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Non-linear Slow-binding Inhibition of *Aerococcus viridans* Lactate Oxidase by Cibacron Blue 3GA

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Lactate oxidase (LOD) was purified from cells of *Aerococcus viridans* by phase partitioning in Triton X-114 (TX-114), ammonium sulphate fractionation and FPLC ion exchange chromatography. The purification achieved from a crude extract of *A. viridans* was 32-fold with a 60% recovery of activity. The isolated enzyme was a true FMN-containing LOD in tetrameric form with a subunit molecular weight of 48,000. The K_M for L-lactate was 175 μM, a 6-fold less value than described in the literature. Among the inhibitors tested, Cibacron Blue 3GA showed the lowest K_i. At low concentrations, Cibacron Blue 3GA behaved as a dye-, pH- and time-dependent inhibitor. A Dixon plot of the steady-state rate showed the time-dependent inhibition to be non-linear, contrary to that described for other slow-binding inhibitors. A model to explain this phenomenon was proposed. The model implies the binding of Cibacron Blue 3GA to the isomerised form of the initial enzyme–inhibition complex (E′I).

Keywords: Lactate oxidase; Cibacron blue 3GA; Slow-binding inhibitor; Affinity; L-lactate; Lactate Oxidase

Abbreviations: LOD, Lactate oxidase; TX-114, Triton X-114; CTAB, Hexadecyl trimethylammonium bromide

INTRODUCTION

In the presence of molecular oxygen, L-lactate oxidase catalyses the conversion of L-lactate to pyruvate and H_2O_2. This enzyme has been reported in various microbial species, including *Tetrahymena pyriformis*,[1] *Streptococcus faecalis*[2] and some species of *Pediococcus*.[3,4] However, some confusion in terminology exists in the field since lactate monooxygenase (E.C.1.13.12.4) has frequently been referred to as lactate oxidase (LOD).[5] Although both enzymes are flavoproteins and use molecular oxygen as the second substrate, the products of lactate monooxygenase are acetate, CO_2 and H_2O.

LOD is used in clinical chemistry for the determination of L-lactate in blood in some pathological diseases such as diabetes, heart diseases and shock syndrome.[6–9] It is also used to test the L-lactic acid secreted into the culture medium as an important index of carbohydrate

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metabolism in animal cells.[10] For the above tests, it is usually co-immobilized with horseradish peroxidase.[11] However, despite these applied studies, no detailed kinetic study on this enzyme has been carried out.

The aim of this paper was to kinetically characterize the first example of a slow-binding inhibition. This phenomenon was observed in LOD, obtained by a new gentle purification method, using Cibacron Blue 3GA, a triazine dye, as inhibitor.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma Quimica (Madrid, Spain) except for Polypropylene glycol and Triton X-114 (TX-114), which were from Aldrich. Substrates and inhibitors were prepared every day.

Media and Procedures for Cell Growth

The *Aerococcus viridans* (ATCC 11563) was obtained from the Spanish type culture collections CECT 978 (Valencia, Spain). The culture media were the same as described by Duncan *et al.*[12] but with some modifications.

The antifoam used was Polypropylene glycol P 2000 and the 2 l. Biostat C fermentator (Braun Biotech.) was operated at 600 rpm, 2 v.v.m. airflow and 30°C. The pH was maintained at 7.0 by addition of 1 M NaOH. Cell growth was estimated by changes in the absorbance at 600 nm and by dry-weight measurements. Samples of 10 ml were filtered onto pre-weighed GF/F filters (Whatman), washed immediately and dried until they reached constant weight. Cells were harvested by centrifugation after 21 h of fermentation and frozen at −20°C.

Purification of LOD

After harversting, 2 g of cells were washed with 50 mM phosphate buffer pH 7.0 and precipitated by centrifugation. The cell pellets were resuspended in 20 ml of phosphate buffer containing 1% TX-114 and 15 mg of lysozyme. The sample was kept for 1 h at 37°C and then centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was cooled to 4°C and then subjected to temperature-induced phase partitioning by warming at 37°C for 15 min. After 10 min, the solution became spontaneously turbid due to the formation of detergent micelles, which contained hydrophobic proteins. This solution was centrifuged at 10,000 *g* for 15 min at 25°C. The detergent-rich phase and the pellet were discarded.

0.1% (w/v) Hexadecyl trimethylammonium bromide (CTAB) was added to the detergent-poor supernatant. The sample was homogenised and centrifuged at 3000 *g* for 30 min at 4°C. The pH of the CTAB supernatant was adjusted to 7.0 and EDTA was added to a final concentration of 1 mM.

