Kinetic characterization of steroid 1,2-dehydrogenase from a new *Mycobacterium sp* mutant of industrial interest

Alina Falero, Nury Llanes, Celso Pérez, Blanca R. Hung, Belinda Aguila, Magdalena Fonseca y Emilia Hervé.

National Center for Scientific Research, Ave. 25, Playa, P.O. Box 6414, Havana City, Cuba.

Recibido: 30 de enero de 2001. Aceptado: 12 de febrero de 2002.

Palabras clave: *Mycobacterium,* mutantes, estudio cinético, actividad enzimática, esteroide 1,2-deshidrogenasa.
Key words: *Mycobacterium,* mutants, kinetic study, enzyme activity, steroid 1,2-dehydrogenase.

RESUMEN. Se realizó un estudio cinético de la enzima esteroide 1,2-deshidrogenasa con células enteras de la cepa 6323, la cual fue aislada por mutagénesis-UV a partir de Ex4, mutante derivada de *Mycobacterium* NRRL-B3683, obtenida previamente por mutagénesis-UV a partir de un aislado del suelo. La esteroide 1,2-deshidrogenasa es una enzima clave para la biotransformación del sistema de anillos de estos compuestos, la cual es esencial en el metabolismo microbiano de los esteroides. Con anterioridad se ha demostrado que esta enzima es inducible y necesita de un aceptor electrónico externo para la transferencia de electrones desde la enzima hasta el oxígeno molecular, el aceptor final de los electrones liberados en la 1,2 deshidrogenación. Se determinaron las constantes cinéticas de Michaelis-Menten (Km_{apa}) y $Vmax._{apa}$. Los valores obtenidos fueron $6{,}92 \cdot 10^{-4}$ mol/L y 2,3 U/mg de biomasa seca, respectivamente. Se estableció el efecto de la concentración de biomasa y la concentración del azul de metileno empleado como aceptor electrónico externo en la reacción enzimática. La actividad máxima se observó entre 5-10 mg/mLde biomasa seca y $1{,}34 \cdot 10^{-4}$ mol/L de azul de metileno. Los resultados obtenidos con azul de metileno, claramente indican la necesidad del uso del aceptor en la 1,2 deshidrogenación de esteroides, como ha sido reportado anteriormente por otros autores. Además se estudió el efecto del pH sobre la velocidad inicial de reacción, obteniéndose la actividad máxima en el intervalo de 8,4-8,6. Los resultados del estudio confirman que esta nueva cepa posee una actividad esteroide 1,2–deshidrogenasa superior con relación a sus parentales. Esto se hace evidente de acuerdo con los parámetros cinéticos obtenidos, los cuales son de gran interés desde el punto de vista industrial.

ABSTRACT. A kinetic study on enzyme steroid 1,2-dehydrogenase from whole cells of the 6323 strain was carried out. This strain was isolated by UV- mutagenesis from Ex4, a derived mutant of the *Mycobacterium* NRRL-B3683 strain. This strain was previously obtained by UV-mutagenesis from a soil isolate. One of the key enzyme for chemical transformation in ring systems of these compounds is the steroid 1,2-dehydrogenase, a key enzyme in microbial steroid metabolism. The enzyme was demonstrated to be inducible and needs an external electron acceptor for transferring electrons from this enzyme to molecular oxygen, the final acceptor of the electrons released in the 1,2-dehydrogenation. The Michaelis-Menten kinetic constants (Km_{apa}) and $Vmax._{apa}$ were determined. The obtained values were: $6.92 \cdot 10^{-4}$ mol/L y 2.3 U/mg dry biomass, respectively. The effect of biomass and methylene blue concentration, employed as an electronic exogenous acceptor in the enzymatic reaction were established. The maximum in activity was observed between 5-10 mg/mL of dry biomass and $1.34 \cdot 10^{-4}$ mol/L of the methylene blue. The obtained results with the methylene blue, clearly indicates the need of using the acceptor in the steroids 1,2-dehydrogenation, as reported before by some authors. Furthermore the pH effect on the initial rate in the reaction was studied, resulting a maximum of activity in the 8.4-8.6 range. The obtained results in this study confirm that this new strain have a higher steroid-1,2-dehydrogenase activity respect to parentals. This is evident according to the kinetic parameters figures, which are interesting from the industrial point of view.

