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Reaction of *Trigonopsis variabilis* D-amino acid oxidase with 2,6-dichloroindophenol: kinetic characterisation and development of an oxygen-independent assay of the enzyme activity

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Abstract

2,6-Dichloroindophenol (DCIP) is shown to be utilised efficiently as electron acceptor replacing dioxygen in the reaction of *Trigonopsis variabilis* D-amino acid oxidase (TvDAO) with D-methionine as the substrate. The specificity constant for DCIP reduction at 30 °C is one-twelfth that of oxygen conversion into hydrogen peroxide. Time course analysis of simultaneous consumption of DCIP and dioxygen, recorded on-line by absorption and non-invasive fluorescence quenching, respectively, pinpoints the preferential utilisation of dioxygen; and reveals a maximum DCIP conversion rate that is independent of the initial concentration of dioxygen. A robust direct assay of TvDAO activity has been developed that does not require anaerobic reaction conditions. It was down-scaled to microtitre plate format and overcomes practical limitations of other assays due to the low affinity of TvDAO for dioxygen ($K_m \approx 0.7$ mmol L^{-1}).

Keywords: Flavoenzyme; Electron acceptor; Oxygen; Biocatalysis; Enzyme assay

1. Introduction

The flavoenzyme D-amino oxidase (DAO) catalyses the oxidative deamination of D-amino acids to the corresponding α-keto acids, coupled to the reduction of dioxygen to H_2O_2 [1]. DAO is absolutely specific for converting D-configured α-amino acids but tolerates a wide range of structural variations of the side chain. Important applications for DAO in biotechnology are biocatalytic synthesis [2–4], resolution of racemic mixtures of amino acids [5], and analytics [6]. The enzyme from the yeast *Trigonopsis variabilis* has been employed commercially to produce 7-aminocephalosporanic acid – a precursor of semisynthetic cephalosporin antibiotics – on a multiton-per-year production scale [7,8], exemplifying the successful implementation of a flavoenzyme into an industrial process.

TvDAO is a relatively stable enzyme considering the high concentration of peroxide it is exposed to during the deamination of cephalosporin C [7]. However, a small loss of enzyme activity inevitably occurs in connection with substrate turnover under the operational conditions. Though the reliable detection of any small changes in activity is crucial to process control, current assays for TvDAO activity which require dioxygen as a co-substrate have a number of clear shortcomings irrespective of whether they are direct [9–11] or use a coupled reaction for detection [12–14]. The apparent affinity of TvDAO for dioxygen is low, and the reported K_m value of approximately 0.7 mmol L^{-1} [15] (for TvDAO from strain *T. variabilis* CBS 4095) corresponds to the maximum solubility of oxygen at 25 °C and atmospheric pressure. Now, because obviously it is not possible to saturate TvDAO in oxygen under these conditions, the

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measurement of the enzyme activity is very sensitive to even small variations in the initial level of oxygen which in turn are very difficult to avoid in practice. Oxygen-dependent assays for TvDAO activity are therefore not well suited for use in high-throughput procedures and might show poor reproducibility along with a high statistical error.

In the present study, we sought for alternative electron acceptors that replace oxygen in the reaction catalysed by TvDAO; and whose conversion can be monitored spectrophotometrically, thus allowing a direct detection of the activity. Prior studies of other oxidoreductases [16–21] that are related to TvDAO by the dependence of their activity on a riboflavin cofactor suggested that 2,6-dichloroindophenol (DCIP) or chelated iron(III) might be candidate co-substrates of TvDAO, representing classes of two- and one-electron acceptors, respectively. We report here reactions of TvDAO with DCIP and iron(III) complexes during the oxidative deamination of D-methionine. The enzymatic reduction of DCIP was characterised kinetically through detailed analysis of initial rates and reaction time courses recorded at different levels of dissolved oxygen. We describe a novel oxygen-independent assay of the enzyme activity that does not require anaerobic conditions and is expected to be useful in practice due to its robustness and good reproducibility. In addition, the results provide some novel insights into the reaction of TvDAO with non-natural electron acceptors.