Ammonium sulphate was added to 35% saturation and the mixture was stirred at 4°C for 30 min. After 1 h, the solution was centrifuged at 120,000 *g* for 30 min and the pellet was discarded. Ammonium sulphate was added to the clear supernatant to give 65% saturation and stirred for 1 h at 4°C. The precipitate obtained between 35 and 65% saturation was collected by centrifugation at the same rotor speed and dissolved in a minimal volume of 50 mM phosphate buffer pH 7.0.

The sample was desalted on a Sephadex G-25 column, applied to a Resource-Q column (Pharmacia) previously equilibrated with 50 mM buffer phosphate pH 7.0 and eluted with a gradient of 0–20% 2 M KCl at a flow rate of 2 ml/min. The active fractions were pooled.

Cibacron Blue 3GA Purification

Cibacron Blue (100 mg) in methanol/water (50:50 (v/v), 10 ml) was passed through a 0.45 μm Millipore cellulose filter and applied to a Sephadex LH-20 (2.5 × 30 cm^2) column equilibrated with

the same solvent. Dye components were eluted at a flow rate of 0.2 ml min. Fractions (1 ml) contained pure product were pooled.

Enzyme Assays

LOD activity was determined by a peroxidase-coupled spectrophotometric assay.[13] The standard reaction medium for the LOD assay contained the following: 7 mM phenol, 0.4 mM 4-aminoantipyrine, L-lactate (sodium salt), 7.6 μg/ml peroxidase (2.2 units) and 0.6 μg/ml LOD in a final volume of 1 ml in 50 mM potassium phosphate buffer pH 7.0. To study Cibacron Blue 3GA inhibition the pH was 6.0 in potassium phosphate buffer.

The concentrations of L-lactate and Cibacron Blue 3GA were varied from 0.10 to 0.89 μM and 0 to 13 μM, respectively.

Assays were started by the addition of enzyme and conducted at 37°C. The absorbance (uv/vis Uvicon 940 Kontron Inst.) at 505 nm was read vs. a reagent blank without enzyme, and the H_2O_2 produced was calculated on the basis of $\varepsilon_{505\,nm} = 13,300\,M^{-1}/cm^{-1}$ for the quinone diimine dye and that 2 mol H_2O_2 is required to produce 1 mol dye. One unit of lactate oxidase activity is defined as that which produced μmol of H_2O_2 per min under the specified conditions.

Preparation of Apoenzyme

The apoenzyme was prepared according to the method described by Duncan *et al.*[12] 2 ml of purified enzyme were added to 18 ml of ice-cold 0.1 M acetic acid, 2.4 M ammonium sulphate (final pH 3.6). The preparation was left on ice for 1 h and then centrifuged at 120,000 *g*. The precipitate was washed twice with 20 ml of cold 0.1 M acetic acid, 2.4 M ammonium sulphate. The washed precipitated was resuspended in 0.2 M KCl, 50 mM potassium phosphate buffer pH 7.0.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein extracts were dissolved in sample buffer containing SDS and 2-mercaptoethanol, denatured by boiling at 100°C for 5 min and subjected to gel electrophoresis in 12.5% acrylamide gel, according to Laemmli's[14] method.

The molecular weight markers used were: carbonic anhydrase (mol wt. 29 KDa), albumin egg (45 KDa), albumin bovine (66 KDa), phosphorylase b (97.4 KDa), β-galactosidase (116 KDa) and myosin (205 KDa).

Determination of the Molecular Weight of the Enzyme

The molecular weight of the enzyme was determined by FPLC gel filtration on G 3000 SW column (Supelco) calibrated with standard proteins. Elution was carried out with 50 mM potassium phosphate buffer pH 7.0. The molecular weight of the standard proteins used were cytochrome c (mol wt. 12.4 KDa), carbonic anhydrase (29 KDa), bovine serum albumin (66 KDa), alcohol dehydrogenase (150 KDa) and β-amylase (200 KDa).

Protein Determination

The protein content were measured by Bradford's dye-binding method.[15]

RESULTS AND DISCUSSION

Purification and Biochemical Characterization

LOD was purified from *A. viridans* by using a modification of Duncan *et al.*'s method.[12] The modifications of this method avoided having to make three passes through a Manton–Gaulin homogenizer by using, instead, the ability of TX-114 to solubilize *A. viridans* membranes in the presence of lysozyme. In addition, only one

ammonium sulphate precipitation was needed due to the clarification of the initial sample achieved by TX-114.[16] The degree of purification (32-fold), specific activity (114.5 U/mg) and recovery (60%) presented in Table I were the same as obtained by Duncan *et al.*'s method,[12] but with a substantial saving of time (72 vs. 8 h) and no special equipment.

