Kinetics of leaf nitrite reductase with Methyl Viologen and ferredoxin under controlled redox conditions

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Some factors that influence the activity of nitrite reductase (EC 1.7.7.1) were investigated, the enzyme from *Cucurbita pepo* (vegetable marrow) being used. The activity with ferredoxin or Methyl Viologen as electron donor was inhibited by certain salts, including NaCl. The steady-state kinetic parameters measured in a commonly used open-tube (aerobic) system were compared with a closed-cell (anaerobic) system in which the redox potential, and thus the concentrations of oxidized and reduced donor, could be controlled. This showed that in the open-tube system the apparent K_m values determined were overestimated (by a factor of 10 for reduced Methyl Viologen), owing to incomplete mediator reduction and competitive inhibition by the oxidized form of the mediator.

An assay of nitrite reductase activity that is frequently cited in the literature (Ramirez *et al.*, 1966) involves the incubation of the enzyme in an open tube with nitrite as the substrate, and dithionite-reduced Methyl Viologen (MV^+) or ferredoxin as electron donor. Samples are withdrawn and assayed spectrophotometrically for nitrite and the rate of disappearance of nitrite calculated. This method is a reliable way of comparing the activity of various samples, but appears to give variable results when used to estimate steady-state kinetic parameters. Apparent K_m values range between 70 and 100 μM for MV^+ (Ramirez *et al.*, 1966; Ida & Morita, 1973), between 10 and 70 μM for ferredoxin (Ramirez *et al.*, 1966; Hewitt *et al.*, 1968; Ho & Tamura, 1973), and between 0.1 and 0.55 mM for nitrite (Ramirez *et al.*, 1966; Ida & Morita, 1973; Shimizu & Tamura, 1974).

We have reinvestigated the kinetic parameters in a cell in which the redox potential could be measured, and thus the concentrations of the reduced dye MV^+ and the oxidized form MV^{2+} could be calculated.

Experimental

Nitrite reductase

The enzyme was purified by the method of Hucklesby *et al.* (1967) from *Cucurbita pepo*

Abbreviations used: MV^+ , dithionite-reduced Methyl Viologen; MV^{2+} , oxidized Methyl Viologen; Mops, 4-morpholinepropanesulphonic acid; BV^+ , reduced Benzyl Viologen; BV^{2+} , oxidized Benzyl Viologen.

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(vegetable marrow) leaves. Preparations had an A_{384}/A_{280} ratio of 0.4 or more. Ferredoxin was obtained from the supernatant after fractionation with $(NH_4)_2SO_4$, by chromatography on DEAE-cellulose with a decreasing gradient of $(NH_4)_2SO_4$ and further purified as described by Scawen *et al.* (1975). Its purity was estimated at 90–98%, with A_{420}/A_{277} of 0.48–0.51.

Enzyme assays

(a) *Open-tube assay.* Nitrite reductase was assayed by the standard method of Ramirez *et al.* (1966) at 30°C. Enzyme (0.1–0.15 μg) was incubated in 50 mM-potassium phosphate buffer, pH 7.0, containing 0.25 mM-nitrite, 0.5 mM- MV^{2+} and 2.5 mg of dithionite in 2.5 ml. Nitrite and MV^{2+} concentrations were varied to determine the K_m values. The enzyme was incubated for periods up to 10 min, after which the dithionite was oxidized by vigorous mixing with a Whirlimixer (Fisons Scientific Apparatus, Loughborough, Leics., U.K.). Oxidation was complete in 15–20 s as judged by the loss of the blue colour of the reduced MV^+ . The concentration of the remaining nitrite was determined by the method of Snell & Snell (1964), by producing a pink diazo compound by the reaction of nitrite with acidic sulphanilamide and naphthylethylenediamine and measuring the A_{540} . The colour yield was standardized with nitrite, to which dithionite was added at an equivalent concentration to that in the enzyme assay, to allow for possible interference by dithionite oxidation products (Senn *et al.*, 1976). In fact this interference was found to be small (about 5% decrease in absorbance).

