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A Study on the Kinetic Mechanism of Apoenzyme Reconstitution from *Aerococcus Viridans* Lactate Oxidase

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The preparation of a reconstitutable apoprotein is widely recognized as an important tool for studying the interactions between protein and coenzyme and also for characterizing the coenzyme-binding site of the protein. Here is described the kinetic analysis of the reconstitution of *Aerococcus viridans* lactate oxidase apoenzyme with FMN and FAD in the presence of substrate. The reconstitution was followed by measuring the increase in catalytic capacity with time. Lactate oxidase activity was easily removed by obtaining its apoenzyme in an acidic saturated ammonium sulphate solution. When the apoenzyme was reconstituted by the addition of FMN or FAD, a marked lag period was observed, after which the system reached a steady state (linear rate). To explain the binding mechanism of the cofactors to the apoenzyme, a kinetic model is proposed, in which the constants, k_3 and k_{-3}, representing the interaction of apoenzyme with cofactor are considered slow and responsible for the lag in the expression of activity. The affinity of apoenzyme was 51-fold higher for FMN than FAD.

Keywords: Lactate oxidase; Apoenzyme; Kinetic; *Aerococcus viridans*

INTRODUCTION

L-lactate oxidase (LOD) from *Aerococcus viridans* is a member of the family of FMN-containing enzymes that catalyze the oxidation of α-hydroxyacids. Under native conditions, purified LOD of *Aerococcus viridans* is a tetramer of 187300 that contains a FMN-like prostetic group.[1] The enzyme has been reported in various microbial species, including *Streptococcus faecalis*,[2] some species of *Pediococcus*[3,4] and *Aerococcus viridans*.[1,5]

Several flavin analogues have stimulated studies, in which the native cofactor is replaced in the enzyme molecule by chemically modified flavin coenzymes.[6–8] The reaction carried out by a particular flavoenzyme is selected and tuned by the kind of interaction between the protein and the isoalloxazine nucleus of the flavin, the portion of the moiety involved in the redox mechanism. In this context, the preparation of a reconstitutable apoprotein is widely recognized as an important tool for studying the interactions between protein and coenzyme and also for characterizing the coenzyme-binding site of the protein.[9]

In this paper, we describe the kinetic mechanism of the LOD apoenzyme from *Aerococcus viridans*, using spectrophotometric techniques for the kinetic reconstitution analysis. The study of the enzyme spectrum[8] requires highly purified and concentrated enzyme. We describe a kinetic analysis of the reconstitution of *Aerococcus viridans* lactate oxidase apoenzyme with FMN and FAD in the presence of substrate. The reconstitution was followed by measuring the increase in catalytic capacity with time. A model for flavoenzyme reconstitution is also presented.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma Quimica (Madrid, Spain).

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Enzyme

The *Aerococcus viridans* (ATCC 11563) was obtained from the Spanish type culture collections CECT 978 (Valencia, Spain). The culture medium used was that commonly used for the cultivation of this microorganism, except that an H_2O_2-decomposing enzyme (catalase) was added to eliminate the peroxide formed during growth. The culture medium and growth conditions have been described previously.[1] The cells were harvested by centrifugation after 14 h of fermentation and then frozen until use. The enzyme purification was described previously by Streitenberger *et al.*[1]

Preparation of Apoenzyme

The apoenzyme was prepared according to the method described by Duncan *et al.*[5] Two 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 120000 × 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.

Enzymatic Assay

Lactate oxidase activity was determined by a peroxidase-coupled spectrophotometric assay.[10] The standard reaction medium for the lactate oxidase 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. Assays were started by the addition of enzyme and conducted at 37°C. The absorbance at 505 nm was read (uv/vis Uvicon 940 Kontron Inst.) vs. a reagent blank without enzyme, and the H_2O_2 produced was calculated on the basis of a value of $\epsilon_{505\,nm} = 13300\,M^{-1}\,cm^{-1}$ for the quinone diimine dye and the fact that 2 moles H_2O_2 are required to produce 1 mole dye. One unit of lactate oxidase activity is defined as that which produced 1 μmole of H_2O_2 per minute under the specified conditions. The kinetics parameters were obtained by linear regression using the program Sigma Plot.

RESULTS AND DISCUSSION

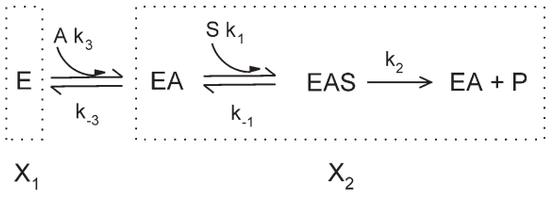
The absorption spectrum of the oxidized enzyme was seen to be typical of flavoproteins, with absorbance maxima at 375 and 458 nm, the latter peak corresponding to the flavine.[11] In common with other members of the family, LOD avidly binds sulphite to form the flavin N(5)-sulphite adduct. However, the K_d for equilibrium formation of the flavin N(5)-sulphite adduct is so low that is cannot be determined accurately by conventional spectral titration methods.