The enzyme purified was a true LOD, which catalysed the conversion of L-lactate in the presence of molecular oxygen to pyruvate and H_2O_2 (Fig. 1A); its activity was easily removed (Fig. 1D) by obtaining its apoenzyme in an acidic saturated ammonium sulphate solution. When the apoenzyme was reconstituted by the addition of the FMN (Fig. 1B) or FAD (Fig. 1C), a marked lag period was observed, after which the system reached a steady state (linear rate). This lag depended on the type and concentration of cofactor (Fig. 1B,C). At high FMN or FAD concentrations, the reconstituted apoenzyme showed no lag period (data not shown). This result is in contrast with the data obtained by Duncan *et al.*,[12] who found that only FMN was able to fully reactivate the enzyme. This contradiction could be due to the gentle purification method used in this work. In addition the affinity of apoenzyme was 40-fold higher for FMN than FAD (data not shown).

The purified enzyme showed an apparent molecular mass of 48,200 in SDS-PAGE (Fig. 2) and the native weight for LOD, as estimated by FPLC gel filtration, was 187,300. The latter molecular weight indicates that LOD exists in tetrameric form after gel filtration, in agreement with the findings of Duncan *et al.*[12] However, these findings contrast with those of Maeda-Yorita *et al.*[17] who reported a dimer form of LOD after gel filtration, when cloned in *E. coli*.

Kinetic Study

The study of the kinetic parameters was carried out at the enzyme's optimum pH (7.0) (Fig. 3, circles), a value which agrees with that mentioned by Duncan *et al.*[12] When L-lactate, the natural substrate of LOD was used, the V_m and K_M values obtained were 93.27 μM/min and 0.157 mM, respectively (data not shown). The K_M value for L-lactate was 6-fold less than that obtained by Maeda-Yorita *et al.*[17] using an LOD overexpressed in *E. coli*.

To further characterise the purified enzyme, a detailed study of its inhibition was carried out using the common inhibitors described for both LOD and lactate dehydrogenase.[18] The inhibition was examined by Dixon plots at three inhibitor concentrations and confirmed by Lineweaver–Burk plots of $1/v$ vs. $1/S$. The inhibition constant K_i of each compound was calculated from the point of interception of the plots and the values obtained are summarized in Table II. Among them, the most effective competitive inhibitor was Cibacron Blue 3GA, a polyaromatic dye, whose Dixon plot is shown in Fig. 4.

A remarkable kinetic feature of Cibacron Blue 3GA inhibition was the shape of the time-course accumulation curves, which depended on inhibitor concentration. Thus, at a concentration higher than 1 μM, Cibacron Blue 3GA behaved as a

TABLE I Partial purification of LOD from *A. viridans*

	Total protein (mg)	*Total activity*	*Specific activity* (U/mg*)	*Purification (fold)*	*Recovery* (%)
Supernatant 1% TX-114	102.34	371.10	3.62	1	100
CTAB supernatant	72.27	316	4.37	1.2	85.15
35–65% $(NH_4)SO_4$	10.95	280.16	25.58	7.06	75.49
FPLC-resource Q	1.94	222.23	114.50	32	60

*The activity was assayed under the standard reaction conditions described in Materials and Methods.

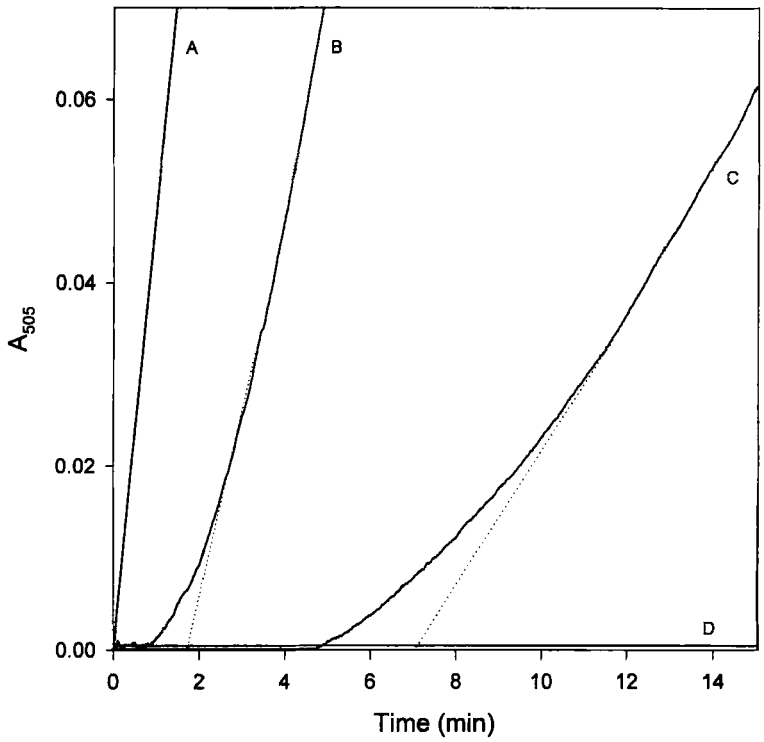


FIGURE 1 Enzymatic activity of LOD (A) Holoenzyme (0.6 μg/ml), (B) apoenzyme (0.6 μg/ml) with 2 μM FMN, (C) apoenzyme (0.6 μg/ml) with 2 μM FAD and, (D) apoenzyme (0.6 μg/ml).