2. Material and methods

2.1. Enzyme preparation

A technical-grade preparation of TvDAO (from strain *T. variabilis* ATCC10679) was kindly provided by Sandoz GmbH (Kundl, Austria). Buffer exchange to a 100 mmol L^{-1} potassium phosphate buffer, pH 8.0, was achieved through repeated cycles of concentration and dilution to original volume using Vivaspin 15 ultrafiltration concentrator tubes (Vivascience AG, Hannover, Germany) with a molecular mass cut-off of 10 kDa. The enzyme solution was aliquoted and stored at −25 °C. It had a specific activity of 41 units mg^{-1} (±8; $N = 15$). The activity was recorded at 30 °C by using a continuous coupled enzymatic assay that is reported elsewhere [14] and based on the lactate dehydrogenase-catalysed NADH-dependent reduction of pyruvate that is produced through oxidation of D-alanine by TvDAO. Enzyme activity is thus monitored by measuring the decrease in NADH absorption at 340 nm. Routinely, air-saturated buffer solutions containing 50 mmol L^{-1} D-alanine and 210 μg mL^{-1} NADH were employed, and a concentration of 210 μg mL^{-1} lactate dehydrogenase from hog muscle (Roche Diagnostics, Mannheim, Germany) was used. Protein concentration was determined using the Bio-Rad Protein Assay (Cat. Nr. 500-0006) and BSA as the reference.

2.2. Screening of artificial electron acceptors

Activity of TvDAO was screened against a series of mixed-ligand complexes of iron(III) and acetylacetonate as the electron acceptor in the presence of 1,10-phenanthroline [22]. After reduction of iron(III), the evolving iron(II) binds to 1,10-phenanthroline, resulting in a red coloured complex which can be spectrophotometrically determined at 510 nm. Additionally, fluorinated derivatives of acetylacetonate, namely 1,1,1-trifluor-2,4-pentanedione (TPD) and 1,1,1,5,5,5-hexafluor-2,4-pentanedione (HPD) which show a lower chelating effect towards iron [23] have been tested. The substrate solutions contained D-alanine (100 mmol L^{-1}), iron(III)–acetylacetonate (0.5 mmol L^{-1}) or the iron(III) complexes of TPD or HPD (0.5 mmol L^{-1} each), and 1,10-phenanthroline (4 mmol L^{-1}) dissolved in a 100 mmol L^{-1} potassium phosphate buffer, pH 8.0. The reaction was initiated by the addition of a suitable amount of TvDAO, and the rate of complex formation was monitored by the increase in absorption at 510 nm using a DU800 UV-VIS spectrophotometer (Beckman-Coulter, Inc., Fullerton, CA, USA). Measurements were performed at 30 °C.

2.3. Initial rates and activity measurements using DCIP as co-substrate of TvDAO

The enzymatic rates were determined using a spectrophotometric assay at 30 °C. The substrate solutions contained D-methionine (20 mmol L^{-1}) and DCIP dissolved in a 100 mmol L^{-1} potassium phosphate buffer, pH 8.0. Quartz cuvettes with a Teflon stopper were used. Depending on the DCIP concentration which varied between 0.1 and 1.0 mmol L^{-1}, the pathlength of the cuvette was varied between 0.1 and 1.0 cm such that the initial absorption at 600 nm did not exceed a value of 2.5 (corresponding to the linear range of the spectrophotometers used). The cuvette containing the reaction mixture was aerated with nitrogen (99.999 pure) or oxygen (99.995 pure) for at least 5 min to achieve depletion or saturation in dissolved oxygen, respectively. The reaction was initiated by the addition of a suitable amount of TvDAO, and the rate of DCIP reduction was monitored by the decrease in absorption at 600 nm using a DU800 UV-VIS spectrophotometer (Beckman-Coulter, Inc., Fullerton, CA, USA) or a Spectronic Genesys 5 photometer (Milton Roy Company, Rochester, NY, USA). The absorption coefficient of DCIP under the conditions was determined to be 20,000 L mol^{-1} cm^{-1}. The enzymatic consumption of oxygen under conditions in which DCIP and oxygen were present at a time was determined using a fibre-optic oxygen microoptode (NTH-L2.5-NS(40 × 1.20 mm)-TS-COB2-YOP, Microx TX3-AOT, PreSens GmbH, Regensburg, Germany) which was placed directly into the quartz cuvette through the Teflon stopper. Calibration of the electrode was performed according to instructions of the supplier. The time courses of conversion of oxygen and DCIP were recorded simultaneously. Graphical differentiation (Δtime = 0.2 min)

of the resultant progress curves was used to calculate slope values ($\Delta C/\Delta t$, where C is the reactant concentration) at the different levels of electron acceptor. The slope provides a rough estimate of the apparent reaction rate under the conditions. It should not be compromised with a true initial rate whose determination is described below.

2.4. Determination of kinetic parameters for the reaction with oxygen

Initial rates of oxygen consumption were measured at 30 °C in a 4.5 mL glass vial that was sealed with a septum and contained a solution of D-methionine (20 mmol L^{-1}) in potassium phosphate buffer (100 mmol mL^{-1}, pH 8.0). Mixing was achieved with a magnetic stirrer. Apparent oxygen concentrations between 0.05 and 1 mmol L^{-1} were adjusted by mixing suitable amounts of phosphate buffers which were aerated with nitrogen or oxygen, respectively. The oxygen microoptode was penetrated through the septum, and the reaction was initiated by addition of a suitable amount of TvDAO using a Hamilton syringe. The time course of oxygen consumption was recorded, and initial rates were calculated from linear plots of apparent $[O_2]$ versus time.