INTRODUCTION

Steroid bioconversion has been used to synthethize a wide variety of allopathic drugs. One of the key enzymes for the biotransformation in ring systems of these compounds is the steroid 1,2-dehydrogenase[1] which is essential in microbial steroid metabolism. The enzyme was demonstrated to be inducible and needs an external electron acceptor for transferring electrons from this enzyme to molecular oxygen, the final acceptor of the electrons released in the 1,2-dehydrogenation.[2,3] The $\Delta^{1,2}$ -dehydrogenation in microbial cells is the most important reaction among various modifications of steroidal compounds. The resulting 1,2-double bond bears new physiological activities thereby enhancing anti-inflammatory and anti-allergic effects,[1] etc. Furthermore, the reaction initiates the chemical degradation of the steroidal nucleus in the breakdown pathway of sterols. The enzyme has been isolated and characterized from various microbials genera, including *Nocardia*, *Rhodococcus*, *Arthrobacter* and *Mycobacterium*.[4,5]

Despite of the specificity of bond saturation in position 1,2 of the ring A, the whole efficiency of this reaction is rather low.

Therefore, new mutants of microorganisms with an enhanced steroid 1,2-dehydrogenase activity must render higher yield in 1,4-androstene-3,17-dione (ADD) amounts from sterols in fermentations. ADD

is considered one of the most valuable precursors in pharmaceutical industry for the synthesis of different drugs of androgenic, estrogenic activities, and others.

Particularly *Mycobacterium* sp. NRRL B-3683 is able to degrade natural sterols up to ADD as a main product. However, the toxicity of this ADD to mycobacterial cells must affect final yields, so that mutants resistant to ADD may bypass this limitation.

An UV-mutant resistant to inhibitory concentrations of ADD, isolated from Ex4 was able to produce higher quantities in ADD fermentations.[6]

This paper shows the kinetics parameters in steroid-1,2-dehydrogenation of AD of this mutant in comparison to parental strains.

MATERIALS AND METHODS

Microorganism

Mycobacterium sp. 6323, an UV mutant was isolated from Ex4, a derived mutant of the *Mycobacterium* NRRL-B3683 strain, which was previously obtained by UV-mutagenesis from a soil isolate.[7]

Media and culture conditions

The cells were previously grown and harvested according to Falero *et al*., 1994.[8] After suspension of cells in buffer tris-HCl 0.1 mol/L, pH 8.4.

The enzymatic activity was assayed as shows in table 1.

The reaction mixture were placed in a rotatory shaker at 30 °C, 150 r/min during 72 h . The catalytic unit was defined as 1 nmol of ADD/(mL · h).

Product extraction and quantification

Each sample was extracted twice with 2 mL of ethyl acetate. After drying at room temperature, residues were suspended in methanol and analyzed by reverse phase chromatography using an HPLC system. UV detection was done at 254 nm, using a mixture methanol-water (65:35) at a flow rate of 1-5 mL/min through RP-8 LichroCART column (125 - 4 mm). Calibration was carried out by response factor with standard solutions of 0.1 mg/mL . 17 α-methyl testosterone was employed as internal standard.

Determination of cellular growth

Cellular growth was determined after biomass-dry weight in Sartorius balance. The results are expressed in percent (w/v).

Determination of kinetic parameters

The effect of biomass, substrate as well as electron acceptor concentration on the initial velocity of the reaction was determined for pH ranging from 7,4-9,4. Throughout this study buffers were tris-HCl 0.1 mol/L (pH 7.4-8.8) and ammonium chloride solution pH (8.2-9.4), ionic strength I = 0.2.

RESULTS AND DISCUSSION

Effect of biomass concentration and electron acceptor on steroid-1,2-dehydrogenase activity in strain 6323

Initial attempts to standardize biomass concentration respect to optical densities in strain 6323 were failed possibly due to heterogeneity of samples. This fact may be explained by the formation of cellular aggregates, typical in Mycobacteria.[8] The problem can be overcome after addition of tween 40 to the culture. Nevertheless, preliminary observations have suggested a further negative effect on the activity of the enzyme. So that the use of tween 40 must be discarded.[9]

The rate biomass concentration/catalytic activity is shown in table 2. Higher values were achieved in the range 5-10 mg/mL biomass dry weigh. It should be noted a significant enzymatic activity decreased for further biomass concentrations. Formations of cellular aggregates in cultures[9] may cause such a behavior, affecting enzyme-substrate interaction. Moreover; decrease of steroid 1,2-dehydrogenase activity in whole free cells in *Arthrobacter simplex* was also reported.[2]

On the other hand, enzymatic activity was shown to be as much as 2.5 times higher than controls if methylene blue was used as an electron acceptor, at the minor concentration ($1{,}34 \cdot 10^{-4}$ mol/L), in the biomass range fixed (5-10 mg/mL).