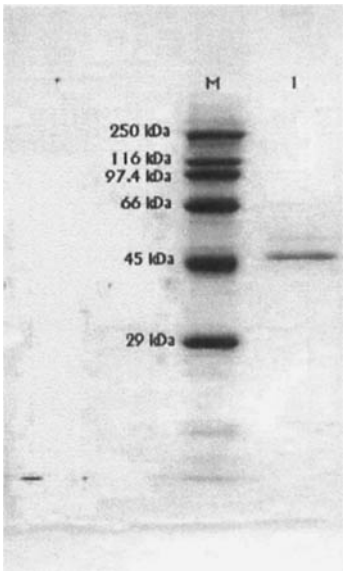


FIGURE 2 SDS-PAGE patterns of purified LOD. Lane M: marker proteins. Lane 1 eluted from FPLC-Resource Q.

normal competitive inhibitor, whereas at concentrations below 1 μM it displayed time-, dye- and pH-dependent inhibition. This last effect is shown in Fig. 3 (triangles) with a marked decrease in inhibition percentage from pH 6.0 to 0 at pH 7.5. For this reason, pH 6.0 was chosen to carry out the time- and dye-dependent study, since at this pH the enzyme showed the highest activity/inhibition ratio. This pH-dependence of the inhibition of LOD by Cibacron Blue 3GA could be due to the protein's tendency to bind to triazine dyes more tightly at lower pH values, presumably because of a contribution from ionic interactions favoured by increasing positive charges on interacting protein molecule.[19]

The dye-dependent inhibition is showed in Fig. 5. When the concentration of Cibacron Blue 3GA in the reaction media was increased to 1 μM, a biphasic response was observed (Fig. 5) with the

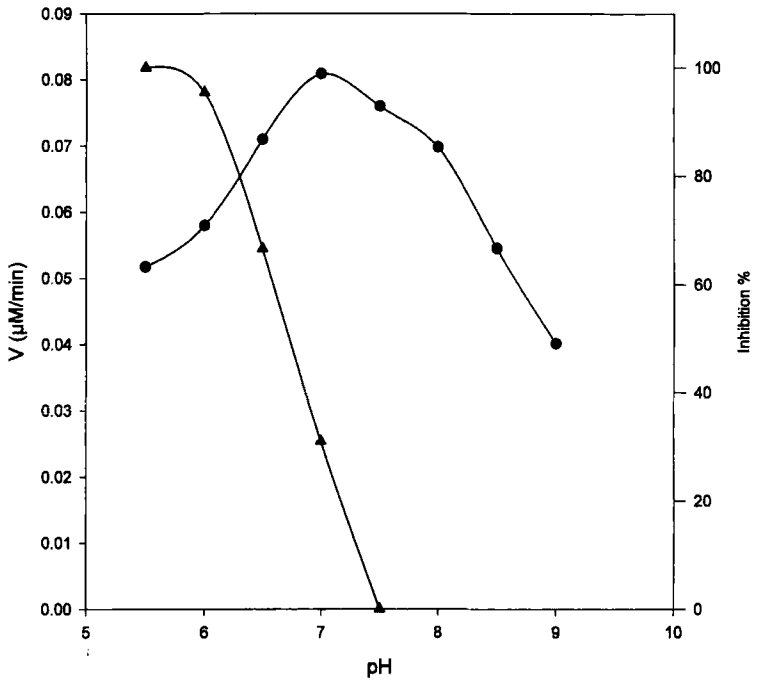


FIGURE 3 Effect of pH on enzymatic activity of LOD (●). The reaction medium at 37°C contained 50 mM potassium phosphate buffer (pH 7.0), 0.445 mM of L-lactate. (▲) pH-dependent inhibition at 0.6 μM of Cibacron Blue 3GA in the above reaction medium.

same initial activity (v_0) followed by a constant rate, which decreased as the inhibitor concentration increased (Fig. 5, curves B–E) from 0.45 to 1 μM. These findings indicate that inhibition occurred slowly, Cibacron Blue 3GA thus behaving as a slow-binding inhibitor, according to the definition given by Morrison.[20] The inhibition expressed is not necessarily tightly bound or stoichiometric since the Cibacron Blue 3GA concentration was always much higher than the enzyme concentration in the experiment.[21]