(b) *Closed-cell assay.* Nitrite reductase was assayed in a closed cell similar to that described by Dutton (1971). The closed cell was continuously flushed with N_2 that had been passed through a 1% (w/v) NaOH/2% (w/v) $Na_2S_2O_4$ solution in a Nilox apparatus (Gilroy & Mayne, 1962) (Jencons Scientific Ltd., Leighton Buzzard, Beds., U.K.) to remove O_2 . Enzyme (0.3–0.5 μg) was incubated at 30°C in 20 mM-Mops buffer, pH 7.0, containing nitrite and MV^{2+} in a volume of 5 ml. $Na_2S_2O_4$ (0.1 M in 0.2 M-Tris/HCl, pH 9.0) was added in microlitre quantities with a screw-driven syringe and the system was slowly stirred. The potential was monitored by a platinum electrode with calomel (Hg_2Cl_2) reference. The potential could be maintained to within ± 2 mV of the poised potential during the course of the experiment. Samples (100 μl) were withdrawn by syringe, diluted 1:20 with water and assayed for nitrite by the method of Snell & Snell (1964). In order to obtain a concentration of a reduced donor such as MV^+ in the presence of a known concentration of the oxidized donor [MV^{2+}], the total amount to be added was:

$$[MV]_t = [MV^+] + [MV^{2+}]$$

and the redox potential was adjusted to

$$E = E^0 + \frac{RT}{nF} \ln \left\{ \frac{([MV]_t - [MV^+])}{[MV^+]} \right\}$$

where R is the gas constant, T the absolute temperature and F the Faraday constant. E^0 , the midpoint potential of the donor, was taken as –440 mV for Methyl Viologen, and –360 mV for Benzyl Viologen (Clark, 1960); $n = 1$ for these donors. The midpoint reduction potential of the ferredoxin was determined by the method of Cammack *et al.* (1977) to be –420 mV. When ferredoxin was used as electron donor, 1 μM -Methyl Viologen was added to act as a mediator to the platinum electrode. In other experiments the cell was left open to the air to compare the effect of an open-tube assay system.

For progress-curve analysis, 0.1–0.15 μg of enzyme was incubated in the cell described above, in 20 mM-Mops buffer, pH 7.0, containing 1 mM-nitrite and 0.25 mM- MV^{2+} . $Na_2S_2O_4$ (100 mM) was added in microlitre quantities to maintain a potential of –450 mV. Samples were withdrawn and assayed for nitrite.

Results

The effect of ionic strength on the enzymic rate was first investigated in the open-tube assay system. Mops (20 mM, pH 7.0) was used as buffer and various concentrations of NaCl were added. With 0.5 mM-Methyl Viologen as electron donor, it was

found that the activity was decreased by 9% on increasing the ionic strength (I) from 0.1 to 0.2 and by 34% at I 0.5. KCl and NaI had similar effects to NaCl, but Na_2SO_4 (at I 0.13) gave a small but consistent stimulation of 10%. With 50 μM -ferredoxin as electron donor the activity was decreased by 50% on increasing I from 0.1 to 0.2 with NaCl, and by 90% at 0.5. In view of these results the NaCl concentration was kept to a minimum in the succeeding experiments under controlled redox conditions.

The enzyme activity was assayed with various concentrations of electron donor in the open-tube system. If it is assumed that the donor is fully reduced, then the K_m values for MV^+ , ferredoxin, nitrite and BV^+ are as shown in the first column of Table 1, which are within the range of values previously reported.

However, this assumption does not appear to be justified. When a solution of dithionite-reduced Methyl Viologen was left in the cell exposed to the air, it was found that the initial potential changed from –480 to –430 mV in 10 min. This corresponds to a decrease from an initial 85 to 37% reduction of Methyl Viologen over the period used for the enzymic open-tube assay system. The cell was slowly stirred, whereas the open-tube system was not, but this experiment shows that changes in the mediator concentration can occur. In an unstirred system there is probably a gradient of MV^+ concentration in the tube which again will result in an overestimation of K_m values.

With the closed cell, the K_m values shown in the second column of Table 1 were obtained. Ferredoxin and MV^+ exhibited classic Michaelis-Menten kinetics, with competitive inhibition by the oxidized form of the mediator, and K_i values could be determined.

With Benzyl Viologen, more complex kinetics were observed. At low concentrations of BV^{2+} (25 μM), the Lineweaver-Burk plots approximated to a straight line, giving an apparent K_m value of $26 \pm 3.5 \mu M$. However, at high BV^{2+} concentrations and low BV^+ concentrations, the rates were anomalously low, giving curved plots, and thus the K_i values could not be calculated. Similar anomalous behaviour of Benzyl Viologen compared with Methyl Viologen has been observed in studies of nitrogenase (Ware, 1972). This might be due to some kind of interaction between BV^+ and BV^{2+} .

