyl viologen–nitrate reductase

The benzyl viologen–nitrate reductase is much more resistant to heat inactivation than is the NADPH activity. As shown in Fig. 1 its thermal inactivation occurs at temperatures above 60°C. This is also illustrated in Fig. 5 which shows the logarithm of residual activity as a function of time when the enzyme was heated at 60, 66 and 68°C. It is apparent that in the first 2 min the enzyme undergoes an inactivation that is greater in rate and extent as the temperature is raised (dotted lines in Fig. 5). After this rapid initial inactivation, whereas at 60° C the activity remains essentially constant, at 66 and 68°C a biphasic loss of activity occurs. Of the two phases, the first is more temperature sensitive than the second, but both are approximated by first order rate constants. At 68°C the rapid and the slow rate constants, specified as k_r and k_s, were 0.053 and 0.024 min^{-1}, respectively. When the logarithm of k_r and k_s determined at 64, 65, 66, 67 and 68°C are plotted *vs.* $1/T$, two straight lines are obtained, and from the slope of each a ΔH_a of approx. 114 kcal was calculated for the inactivation reaction (Fig. 6).



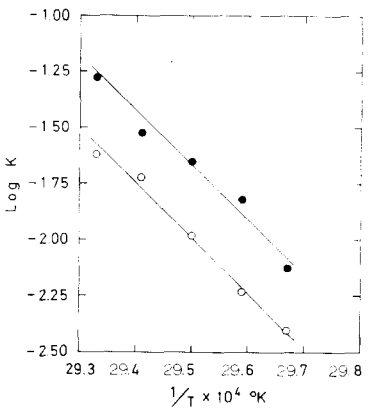


Fig. 5. Time course of thermal inactivation of fully expressed benzyl viologen–nitrate reductase activity at 60 (●—●), 66 (▲—▲) and 68°C (○—○). The enzyme (0.025 ml) was heated as described in the legend of Fig. 4. The residual activity was assayed at 30°C. Activity, nmoles of nitrite produced per min per system.

Fig. 6. Determination of the energy of activation for inactivation of fully expressed benzyl viologen–nitrate reductase. The log of the first-order rate constants of the rapid (●—●) rate, K_r, and slow (○—○) rate, K_s, computed in min^{-1} at 64, 65, 66, 67 and 68°C are presented as a function of $1/T$. ΔH_a calculated from each slope is 114 kcal.

Optimal temperature for the enzyme activity and Arrhenius activation energies.

To analyse more closely the temperature optimum of the nitrate reductase, activity was determined at various temperatures (with an interval of 3°C) in the range between 15 and 60°C, and the patterns shown in Fig. 7 were obtained. The temperature–velocity profile exhibits a characteristic shoulder at 24°C for both benzyl viologen– and NADPH–nitrate reductase activity. It should be noted that, whereas below 24°C the increase in activity with temperature for both activities is about the same, above 24°C the increase observed was more pronounced for the

NADPH activity. The temperature optimum for the NADPH activity is 37°C, *i.e.*, several degrees lower than that found for the benzyl viologen activity which occurs at 45°C. At the latter temperature the NADPH–nitrate reductase was not measurable. It seems that, with its higher energy of activation (see later) the NADPH activity reaches the temperature optimum for activity and the temperature of denaturation before (*i.e.* at lower temperature) the benzyl viologen activity does.

When corresponding values of activity obtained at various temperatures (with an interval of 3°C, from 15 up to 42°C) are plotted (as logarithm) against the reciprocal of absolute temperature according to the Arrhenius equation, the graphs shown in Fig. 8 were obtained. It should be noted that a break in the Arrhenius plot occurs at 24°C, which corresponds to the characteristic shoulder observed in the enzyme velocity *vs.* temperature curves shown in Fig. 7. The energies of activation calculated are: for the benzyl viologen activity, 9.6 kcal below 24°C and 6.15 kcal above 24°C; for the NADPH-nitrate reductase, 9.8 and 7.55 kcal below and above 24°C, respectively.

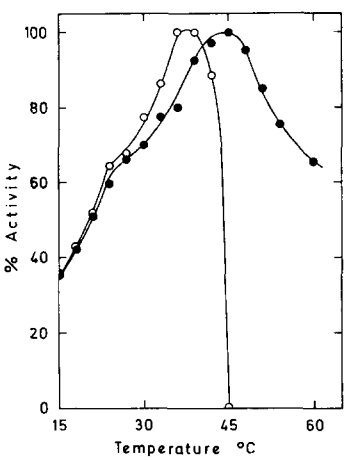


Fig. 7. Determination of the temperature optimum for NADPH (○—○) and for benzyl viologen (●—●) activities. All determinations were done at the reported temperatures. Fully active-enzyme was used.

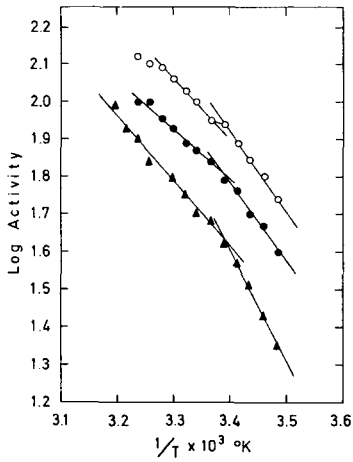


Fig. 8. Arrhenius plots of the NADPH (○ - ○) and of the benzyl viologen (●—●) nitrate reductase activities. (▲- -▲), Arrhenius plot obtained with extract heated at 60°C for 3 min. For benzyl viologen and NADPH, 0.02 and 0.08 ml of fully active enzyme respectively were used. Activity, nmoles of nitrite produced per min per system.

