Kinetics of Befunolol Reductase from Rabbit Liver

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The kinetic mechanism for the reduction of befunol catalyzed by befunol reductase from rabbit liver was investigated. From the initial velocity analysis, product inhibition and coenzyme binding studies, the reduction of befunol was found to proceed through an ordered Bi Bi mechanism, in which β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), binds to the enzyme firstly and NADP⁺ leaves lastly. NADPH bound to the free enzyme at a molar ratio of 1:1. Furthermore, the result of dead-end inhibition by Cibacron blue F3GA, a nucleotide analogue which binds to many enzymes, was consistent with the ordered Bi Bi mechanism for the enzyme.

Keywords befunol reductase; befunolol; enzyme reaction; kinetic mechanism; ordered Bi Bi mechanism; initial velocity; product inhibition; fluorescence titration

Carbonyl reductase (EC 1.1.1.184) is well known to catalyze the reduction of xenobiotic and endogenous aldehydes and ketones in the presence of β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). Several investigators have recently described the kinetics of carbonyl reductases from various tissues of mammalian species. However, no attempt has been made to elucidate the kinetic mechanism for the reduction of drugs containing a ketone group such as befunolol (BF) and acetohexamide catalyzed by carbonyl reductase.

BF, which is widely used in the treatment of arrhythmia,12,13) is a typical ketone-containing drug. Recently, this drug has been reported to be reduced to dihydrobefunolol (DBF), a pharmacologically active metabolite, in human14) and some animals.15-18) Furthermore, an enzyme which catalyzes the metabolic reduction of BF, designated as BF reductase, has been purified from rabbit liver cytosol19) and its characteristics have been examined in this laboratory. This enzyme had the ability to reduce a variety of aldehydes and ketones to the corresponding alcohols, and was classified as a carbonyl reductase. We have studied in detail the kinetic mechanism for the reduction of BF catalyzed by BF reductase from rabbit liver.

Experimental

Materials BF [2-acetyl-7-(2-hydroxy-3-isopropylaminoproxy) benzofuran] hydrochloride and DBF were supplied by Kaken Pharm. Co., Ltd., (Tokyo). Nicotinamide nucleotides were obtained from Oriental Yeast Co., (Tokyo). Cibacron blue F3GA was purchased from Sigma Chemical Co., (MO, U.S.A.). All other chemicals used in this study were guaranteed reagents. The BF reductase used in this study was purified from rabbit liver according to the method reported previously.20)

Enzyme Assay and Kinetic Study BF reductase activity was determined spectrophotometrically by monitoring NADPH oxidation at 340 nm. The reaction mixture in a final volume of 2.8 ml contained 100 mM Na2HPO4 buffer (pH 6.5), NADPH, BF and enzyme. The concentrations of NADPH, BF and Cibacron blue F3GA are given in the text as the specific experiments. All reactions were initiated by the addition of NADPH. Controls contained all reactants except the enzyme. The initial velocity of the reaction was measured in a Shimadzu UV-240, and the results are given in unit of μmol NADPH oxidized/min at 30°C. Protein concentration was determined by a modification of the method of Lowry et al., using bovine serum albumin as the standard.

Data Analysis Initial velocities in the asymptotic region that follows Michaelis kinetics were fitted to Eq. 1:

\[ v = V_{MAX}[A][B]/([A][B] + K_{M1}[A] + K_{M2}[B] + K_{M3}[A][B]) \]  

by using a computer program for least-squares linear regression, where [A] and [B] represent the concentrations of each substrate and \( K_{M1}, K_{M2}, \) and \( K_{M3} \) are the respective limiting Michaelis constants. \( K_{M} \) is the equilibrium dissociation constant of the binary enzyme-substrate complex assuming that this complex undergoes no transformation before the binding of another substrate. Kinetic studies in the presence of inhibitors were carried out in an identical manner. Data for linear competitive and linear noncompetitive inhibition were described by Eqs. 2 and 3, respectively:

\[ \frac{v}{v_i} = \frac{[S][I]}{[S][I] + K_i(1 + [I]/K_i)} \]  

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where \( K_i \) (slope effect) and \( K_c \) (intercept effect) are inhibition constants for the inhibitor with respect to a varied substrate and [I] is the concentration of inhibitor. \( S \) represents the concentration of the varied substrate and \( K_i \) is the limiting Michaelis constant for the substrate. All values are the means of three determinations.

BF has an absorption at 340 nm (\( ε = 1.37 \times 10^4 \)), and so it was necessary to determine a correction factor for the initial rates observed in the presence of this compound. The correction factor was calculated according to Eq. 4:

\[ \text{correction factor} = \frac{ε_{NADPH} - ε_{BF}}{ε_{NADPH} + ε_{BF}} \]

Binding Study The binding of coenzyme or Cibacron blue F3GA to the enzyme was analyzed by fluorometry. The fluorescence of the enzyme and NADPH were measured in a JASCO FP-770 with 1 cm quartz cuvettes. All experiments were performed in 100 mM Na2HPO4 buffer (pH 6.5) at 25°C. Binding of coenzyme was determined by monitoring the fluorescence of NADPH or BF reductase. In a typical binding experiment, 2.0 ml of enzyme solution (2.1 μM) and reference buffer were titrated with a coenzyme stock solution (0.5 mM) and the fluorescence intensity of NADPH was measured. Dissociation constants were estimated according to the method of Attallah and Lata,24)

Results

Initial Velocity Measurements At fixed concentrations of BF, the double-reciprocal plots of initial rate versus the