2.5. Reaction of TvDAO under fully anaerobic conditions

The substrate solution (2 mL) containing D-methionine (20 mmol L^{-1}) and DCIP (0.15 mmol L^{-1}) in potassium phosphate buffer (100 mmol L^{-1}, pH 8.0) was aerated with nitrogen (99.999 pure) 5.0 for at least 5 min. Then, it was placed in an anaerobic quartz cuvette with a reservoir containing a suitable amount of TvDAO. After repeated cycles of evacuation and flushing with nitrogen, the enzymatic reaction was initiated by mixing the content of the cuvette, and the rate of DCIP reduction was monitored spectrophotometrically.

2.6. DCIP-based assay for TvDAO in microtitre plates

Measurements were carried out with a SPECTRAmax PLUS 384 micro-plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). One hundred microliter aliquots of an appropriately diluted TvDAO solution were transferred into a 96-well microtitre plate. After addition of 100 μL of assay mixture containing 300 μmol L^{-1} DCIP and 40 mmol L^{-1} D-methionine in phosphate buffer, pH 8.0, the decrease of absorption was measured at 600 nm and 30 °C. All measurements were performed in duplicate.

2.7. Inactivation studies and effects of additives on stability

Enzyme solutions (1.5 mL) containing 20 μmol L^{-1} TvDAO and one of the additives listed below in 100 mmol L^{-1} potassium phosphate buffer, pH 8.0, were incubated in Eppendorf vessels, using an Eppendorf thermomixer at 50 °C and 300 rpm. As a reference, the solution of TvDAO without additive was used. Samples (50 μL) were taken at different times up to 8 h, diluted with potassium phosphate buffer and immediately added to the enzyme assay. The residual activity of TvDAO was measured with the micro-plate spectrophotometer as described above. The influence on TvDAO thermal stability of hydrogen peroxide (3.3 mmol L^{-1}) as well as of common stabilisers of proteins was investigated: bovine serum albumin (20 mg mL^{-1}), flavin adenine dinucleotide (FAD, 130 μmol L^{-1}), 2-mercaptoethanol (500 μmol L^{-1}), trehalose (450 mmol L^{-1}) and glycerol (1.5%, v/v).

3. Results and discussion

3.1. Alternative electron acceptors of the reaction catalysed by TvDAO

Earlier studies of D-amino acid oxidase from hog kidney have shown that this enzyme reduces a range of artificial electron acceptors replacing oxygen, among them phenazine methosulfate, DCIP and ferricyanide [24]. During an initial screening we found that TvDAO also accepts these previously reported co-substrates (data not shown) and has particularly good activity with DCIP (see later). TvDAO reduces iron(III) in a series of metal–ligand complexes including acetylacetonate and fluorinated derivatives thereof, the activities of the fluorinated derivatives being about one order of magnitude lower than that of acetylacetonate. The characterisation of the enzymatic reactions with DCIP and iron(III)–acetylacetonate revealed that there is a linear relationship between the observed spectrophotometric rates in each assay and the protein concentration (Fig. 1). However, in comparison with DCIP, the Fe^{3+}-linked activity of TvDAO is very low. Fig. 1 also indicates that a Fe^{3+}-based assay tends to overestimate TvDAO activity in the low protein concentration range, in contrast to a DCIP-based assay. This result arguably indicates kinetic complexity in the steps from the enzymatic reduction

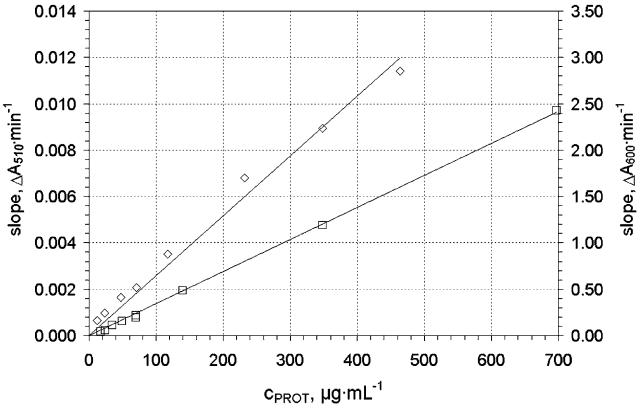

Fig. 1. Linearity of TvDAO reaction with iron(III) acetylacetonate/phenanthroline and DCIP. Open diamonds: iron(III) (left axis); open squares: DCIP (right axis).

of iron(III) to formation of the detectable complex of iron(II) and 1,10-phenanthroline.