In addition, the higher concentration of the methylene blue, the smaller the catalytic activity of the enzyme. The effect might be related to a hydrogen concentration raise as a result of electron flux from respiratory chain which provokes a subsequent raise of peroxides, toxic to the cell. Similar results were observed in *Arthrobacter simplex* free cells extracts[10] with an inhibition of 20 % at a concentration of 10^{-3} mol/L hydrogen peroxide

H^+ ionic concentration on enzymatic activity

Enzymatic activity in strain 6323 was favored at basic pH, showing maximal figures in pH 8.4 (tris-HCl) and 8.6 (NH_4Cl); 25 °C (Fig. 1).

It seems reasonable thinking that buffer nature can influence thermodynamic and kinetically over the enzymatic reaction. Besides, buffer plays an important role in self-stabilization of the catalyzer.[11]

On the other hand, it was known that buffers having the same pH, do not harbor either the same superfi-

Tabla 2. Effect of biomass on 1,2 dehydrogenation of AD in the strain 6323.

Biomass (mg)	Specific activity (U/mg dry biomass)
5	1,63 a
10	1,60 a
15	1,09 b
20	1,03 b
25	1,05 b

Table 3. Effect of methylene blue on steroid-1,2-dehydrogenation of AD by strain 6323.

Methylene blue (mol/L)	Specifical activity (U/mg dry-weight)
$1.34 \cdot 10^{-4}$	1.65 a
$2.67 \cdot 10^{-4}$	1.52 b
$4.01 \cdot 10^{-4}$	1.48 bc
$5.35 \cdot 10^{-4}$.	1.44 c
$6.69 \cdot 10^{-4}$	1.44 c
$8.02 \cdot 10^{-4}$	1.45 c
Control	0.66 d

Table 1. Experimental conditions in steroid 1,2-dehydrogenase reaction.

Reaction mixture (V_T = 1 mL)	Substrate	Sample (μL)	Reaction
Buffer	50	940	40
Methylene blue	50	50	50
AD ($1.04 \cdot 10^{-3}$ mol/L)	–	10	10
Cellular suspension	900	–	900

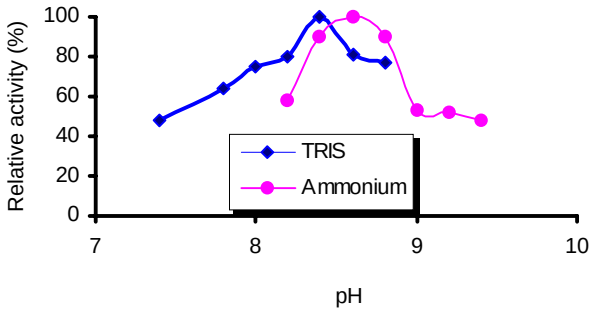

***Fig. 1**. Effect of pH on steroid-1,2-dehydrogenase activity in the strain 6323.*

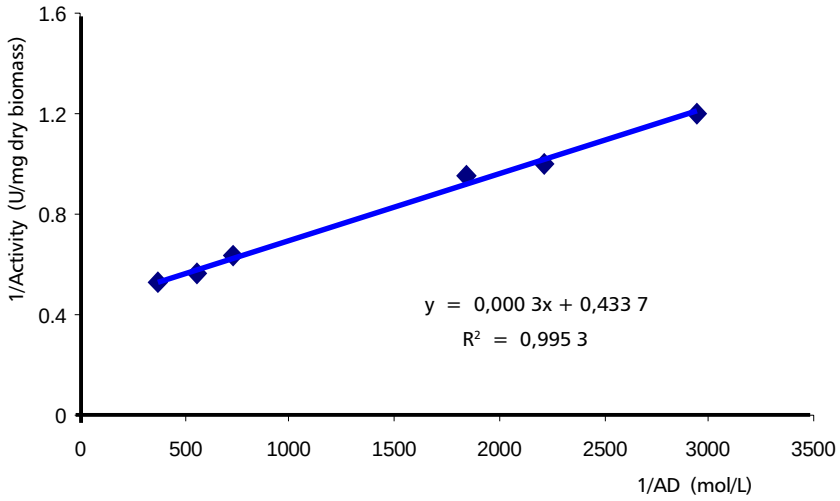

***Fig 2**. Effect of substrate concentration on 1,2-dehydrogenase activity of strain 6323.*

cial tension or the same charge, so that three-dimensional conformation of the enzyme must be affected and consequently its activity and stability.

A like behavior was also noticed in whole cell enzymatic assays of *Mycobacterium* B-3683 and Ex4[12] and crude extract of *Mycobacterium fortuitum* ATCC 6842.[13]

Effect of substrate concentration on steroid-1,2-dehydrogenase activity

The effect of AD concentration between $2{,}04 \cdot 10^{-5}$ and $1{,}81 \cdot 10^{-3}$ mol/L; on the activity of the enzyme was investigated. Assay were done under the best conditions of buffer and pH found, that is, buffer tris-HCL, pH 8,4. as a results, a Km (Michaelis-Menten) of $6{,}92 \cdot 10^{-4}$ mol/L and Vmáx. of 2.3 U/mg dry-biomass were found (Fig. 2).