Determination of the K_m values for nitrite by initial-velocity measurements involved the measurement of small concentrations of nitrite. This led to an increase in the errors during nitrite estimation. Also the assumption that substrate limitation may be ignored was no longer valid. Hewitt *et al.* (1968) found apparent K_m values to range between 1 and 300 μM in different experiments. The use of progress-

Table 1. Summary of K_m values for nitrite and reduced donors, and K_i values of oxidized donors, for *C. pepo* nitrite reductase

K_m and K_i values are shown for (a) the aerobic open-tube system and (b) the anaerobic-cell system. Errors were calculated by a least-squares fit to the rate data (Wilkinson, 1961).

Substrate	Method of measurement	K_m values (μM)		K_i values (μM)
		(a)	(b)	
Ferredoxin	Initial rate	13.3 ± 1.5	3.4 ± 0.4	15.6 ± 1.6
Benzyl Viologen	Initial rate	—	26.0 ± 3.5	—
Methyl Viologen	Initial rate	106 ± 11	7.6 ± 0.8	490 ± 50
Nitrite*	Initial rate	110 ± 12	—	—
Nitrite*	Progress curve	73 ± 8	40 ± 5	—

* With reduced Methyl Viologen as electron donor.

curve analysis allowed more accurate estimates of K_m values for nitrite, as larger initial nitrite concentrations could be used, and changes more precisely measured. NH_4^+ , the product of the reaction, was found not to inhibit the enzyme at concentrations up to 50 mM, so that a simplified integrated rate equation could be used.

Fig. 1 shows the progress curve of disappearance of nitrite, plotted as described by Cornish-Bowden (1976). The values for K_m for nitrite derived from this plot were $40 \mu\text{M}$ for controlled redox conditions and $70 \mu\text{M}$ for the uncontrolled open-tube system. The difference may be partly due to the different buffers employed, phosphate in the open-tube system and Mops in the closed system. The values are somewhat smaller than those obtained by

Ramirez *et al.* (1966), Ida & Morita (1973) and Shimizu & Tamura (1974), but much larger than the value of $1 \mu\text{M}$ reported by Cresswell *et al.* (1965) for the *C. pepo* enzyme. The latter report is difficult to explain, but may have been due to side reactions between nitrite and BV^+ (Hewitt *et al.*, 1975).

The maximum rate of turnover, with MV^+ or ferredoxin as mediator, corresponds to between 9.5 and 10.5×10^3 mol of nitrite reduced/min per mol of enzyme, assuming an ϵ_{384} for the enzyme of $76 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Lancaster *et al.*, 1979). Values determined with the open-tube system were more varied, ranging between 4 and 7.5×10^3 mol of nitrite/min per mol of enzyme. If ϵ for the enzyme is taken as $37 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Hucklesby *et al.*, 1976) or $40 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Vega & Kamin, 1977), the activity is correspondingly lower.

Discussion

A number of different methods have been used to study the kinetics of enzymes that use MV^+ as electron donor, notably hydrogenase. For example, Van Dijk *et al.* (1981) used the dithionite/bisulphite couple to reduce the MV^{2+} , calculating the proportion reduced from a knowledge of the respective midpoint potentials and pH. Arp & Burris (1981) measured the concentration of MV^+ spectrophotometrically. The advantages of the potentiometric method employed in the present work are that there is no limit on the concentration of MV used or pH, and that the ratio $\text{MV}^{2+}/\text{MV}^+$ is continuously monitored.

The results of the closed-cell system indicate that it gives higher specific activities than the open-tube system. Apart from the use of a lower-ionic-strength buffer, the main factor is probably the more efficient reduction of the mediator. This in turn is due to the use of the correct amount of dithionite (too much dithionite can increase the redox potential; Mayhew, 1978) and to the exclusion of O_2 . The assay of nitrite

Fig. 1. Progress-curve analysis of the effect of nitrite concentration on nitrite reductase activity, under aerobic conditions (closed circles) and anaerobic conditions (open circles) as described in the Experimental section

Lines of regression were calculated for the points to determine the K_m values. s_0 is the initial substrate concentration, and s the concentration at time t .

reductase in general would therefore be assisted by allowing the reaction to proceed under an anaerobic atmosphere, as recommended by Hewitt *et al.* (1968). It should also be noted that the assay rate will be inhibited if the nitrite reductase preparation, or ferredoxin, introduces significant amounts of NaCl into the assay mixture.

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