For reasons of sensitivity and practical use, DCIP was selected for further experiments. We also considered that others have used DCIP previously to characterise oxidoreductases that are promiscuous in respect to the terminal electron acceptor of their reaction [25,17,26,27,20,28].

3.2. Simultaneous measurements of oxygen and DCIP consumption

It is not practical to work under strictly anaerobic conditions. Therefore, we were interested to determine (1) the relative timing of consumption of oxygen and DCIP when D-methionine is converted by TvDAO and both electron acceptors are present at a time; and (2) the effect of dissolved oxygen on the experimental rate of DCIP reduction.

Fig. 2 shows a typical time course of TvDAO-catalysed conversion of oxygen and DCIP. After addition of the enzyme (indicated by the arrow in Fig. 2) the apparent concentration of oxygen decreases rapidly. DCIP is utilised, however, at a slower rate. A linear decrease of the absorption is attained not until oxygen is completely depleted. It has to be pointed out that due to the non-invasive character of detection method, no oxygen is consumed because of its measurement. The results indicate a strong preference of TvDAO for reaction with oxygen over reaction with DCIP.

If electron acceptors are reduced by TvDAO in a truly sequential manner, i.e. oxygen before DCIP, the apparent level of dissolved oxygen in the enzyme assay will hardly influence the rate of DCIP reduction and hence the measured activity. DCIP will be utilised by the enzyme only until oxygen has been almost completely reacted. Results illustrated in Fig. 3 are in agreement with this notion: maximum DCIP conversion rates were not changed within limits of ±1.7% as the apparent concentration of dissolved oxygen was varied from below 5 μmol L^{-1} to approx. 1100 μmol L^{-1}. This clearly validates the use of DCIP for assaying the enzyme activity under aerobic conditions.

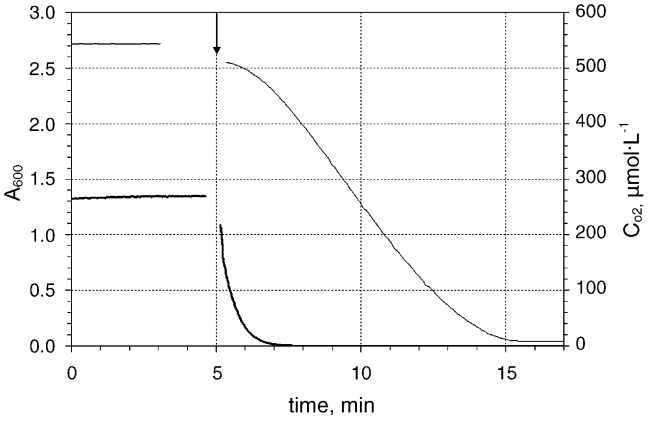

Fig. 2. Simultaneous measurement of TvDAO-catalysed reductions of DCIP and oxygen. DCIP is indicated as thin line (left *y*-axis), oxygen as thick line (right *y*-axis). TvDAO was added after approx. 5 min of incubation at 30 °C. The measurement was carried out as described in Section 2, using 150 μmol L^{-1} DCIP and a pathlength of 1 cm. The gap at approx. 5 min is due to the necessary removal of the oxygen sensor during addition of TvDAO.

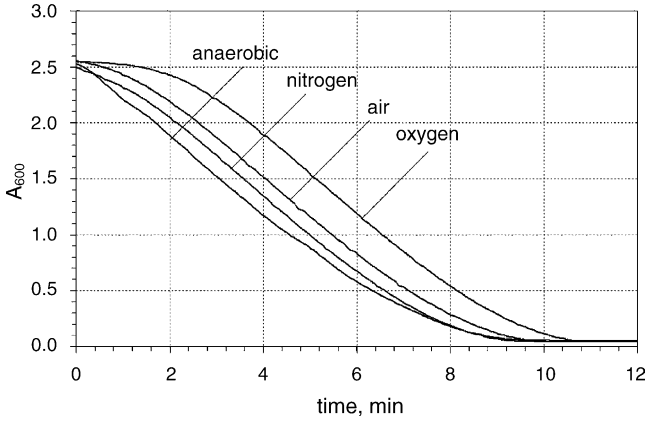

Fig. 3. TvDAO-catalysed DCIP reduction at different initial apparent oxygen concentrations. Approximate initial apparent oxygen concentrations are indicated: anaerobic (<5 μmol L^{-1}), nitrogen (50 μmol L^{-1}), air (260 μmol L^{-1}), oxygen (1100 μmol L^{-1}).