These figures clearly show a higher steroid-1,2-dehydrogenase activity of strain 6323 respect to parentals (*Mycobacterium* B-3683 and Ex4). In the latter cases values of Km 1,87 and 2,52 . 10^{-3} M and 1.17 and 1.88 U/mg dry-biomass were respectively observed in whole cells experiments[9].

It was also determined that Km values were higher in whole cell assays than in case of crude extracts, for parental strains. In fact, a difusional effect much more higher in whole cells could hamper enzyme-substrate interaction, need for the catalysis. The effect must be enhanced in *Mycobacterium* due to typical aggregates growing cultures.

CONCLUSIONS

The enzyme steroid 1,2-dehydrogenase shows maximal values of activity at 5-10 mg dry biomass/mL and a methylene blue concentration of $1{,}34 \cdot 10^{-4}$ mol/L, pH 8,4-8,6. Km was reached at an AD concentration of $6.92 \cdot 10^{-4}$ mol/L and the Vmax. of the reaction was 2.3 U/mg dry biomass.

The mutant 6323 shows a higher steroid-1,2-dehydrogenase activity respect to parentales.

BIBLIOGRAPHY

1. Itagaki E., Wakabayashi T. and Hatta T. Purification and characterization of 3-ketosteroid-Δ^1-dehydrogenase from *Nocardia corallina*. **Biochimica et Biophysica Acta, 1038**, 60, 1990.
2. Pinheiro H.M., Cabral J.M.S. and Adlercreutz P. Quinones as external electron acceptors in steroid Δ^1-dehydrogenation with entrapped cells in organic medium. **Biocatalysis, 7**, 83, 1993.
3. Choi K.P., Molnár I. and Murooka Y. Secretory overproduction of *Arthrobacter simplex* 3-ketosteroid Δ^1-dehydrogenase by *Streptomyces lividans* with a multi-copy shuttle vector. **Appl. Microbiol. Biotechnol., 43**, 1044, 1995.
4. Kaufman G., Thole, H., Kraft R. and Atrat P. Steroid-1-dehydrogenase of *Rhodococcus erytropolis*: purification and N-terminal amino acid sequence. **J. Steroid Biochem. Molec. Biol., 43**, 297, 1992.
5. Fujui Ch., Morii S. Kadode M., Sawamoto S. Iwami, M. and Itagaki E. Essential tyrosine residues in 3-ketosterroid-Δ^1-dehydrogenase from *Rhodococcus rhodochrous*. **J. Biochem., 126**, 662, 1999.
6. Pérez C., Falero A., Llanes N., Hung B.R., Hervé M.E., Palmero A. Resistance to androstanes as an approach for androstandienedione yield enhancement in industrial mycobacteria. **Journal of Industrial Microbiology & Biotechnology,** (in press), 2003.
7. Marsheck W.J. Kraychy S. and Muir R. Microbial degradation of sterols**. Applied Microbiology,** 72-77, 1972.
8. Borrego S., Niubó E., Ancheta O. and Espinosa M.E. Study of the microbial aggregation in *Mycobacterium* using image analysis and electron microscopy. **Tissue and Cells, 32**, 494, 2000.
9. Falero A., Hung B.R., Aguila B., Fonseca M. y Díaz M.A. Estudio cinético de la esteroide 1,2-deshidrogenasa de una cepa mutante. **Revista CENIC Ciencias Biológicas, 25**, 1994.
10. Montes M.C. and Magaña I. Δ^1-dehydrogenation of steroids by *Arthrobacter simplex* immobilized in calcium polygalacturonate beads. **Journal of Industrial Microbiology, 8**, 259, 1991.
11. Carr P.W. and Bowers L.D. Immobilized enzymes in analytical and clinical chemistry. **Chemical Analysis Interscience Publication, 56**, 51, 1980.
12. Falero A. Estudios Bioquímicos de la esteroide 1,2-deshidrogenasa en mutantes de *Mycobacterium* sp. biotransformadoras de esteroides**.** Tesis de maestría en Bioquímica de las Proteínas. Facultad de Biología, Universidad de la Habana, 2001.
13. Hung B.R., Benítez J. y Aguila B. Estudio cinético de la esteroide 1,2-deshidrogenasa de la cepa *Mycobacterium fortuitum* ATCC 6842. **Revista CENIC Ciencias Biológicas, 25**, 1994.