The progress curves shown in Fig. 5 can be described by the general [22] for a first-order process:

$$P = v_{ss}t + (v_0 - v_{ss})(1 - e^{-k_{app}t})/k_{app} \qquad (1)$$

where v_o, v_{ss} and k_{app} represent, respectively, the initial rate, the steady-state rate and the apparent first-order rate constant, the value of the last depending on the mechanism under study. Data analysis of these product accumu-

TABLE II Effect of some inhibitors on the activity of LOD (Assayed under the standard reaction condition (pH 7.0) with the appropriate concentration of inhibitor and L-lactate as substrate)

Inhibitors of LOD	K_i (mM)	Inhibitors of lactate dehydrogenase	K_i
Glycolate	0.317	Cibacron blue 3GA†	1.69 μM*
D-Lactate	9.04	Oxalate†	0.50 mM
DL-α-hydroxy butyrate	42.35	L-Mandelate†	0.30 mM

*Assayed at pH 6.0.
†Known also as inhibitors.

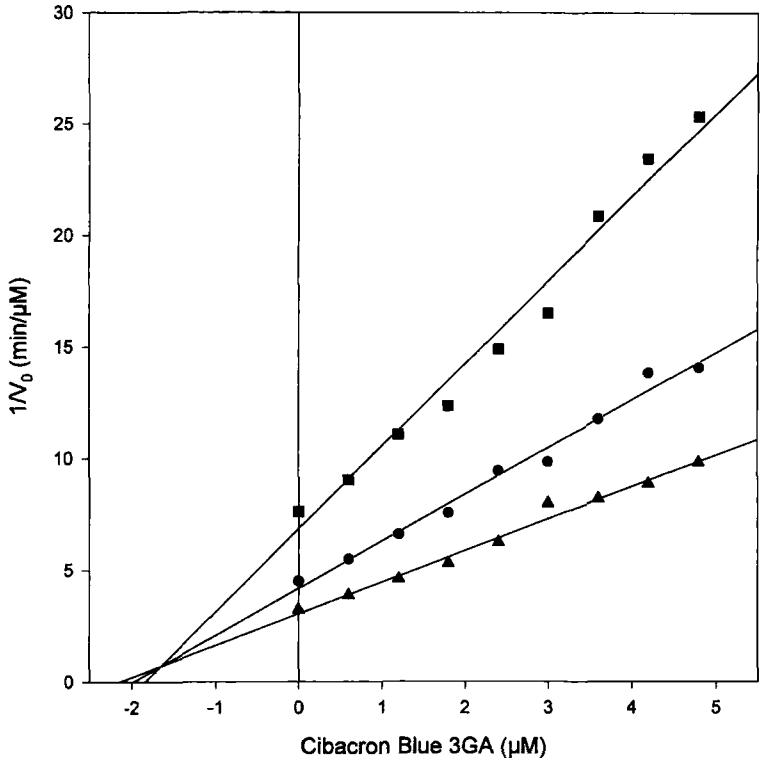


FIGURE 4 Dixon plot of the effect of Cibacron Blue 3GA on initial rate of LOD (v_0) at different L-lactate concentrations. The reaction medium at 37°C contained 50 mM potassium phosphate buffer (pH 7.0), 2.4 μg/ml LOD and L-lactate concentrations of (■) 0.15, (●) 0.225 or (▲) 0.89 mM.

lation curves can be performed by making an overall fit of the experimental data to Eq. (1) by non-linear regression, as has been described by Morrison.[20]

To further characterise the inhibition, Dixon plots in the range of 0–1 μM were analysed according to Eq. (1). The curves obtained for v_{ss} are shown in Fig. 6, in which a hyperbolic response was obtained in contrast to the linear response for other time-dependent inhibitors.[23–27]

To explain this non-linear behaviour, the following inhibition mechanism is proposed (Scheme 1), in which, LOD binds sequentially to a Cibacron Blue 3GA molecule to give $E'I$ and $E'I_2$ forms, respectively.

The slow-transition constant was evaluated by using Cha's method[28] with the following assumptions: steady-state conditions are reached instantaneously between E, EI and ES; steady-state conditions are also reached instantaneously between $E'I$ and $E'I_2$; substrate concentration is much greater than enzyme concentration, thus the depletion of free substrate by binding to the enzyme is negligible; experimental observations are carried out only while the effects of substrate depletion and product inhibition on the velocity are negligible, and, finally, the reaction is started by the addition of enzyme or substrate.