3.3. Time course analysis

Fig. 3 shows that the extent (expressed as a reaction time) to which the enzymatic conversion of DCIP lags behind the utilisation of oxygen increases with increasing initial concentration of oxygen. In the likely instance that the two-electron acceptors compete for the same enzyme active site, the presence of oxygen will inhibit the reaction with DCIP, arguably causing the lag. However, when progress curve analysis is used to calculate the enzymatic rates at the respective residual concentration of DCIP, a more complicated picture is obtained which is illustrated in Fig. 3. The results suggest that the consumption of DCIP occurs in three phases A–C.

In the phase A, DCIP reduction is inhibited by the presence of oxygen. In the ensuing phases B and C, oxygen is completely depleted. Interestingly, therefore, the maximum rate of DCIP consumption is not reached in phase B but in phase C which is used for the determination of enzymatic activity. To explain the occurrence of a phase in which DCIP is converted at an intermediate rate when no oxygen is present, we examined the influence of the H_2O_2 level. Both addition of peroxide to an end concentration of 5 mmol L^{-1} or its removal in situ through supplemented catalase (1900 U mL^{-1}) failed to produce significant changes in the three-phase pattern of DCIP reduction (Fig. 3). Experiments in which the concentration of D-methionine was varied revealed that the lag-time increased with increasing levels of substrate; and nearly disappeared when [D-methionine] was limiting (Fig. 4A), irrespective of the availability of dissolved oxygen. Based on these results

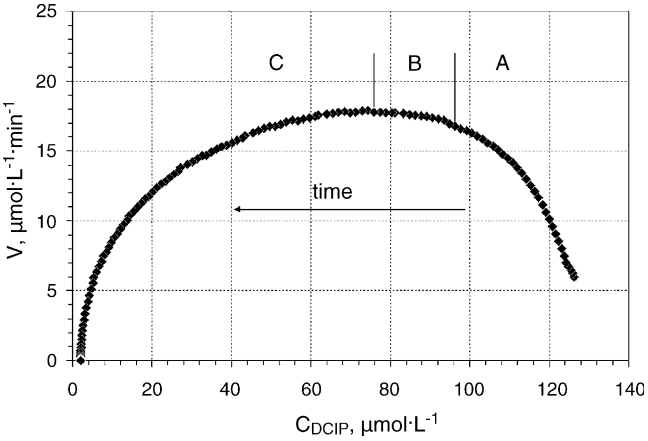

Fig. 4. Michaelis–Menten chart of TvDAO-catalysed DCIP reduction obtained from progress curve analysis. The rate of DCIP reduction corresponds to the slope of the progress curve, calculated from adjacent data points. Phase A indicates dissolved apparent oxygen concentrations above zero, during phases B and C no oxygen is present.

we hypothesise that a non-productive complex of TvDAO, D-methionine, and DCIP is formed at high substrate concentrations which must regenerate the free enzyme by slow dissociation.

3.4. Stoichiometry of the reaction of TvDAO with DCIP

DCIP is a known two-electron acceptor in the reactions catalysed by different oxidoreductases. Thus, it was expected that D-methionine and DCIP are converted by TvDAO stoichiometrically. However, there are cases in which DCIP takes part in one-electron transfer reactions [29]. Thus, 2 mol of DCIP might be utilised by the enzyme per each mole of D-methionine transformed. Therefore, experiments were carried out in which the total concentration of electron acceptors (i.e. the apparent levels of oxygen and DCIP) exceeded the concentration of the substrate. Under these conditions, DCIP cannot be completely reduced if it truly functions as a two-electron acceptor. In that case, a constant absorption value at 600 nm, reflecting the residual DCIP that has not reacted, will be reached after exhaustive conversion of D-methionine. Furthermore, an increase in absorption after D-methionine has gone to completion might indicate reoxidation of DCIP.

Fig. 5A and B shows that the reduction of DCIP is only partial when the substrate concentration is limiting. By contrast, oxygen is depleted completely when 570 µmol L^{-1} D-methionine is present (Fig. 5A). Residual oxygen is, however, detected at the lower substrate concentration of 285 µmol L^{-1}. Within limits of ±5%, the experimental data clearly reveal a stoichiometric relationship between substrate converted and electron acceptor consumed (i.e., oxygen and DCIP reduced). The results also indicate that a non-enzymatic reoxidation of DCIP takes place, however, at a rate that is clearly negligible under the conditions used.