LOD activity (Fig. 1A) 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 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.*,[5] 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.

To explain the binding mechanism of the cofactors to the apoenzyme, the following mechanism is proposed (Scheme 1), in which the constants, k_3 and k_{-3}, representing the interaction of apoenzyme with cofactor (A) are considered slow and responsible for the lag in the expression of activity.

The scheme was evaluated by using Cha's method[12] with the following assumptions: steady-state conditions are reached instantaneously between *EA* and *EAS*; 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).



SCHEME 1

where

$$[X_1] = [E] \tag{1}$$

and

$$[X_2] = [EA] + [EAS]. \tag{2}$$

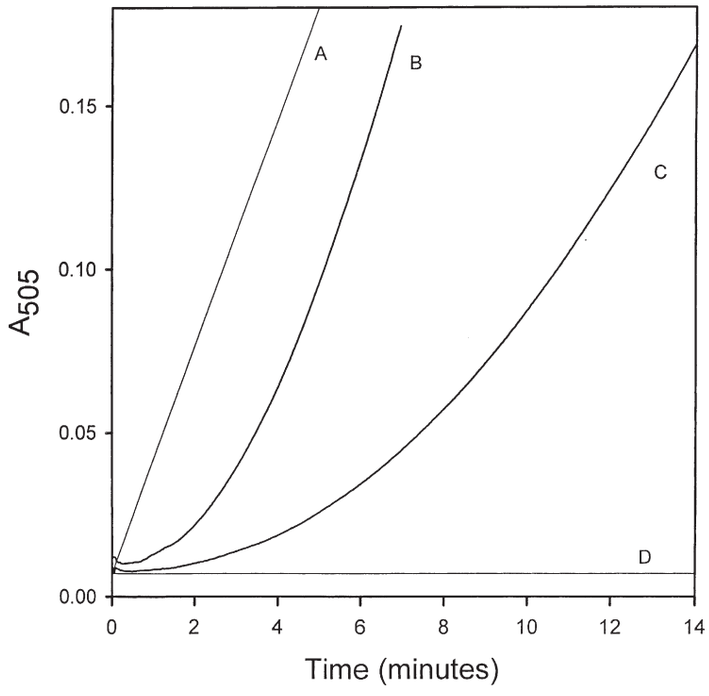


FIGURE 1 Enzymatic activity of LOD. (A) holoenzyme (0.6 μg/ml), (B) apoenzyme (0.6 μg/ml) with 2 μM FMN, (C) apoenzyme (0.6 μg/ml) with 2 μM FAD and (D) apoenzyme (0.6 μg/ml).

By using K_M as Michaelis constant for *EA* and *EAS* the factors are:

$$f_{EA} = \frac{K_M}{K_M + S} \tag{3}$$

$$f_{EAS} = \frac{S}{K_M + S}. \tag{4}$$

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

$$\frac{dX_2}{dt} = k_3[A][X_1] - k_{-3} f_{EA}[X_2]. \tag{5}$$

According to Eq. (5) and keeping in mind that $E_T = [X_1] + [X_2]$, the variation of $[X_2]$ with time can be defined as follows:

$$[X_2] = \frac{k_3 A E_T}{k_3 A + \frac{k_{-3}K_M}{K_M+S}} \left[1 - e^{-\left(k_{-3}A + \frac{k_3 K_M}{K_M+S}\right)t}\right]. \tag{6}$$

When the time tends to infinite:

$$[X_2]_s = \frac{A E_T}{A + K_d\left(\frac{K_M}{K_M+S}\right)} \tag{7}$$

where,

$$K_d = \frac{k_{-3}}{k_3}. \tag{8}$$

If $[X_2]$ is expressed as the rate, we obtain,

$$V = V_f(1 - e^{k_{ap}t}) \tag{9}$$

where,

$$V_f = \frac{V_m[A]}{[A] + K_d\left(\frac{K_M}{K_M+S}\right)} \tag{10}$$

$$K_{ap} = k_{-3}A + \frac{k_3 K_M}{K_M + S}. \tag{11}$$

Equation (9) can be integrated to give:

$$P = V_f t - \frac{V_f}{k_{ap}}(1 - e^{-k_{ap}t}). \tag{12}$$

When time tends to infinite

$$P_s = V_f t - \frac{V_f}{k_{ap}}. \tag{13}$$

Defining L as the intersection on the abscissa of the product accumulation straight line in stationary state, the following equation is obtained:

$$\frac{1}{L} = k_{-3}A + \frac{k_3 K_M}{K_M + S}. \tag{14}$$

To evaluate cofactor affinity for apoenzyme, increasing concentrations of FMN or FAD were added until the LOD catalytic activity was totally recovered (Fig. 2). Using Eq. (10), the dissociation constant (K_d) was $5 \times 10^{-7} \pm 1.8 \times 10^{-7}$ and