Based on these premises, this model can be simplified (see squares in Scheme 1) to Scheme 2 where,

$$[X_1] = [E'I] + [E'I_2] \quad (2)$$

and

$$[X_2] = [E] + [ES] + [EI] \quad (3)$$

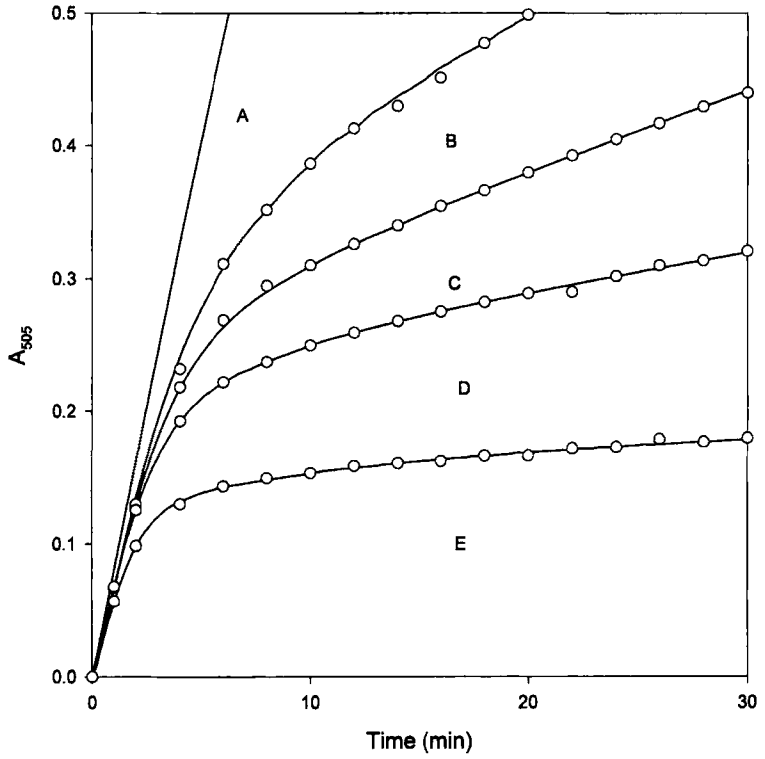


FIGURE 5 Progress curves for the inhibition of LOD by Cibacron Blue 3GA. The reaction medium at 37°C contained 50 mM potassium phosphate buffer (pH 6.0) and increasing concentrations of Cibacron Blue 3GA; (A) 0, (B) 0.45, (C) 0.60, (D) 0.75 and (E) 1 μM. O represents the experimental data. The reaction was started by the addition of 0.6 μg/ml LOD.

The coefficients for Scheme 2 are

$$a_2 = k_4 f_{EI} \tag{4}$$

$$a_1 = k_{-4} f_{E'I} \tag{5}$$

$$b_2 = k_2 f_{ES} \tag{6}$$

by using:

$$K_M = \frac{k_{-1} + k_2}{k_1} \text{ as Michaelis constant for } ES \tag{7}$$

$$K_i = \frac{k_{-3}}{k_3} \text{ as dissociation constant for } EI \text{ and} \tag{8}$$

$$K_i' = \frac{k_{-5}}{k_5} \text{ as dissociation constant for } E'I \tag{9}$$

k_4 and k_{-4} are the rate constant of slow transition between $E'I$ and $E'I_2$.

The factors are:

$$f_E = \frac{1}{1 + ([S]/K_M) + ([I]/K_i)} \tag{10}$$

$$f_{ES} = \frac{[S]/K_M}{1 + ([S]/K_M) + ([I]/K_i)} \tag{11}$$

$$f_{EI} = \frac{[I]/K_i}{1 + ([S]/K_M) + ([I]/K_i)} \tag{12}$$

$$f_{E'I} = \frac{1}{1 + ([I]/K_i')} \tag{13}$$

$$f_{E'I_2} = \frac{([I]/K_i')}{1 + ([I]/K_i')} \tag{14}$$

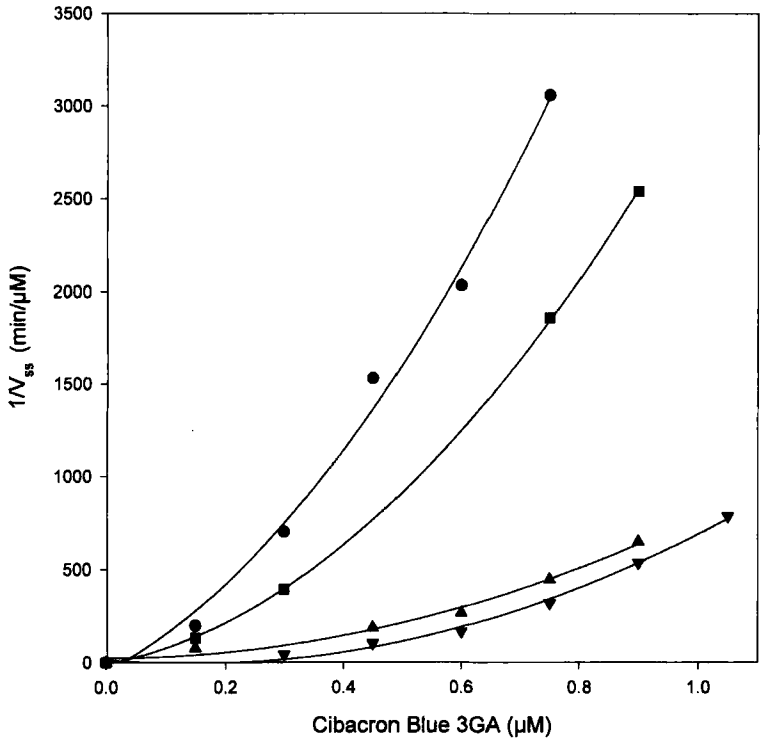


FIGURE 6 Dixon plot of the effect of Cibacron Blue 3GA on the steady-state rate of LOD at different L-lactate concentrations. The reaction medium at 37°C contained 50 mM potassium phosphate buffer (pH 6.0), 0.6 μg/ml LOD and four concentrations of L-lactate (●) 0.10 mM, (■) 0.15 mM, (▲) 0.225 mM or (▼) 0.89 mM.

The kinetics of interconversion between X_2 and X_1 may then be expressed as,

$$\frac{dX_1}{dt} = a_2X_2 - a_1X_1 = a_2(E_t - X_1) + a_1X_1 \quad (15)$$

v_{ss} can be defined as

$$v_{ss} = \frac{k_2k_{-4}K_iK_i'SE_t}{k_{-4}K_MK_iK_i' + k_{-4}SK_iK_i' + (k_{-4} + k_4)K_MK_i'I + k_4K_MI^2} \quad (16)$$

and k_{app} as

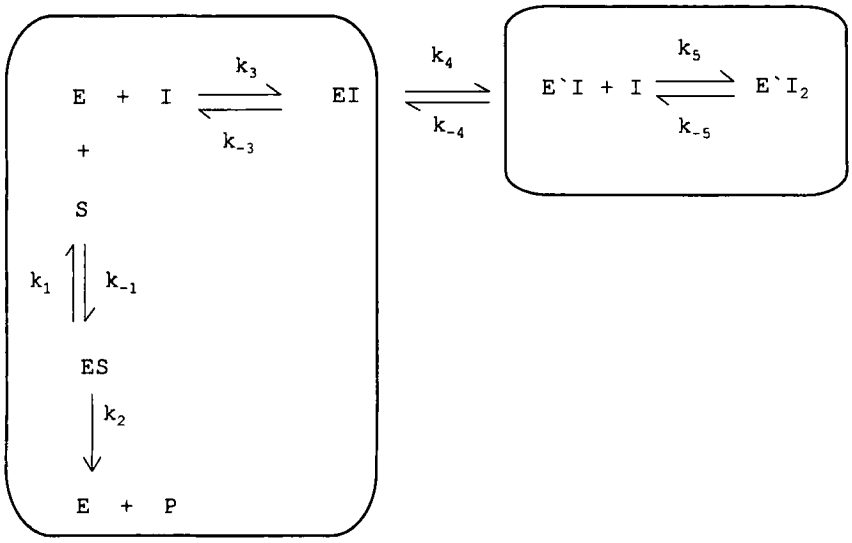
$$k_{app} = \frac{k_{-4}K_MK_iK_i' + k_{-4}SK_iK_i' + k_{-4}K_MK_i'I + k_4IK_MK_i' + k_4I^2K_M}{K_MK_iK_i' + IK_MK_i + SK_iK_i' + SIK_i + IK_i'K_M + I^2K_M} \quad (17)$$

Equation (16) clearly gives a quadratic relation between $1/v_{ss}$ and Cibacron Blue 3GA. This means, according to Scheme 1, that the sequential binding of two molecules of inhibitor was responsible for the non-linear Dixon plots of Fig. 4.

The progress curves of Fig. 5 were analysed according to Eq. (1). The curves obtained for k_{app} are shown in Fig. 7. In addition, two rate constants, k_{-4} and k_4, can be evaluated from Eq. (17). When Cibacron Blue 3GA tends to infinity, k_{app} tends asymptotically to k_4, as can be seen in Fig. 7. On the other hand, when Cibacron Blue 3GA tends to zero (Fig. 7, inset), the intercept with the ordinate axis corresponds to k_{-4}, with a value of 1.4 s.