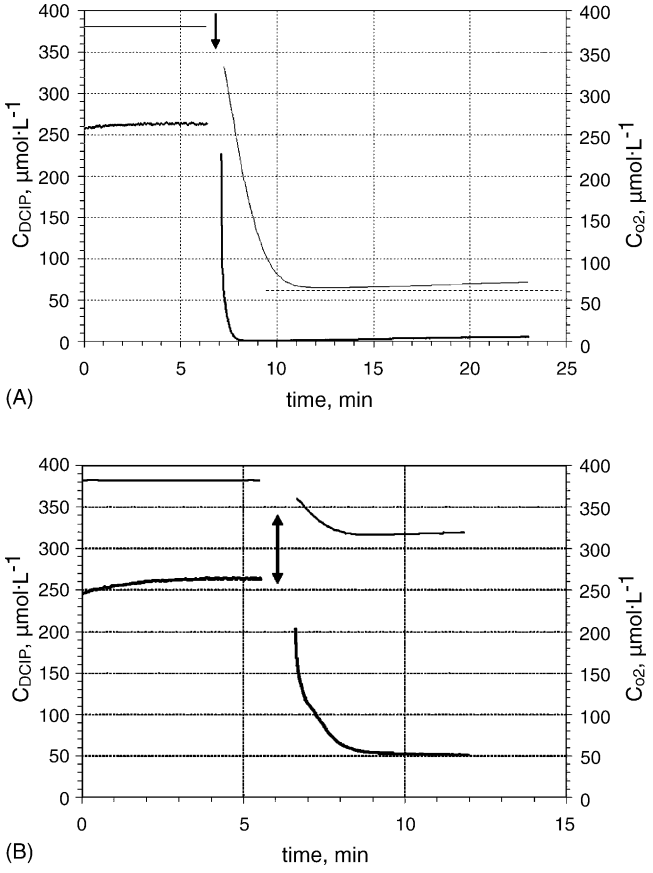

Fig. 5. TvDAO-catalysed DCIP reduction at limiting concentrations of D-methionine. DCIP is indicated as thin line, oxygen as thick line. The arrow indicates the addition of TvDAO to the assay mixture. The measurement was carried out as described in Section 2, using 350 µmol L^{-1} DCIP and a pathlength of 0.2 cm. The gap after approx. 6 min is due to the necessary removal of the oxygen sensor during addition of TvDAO: (A) 570 µmol L^{-1} D-methionine; (B) 285 µmol L^{-1} D-methionine.

3.5. Kinetic parameters for TvDAO-catalysed reduction of electron acceptors

Using a constant concentration of D-methionine of 200 mmol L^{-1}, initial rates of reduction of oxygen and DCIP were recorded at varied concentrations of the respective electron acceptor. The maximum rates of DCIP conversion (i.e. phase C in Fig. 4) were used. The data shown in Fig. 6 were fitted to the Michaelis–Menten equation using unweighted non-linear regression and apparent kinetic parameters K_m and k_{cat} were determined. SDS PAGE of TvDAO and qualitative analysis of Coomassie-stained gels suggested a minimum purity of >80% for the enzyme (Fig. 7), justifying the use of turnover numbers.

The values of K_m and k_{cat} for the reaction with oxygen were 755 ± 76 µmol L^{-1} and 229 ± 12 s^{-1}, respectively, and yield a specificity constant (k_{cat}/K_m) of $(303 \pm 35) \times 10^3$ L mol^{-1} s^{-1}. Kinetic parameters for the reaction with DCIP were 182 ± 8 µmol L^{-1} and 4.6 ± 0.1 s^{-1} for K_m and k_{cat}, respectively, and give a specificity constant of $(25 \pm 1) \times 10^3$ L mol^{-1} s^{-1}. Comparison of k_{cat}/K_m val-

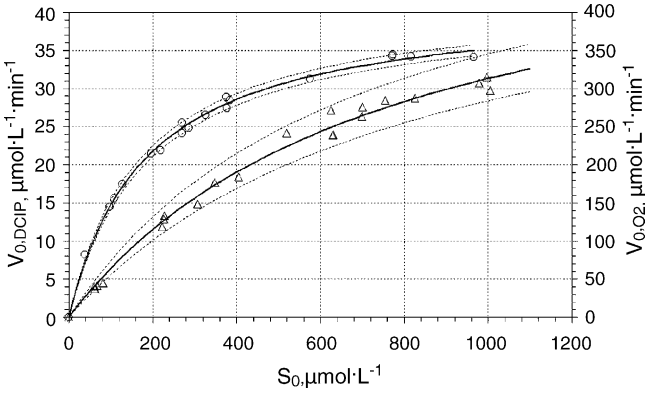

Fig. 6. Determination of kinetic parameters of TvDAO for DCIP and oxygen. Open circles: DCIP (left axis); open triangles: oxygen (right axis). For experimental conditions see Section 2. The full lines show the non-linear fit of the data to the Michaelis–Menten equation. The dotted lines contour the 95% confidence level.

ues shows a (12.0 ± 1.5)-fold higher preference of TvDAO for reaction with oxygen over reaction with DCIP, fully explaining the sequential utilisation of the electron acceptors in a simultaneous conversion experiment (cf. Fig. 3). Fig. 8 shows that DCIP inhibits the use of oxygen for D-methionine oxidation by TvDAO, as expected for the case of two competing co-substrates.