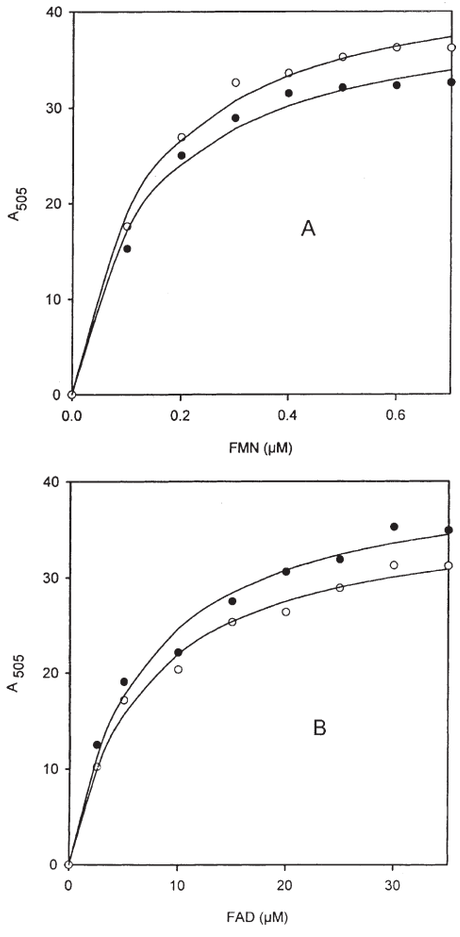


FIGURE 2 Effect of the concentration of FMN (A) and FAD (B) on apo-LOD activity. The reaction medium at 25°C in potassium phosphate buffer 50 mM pH 7.0 contained 1.4 μg/ml of apo-LOD, (●) L-lactate 0.225 mM, (○) L-lactate 0.445 mM and increasing concentrations of FMN or FAD.

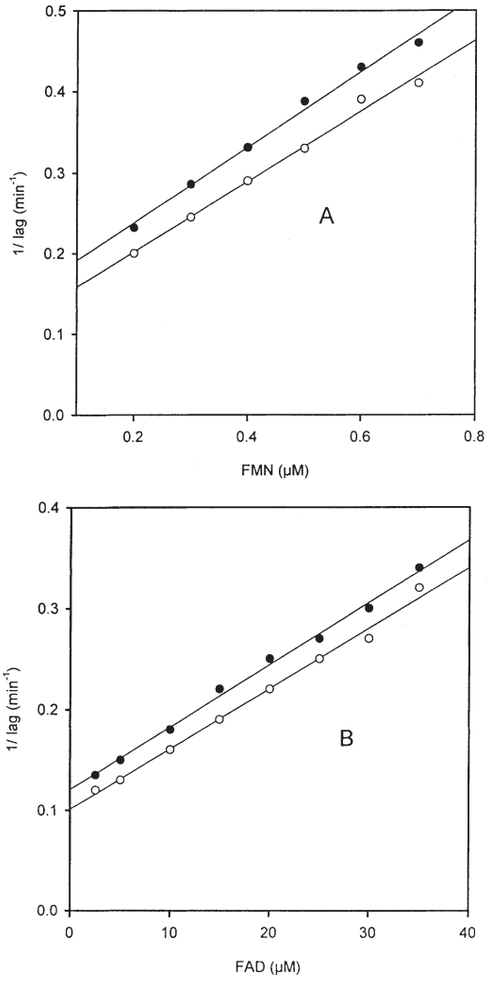


FIGURE 3 Effect of FMN (A) and FAD (B) concentration on the reconstitution of apo-LOD. The reaction medium at 25°C in potassium phosphate buffer 50 mM pH 7.0 contained 1.4 μg/ml of apo-LOD, (●) L-lactate 0.225 mM, (○) L-lactate 0.445 mM and increasing concentrations of FMN or FAD.

$2.55 \times 10^{-5} \pm 0.8 \times 10^{-5}$ M for FMN and FAD, respectively. In addition the affinity of apoenzyme was 51-fold higher for FMN than FAD.

The lag period observed during the reconstitution of apoenzyme was analysed according to Eq. (14) and the constants k_{-3} and k_3 were calculated as: $0.136 \pm 0.06\,min^{-1}$ and $0.149 \pm 0.08\,\mu M^{-1}\,min^{-1}$ for FMN and $1.23 \pm 0.80\,min^{-1}$ and $0.118 \pm 0.05\,\mu M^{-1}\,min^{-1}$ for FAD respectively (Fig. 3).

Acknowledgements

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