CONCLUSIONS

The results of the present paper shows that LOD can easily be purified by using TX-114 phase



SCHEME 1

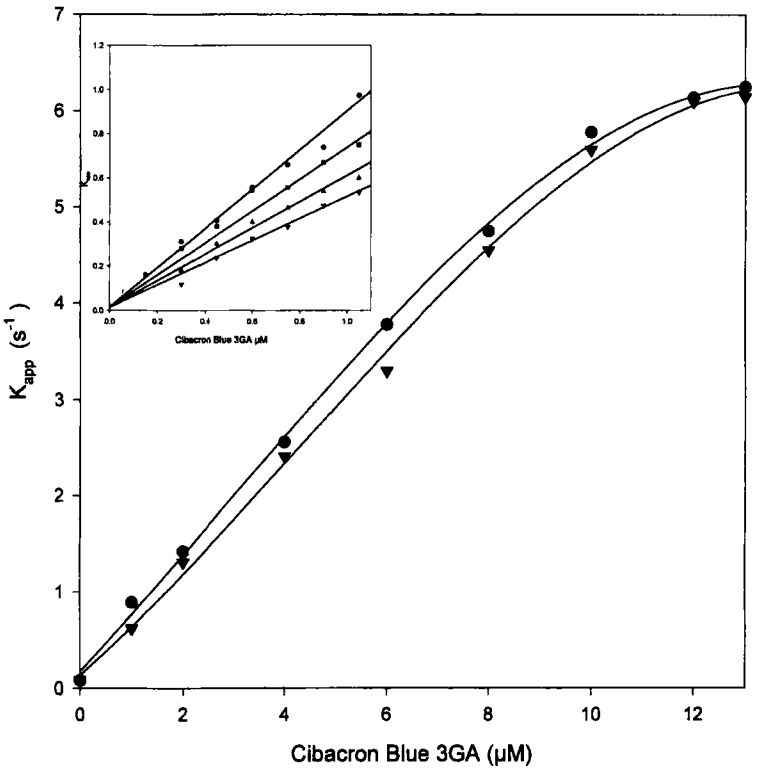
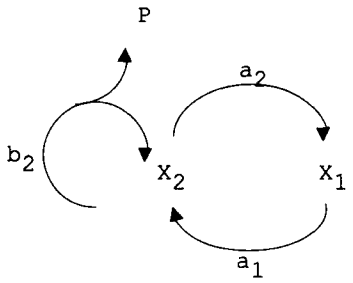


FIGURE 7 Effect of Cibacron Blue 3GA on k_{app}. The reaction medium at 37°C contained 50 mM potassium phosphate buffer (pH 6.0), 0.6 μg/ml LOD, L-lactate at (●) 0.10 mM or (▼) 0.89 mM and increasing concentrations of Cibacron Blue 3GA. (Inset) Detailed effect of Cibacron Blue 3GA on k_{app} at Cibacron Blue 3GA concentrations below 1 μM. The reaction medium at 37°C contained 50 mM of potassium phosphate buffer (pH 6.0), 0.6 μg/ml LOD, increasing concentrations of Cibacron Blue 3GA from 0.01 to 1.0 μM and L-lactate at (●) 0.10, (■) 0.15, (▲) 0.225 or (▼) 0.89 mM.



SCHEME 2

separation, to give a true tetrameric FMN-containing LOD.

The interaction of Cibacron Blue 3GA with LOD is the first described example involving an FMN-containing oxidase. Only one other example of the interaction between triazine dyes and FMN-requiring enzyme has been described, that by Pompom *et al.*[29] involving flavocytochrome b_2. However, this interaction is not general since apoflavodoxin, another FMN-dependent electron carrier, showed no binding of Cibacron Blue 3GA.[30]

In addition, this paper represents the first example of a slow-binding inhibitor in which the isomerised form of the initial enzyme–inhibitor ($E'I$) complex is able to bind another inhibitor (Cibacron Blue 3GA) molecule. The slow-binding inhibitors described in the literature[20,21] and in several papers of our group[23–27] show that the first binding between enzyme and inhibitor involves the rapid formation of an initial complex (EI) that subsequently undergoes a slow isomerization reaction to give ($E'I$). The progress curves for this type of inhibition represent an initial burst of reaction (Fig. 5) followed by a slower steady-rate, which decreases with increasing concentrations of the slow-binding inhibitors. Thus, for an inhibition that conforms to the mechanism described above, K_i' must be lower than K_i (k_{-3}/k_3). The way of calculating both dissociation constants is by Dixon plots on the steady-state and initial rate, respectively. In both cases, the data are a set of straight lines. However, in the slow-binding inhibition of LOD by Cibacron Blue 3GA, the Dixon plot for the initial rate was a set of straight lines (Fig. 4), but for the steady-state rate it was a set of hyperbolic lines (Fig. 6), thus indicating the isomerised $E'I$ complex.

This feature of double binding of Cibacron Blue 3GA to both E and $E'I$ forms of LOD opens up the opportunity to develop new affinity purification methods for this enzyme based on its interaction with triazine dyes.

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