3.6. Scale-down of the DCIP-based assay to microtitre plate format and its use to analyse TvDAO stability

To demonstrate the applicability of the DCIP-based assay for high-throughput screening, we examined the thermal stability of TvDAO at 50 °C and used microtitre plates to measure the residual enzyme activity in samples taken from the incubation mixtures. Diffusion of oxygen into microtitre wells cannot be avoided in practice. Therefore, it was particularly important to characterise the performance of the oxygen-independent assay under the 'open' conditions in which reoxidation of DCIP could occur and lead to incorrect activity values.

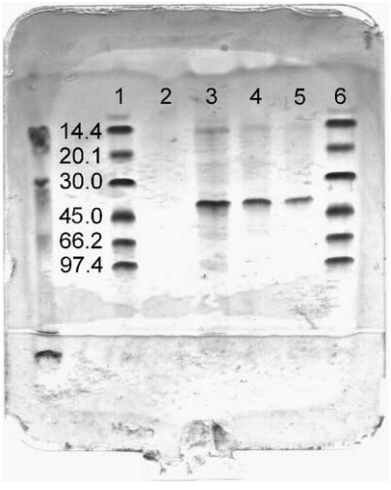

Fig. 7. Analysis by SDS/PAGE of used TvDAO preparation. Coomassie staining was used for the visualisation of protein bands. Lanes 1 and 6 show molecular mass standards. Lanes 3, 4, and 5 show different dilutions of TvDAO: 1.72, 0.86 and 0.43 mg L^{-1}, respectively.

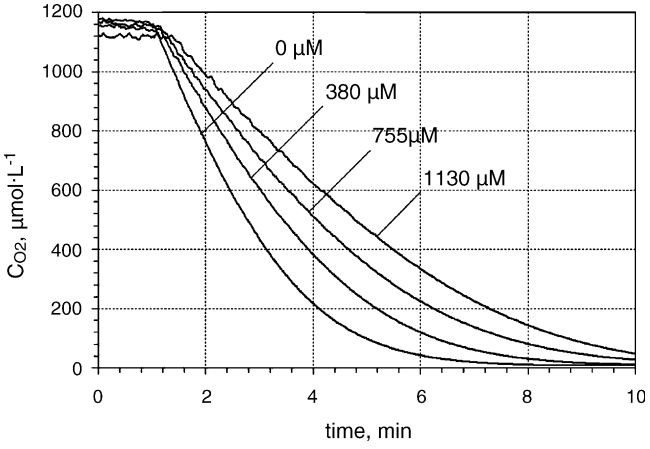

Fig. 8. DCIP inhibits TvDAO-catalysed oxygen consumption. The initial DCIP concentrations are indicated in the chart.

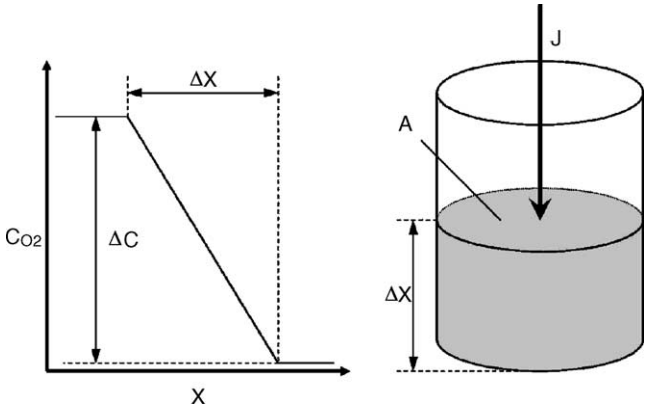

Fig. 9. DCIP-based assay in the microtitre plate: diffusion of oxygen into one well. For explanations see text.

For this reason, we performed a rough estimation of the mass transfer of oxygen which is based on Fick's law describing one-dimensional diffusion in the steady state:

$$J\,(\mu\text{mol/s}) = D\,(\text{cm}^2/\text{s}) \cdot A\,(\text{cm}^2) \cdot \frac{\Delta c}{\Delta x}\,(\mu\text{mol/cm}^3\,\text{cm}) \quad (1)$$

The diffusion coefficient D for oxygen (2.8×10^{-5} cm^2 s^{-1} at 30 °C) was obtained by linear extrapolation from known data [30]. The interface area of the well A was determined to be 0.35 cm^2. With the assumption that the apparent concentration of dissolved oxygen decreases from a value reflecting air saturation at the surface to essentially zero at the bottom of the well, we can expect an oxygen gradient Δc of 0.260 µmol cm^{-3} over a distance Δx of 0.5 cm (Fig. 9). The flux J of oxygen into the vial then corresponds to 1.75×10^{-3} µmol min^{-1} mL^{-1} which is – considering the ratio of specificity constants for DCIP and oxygen – equivalent to an enzyme activity of

0.146×10^{-3} μmol min^{-1} mL^{-1} towards DCIP. This value represents a maximum and constant amount of additional activity not detected by the DCIP-based assay. As a consequence, TvDAO activities down to 0.006 μmol min^{-1} mL^{-1} are affected by oxygen diffusion less than 5%.