The Arrhenius plot for the benzyl viologen–nitrate reductase obtained with enzyme heated at 60°C for 3 min, exhibits the characteristic break at 24°C, but the energies of activation calculated are different from those found with unheated extract. They are: 13 and 7.8 kcal below and above 24°C, respectively.

DISCUSSION

As with nitrate reductase from spinach[1], *Chlorella fusca*[3] and *Ankistrodesmus braunii*[4], the enzyme studied by us exhibits both pryidine nucleotide and benzyl

viologen activity. The former is more sensitive to the action of heat than the latter. Furthermore, as previously reported[5], NH_4^+ has the same repressive effect on the synthesis of nitrate reductase of Cyanidium as in other unicellular algae[4]. We may thus conclude that in all significant respects the Cyanidium nitrate reductase system resembles that found in other photosynthetic organisms, with the exception[5] that: it is more heat stable, utilises as substrates NADPH and to a lesser extent NADH (other nitrate reductases utilise NADH only) and shows a higher pH optimum.

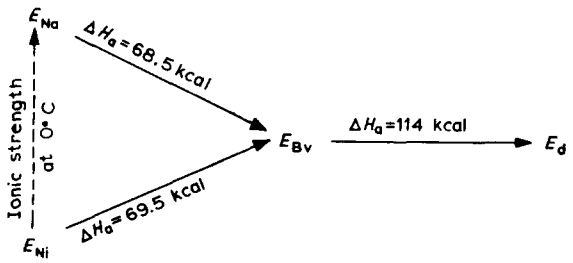
Another interesting action of NH_4^+ observed in *Chlorella fusca* by LOSADA *et al.*[6] and in Cyanidium in our laboratory, is the *in vivo* inactivation of the nitrate reductase system. If fully induced cells are supplied with NH_4^+, the extract derived from these contains 75 % or more of an enzyme which is unable to reduce nitrate either with benzyl viologen or with NADPH. Simply by keeping the extract at 0°C for some hours, the inactive enzyme is reactivated and both benzyl viologen and NADPH-dependent activities are restored. As reported above, the reactivation of Cyanidium nitrate reductase at 0°C is dependent on the ionic strength of the medium.

When extracts of Cyanidium containing inactive nitrate reductase are heated, the benzyl viologen-dependent activity is increased up to 4 fold. However, this heat activated enzyme lacks totally the NADPH activity.

The apparent activation of enzyme by heat has in some instances been related to the presence of a thermolabile inhibitor[7]. At this stage of our work we cannot exclude the existence of a nitrate reductase–inhibitor complex which is dissociated by heat treatment or by the ionic strength of the buffer, but some observations suggest that the activation of latent nitrate reductase may have another explanation.

The activation of benzyl viologen–nitrate reductase by heat is a first order process, is irreversible and exhibits a ΔH_a of 69.5 kcal. The loss of NADPH activity is also a first order irreversible process and exhibits a ΔH_a of the same magnitude. These thermodynamic quantities suggest that, when latent nitrate reductase is heated, while the benzyl viologen activity is increased, the latent NADPH activity is lost, and this explains why the heat-activated nitrate reductase is operative with benzyl viologen but not with NADPH.

A minimal mechanism which explains the distinctive features of heat on nitrate reductase may be proposed:



where E_{Na} = native enzyme active with both benzyl viologen and NADPH; E_{Ni} = native enzyme, but inactive with both benzyl viologen and NADPH; E_{Bv} = modified enzyme active only with benzyl viologen; E_d = denatured enzyme.

With reference to the proposed scheme we must conclude that the heat inactivation of the NADPH activity and the heat activation of the benzyl viologen

activity exhibit similar kinetic properties because both processes are essentially identical, and consist of the irreversible conversion of the native enzyme (which may possess latent or fully expressed activity) into a modified form whereby the enzyme has lost the ability to utilise NADPH while retaining completely the ability to utilise benzyl viologen. This modified enzyme is then destroyed at higher temperatures with a ΔH_a of 114 kcal.

There are many examples of enzymes activated by heat through a change in conformation. A similar effect of temperature was found by NEECE AND FRIDOVICH[8] for a latent acetoacetate decarboxylase, and FARRON AND RACKER[9] have described a coupling factor from spinach chloroplast which lost coupling activity but gained ATPase activity on heating.

There are reports[10,11] that the presence of a break in the Arrhenius plot is associated with a protein conformational change. The Arrhenius plot of nitrate reductase activity exhibits such a break; the transition temperature occurs at 24°C. It is therefore possible that, at this temperature a structural change in the nitrate reductase molecule occurs. The occurrence of such a change is suggested also by comparing the energy of activation values found with benzyl viologen and NADPH. Below 24°C the nitrate reductase activity exhibits energy of activation values which are almost the same for both reductants; by contrast, above 24°C, the energy of activation values differ for each reductant (see RESULTS). These results are easily explained by supposing that temperature induces the formation of certain conformation(s) of enzyme molecules each possessing characteristic kinetic properties. These differences found in the Arrhenius energy of activation above 24°C with respect to the electron donors, anticipate the differences found at still higher temperature during heat denaturation.

All these observations on the kinetic quantities for the enzyme indicate indirectly but strongly that the differential effect of temperature on NADPH– and benzyl viologen–nitrate reductase activity may be explained by some structural change in the enzyme molecule whereby the two activities are differently affected.

ACKNOWLEDGEMENTS

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