Results of microtitre plate measurements are depicted in Fig. 10A and B. They show the time course of TvDAO activity in the absence and presence of compounds known to stabilise many labile proteins against thermal denaturation. The addition of 2-mercaptoethanol and FAD significantly increased the stability of TvDAO whereas BSA, glycerol and, interestingly, hydrogen peroxide had no detectable effect. The observed good stability of TvDAO in the presence of hydrogen peroxide may warrant further investigation as it could indicate a stabilising effect of the DCIP. Unexpectedly, α,α-trehalose seemed to destabilise the enzyme activity. Suitable controls along with statistical data analysis using a two-tailed *T*-test proved that the additives did not influence the photometric assay at a significance level α of 0.025. All time courses of TvDAO deactivation could be described satisfactorily by assuming double exponential decay (Fig. 10) and, clearly, did not follow pseudo-first order kinetics. The kinetic evidence would be consistent with a two-step mechanism of denaturation or it could imply the presence of *iso*-forms with different activities and stabilities.

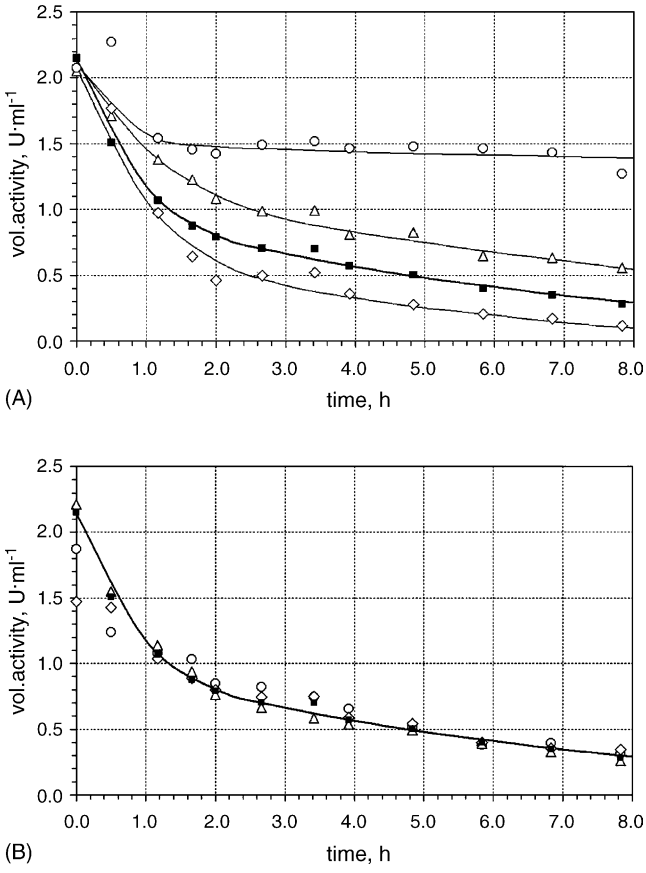

Fig. 10. DCIP-based assay in the microtitre plate: influence of additives on TvDAO stability. (A) Additives which showed a significant effect are indicated as follows: full squares (TvDAO without additive); circles (2-mercaptoethanol, 500 μmol L^{-1}); triangles (FAD, 130 μmol L^{-1}); diamonds (trehalose, 450 mmol L^{-1}). (B) Additives which showed no effect are indicated as follows: full squares (TvDAO without additive); circles (BSA, 20 mg mL^{-1}); triangles (glycerol, 1.5%, v/v); diamonds (hydrogen peroxide, 3.3 mmol L^{-1}). One unit of enzyme activity refers to the reduction of 1 μmol of DCIP per minute, using an initial DCIP concentration of 150 μmol L^{-1}.

In conclusion, we have characterised the reaction of TvDAO with DCIP in the absence and presence of oxygen. The results show clearly that the enzymatic conversion of DCIP is useful to determine the enzyme activity in a robust assay that does not require oxygen as a co-substrate and is not compromised by the presence thereof in buffer solutions. The conversion of DCIP could be a practical method to analyse DAO activity and stability in studies that depend on high-throughput screening capability.

Acknowledgement

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