![Fig. 1. Initial Velocity Analysis of the Reduction of BF by BF Reductase](image-url)

(A) The concentrations of BF were 0.2 mM (C), 0.3 mM (●), 0.5 mM (▲), 0.75 mM (△), 1.0 mM (□) and 1.25 mM (☆). Velocity is expressed as unit/mg. (B) Replots of the slopes (C) and intercepts (●).
varied concentrations of NADPH yielded a series of intersecting lines as shown in Fig. 1A. When the slopes and intercepts against the reciprocal of BF concentration were again plotted for each of these experiments, the plots were linear (Fig. 1B). Similar patterns of initial velocity were observed when the fixed substrate was NADPH (data not shown). These results are consistent with a reaction mechanism that proceeds in a sequential manner. The kinetic constants for BF and NADPH are shown in Table I.

**Product Inhibitions** Product inhibition studies for BF reductase were carried out to determine the substrate binding and product dissociating order. The inhibition patterns of products are summarized in Table II. When DBF was used as the product inhibitor, BF reductase was inhibited noncompetitively with respect to BF at saturating and unsaturating concentrations of NADPH, or with respect to NADPH at unsaturating concentration of BF. Similarly, product inhibition of this enzyme by NADP⁺ was noncompetitive with respect to BF at saturating and unsaturating concentrations of NADPH. However, NADP⁺ was a competitive inhibitor of this enzyme with respect to NADPH at unsaturating concentration of BF. We could not carry out the determination with a saturated concentration of BF because the drug had an absorption at 340 nm and this interfered with the kinetic analysis. These findings suggest that the kinetic mechanism for BF reductase is an ordered Bi Bi mechanism, where NADPH binds to the free enzyme before BF, and DBF leaves firstly, followed by NADP⁺.

In order to elucidate further the kinetic mechanism of BF reductase, the inhibitory effect of Cibacron blue F3GA on this enzyme was examined. As shown in Fig. 2, the inhibition was competitive with respect to NADPH and noncompetitive with respect to BF. According to the Cleland rule for dead-end inhibition, the inhibition patterns by Cibacron blue F3GA also support the hypothesis that the enzymatic reaction of BF reductase follows the ordered Bi Bi mechanism.

**Binding of Coenzymes and Cibacron Blue F3GA** Figure 3A shows the fluorescence spectra of BF reductase in the absence and the presence of NADPH, NADP⁺ or Cibacron blue F3GA. The fluorescence intensity of the enzyme was decreased by adding these compounds, indicating that free BF reductase binds NADPH, NADP⁺ or Cibacron blue F3GA. Moreover, when BF reductase was added to NADPH, the fluorescence intensity of NADPH was increased and its emission peak was shifted from 465 to 450 nm, as shown in Fig. 3B. This suggests that the NADPH molecule binds at a hydrophobic region of BF reductase, as pointed out for drug-protein interaction. Although NADP⁺ or Cibacron blue F3GA did not directly affect the fluorescence of NADPH, the addition of NADP⁺

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**Table I. Kinetic Constants of BF Reductase**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied substrate</th>
<th>Fixed substrate</th>
<th>Inhibition pattern</th>
<th>Inhibition constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH⁺</td>
<td>BF</td>
<td>10 μM NADPH</td>
<td>Noncompetitive</td>
<td>30.8</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>BF</td>
<td>100 μM NADPH</td>
<td>Noncompetitive</td>
<td>71</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>NADPH</td>
<td>1 mM BF</td>
<td>Competitive</td>
<td>16.0</td>
</tr>
<tr>
<td>DBF</td>
<td>BF</td>
<td>10 μM NADPH</td>
<td>Noncompetitive</td>
<td>4171</td>
</tr>
<tr>
<td>DBF</td>
<td>BF</td>
<td>100 μM NADPH</td>
<td>Noncompetitive</td>
<td>58577</td>
</tr>
<tr>
<td>Cibacron blue F3GA</td>
<td>BF</td>
<td>100 μM NADPH</td>
<td>Noncompetitive</td>
<td>22.4</td>
</tr>
<tr>
<td>Cibacron blue F3GA</td>
<td>NADPH</td>
<td>1 mM BF</td>
<td>Competitive</td>
<td>4.19</td>
</tr>
</tbody>
</table>

The concentrations of NADP⁺ were 0, 50 and 100 μM. DBF concentrations were 0, 2.0 and 5.0 mM and Cibacron blue F3GA concentrations were 0, 5.0 and 20 μM. The concentrations of the varied substrates were the same as those shown in Fig. 1A.
or Cibacron blue F3GA to the enzyme–NADPH complex resulted in a decrease of the fluorescence intensity. Figure 4 shows the fluorescence titration of BF reductase with NADPH at emission wavelengths of 340 nm (A) and 450 nm (B). From these findings, it appears that 1 mol of NADPH binds to 1 mol of BF reductase. In addition, the dissociation constant for the BF reductase–NADPH complex determined spectrophotometrically was 1.04 μM.

Discussion

The intersecting lines obtained in the initial velocity studies indicate that the reduction of BF catalyzed by BF reductase proceeds in a sequential kinetic mechanism. A ping-pong kinetic mechanism seems unlikely, since this mechanism yields parallel lines in double-reciprocal plots. We have determined kinetically a dissociation constant for the BF reductase–NADPH complex of 0.82 μM. This value is in good agreement with that of 1.04 μM obtained spectrophotometrically. When DBF was tested as a substrate in the reverse reaction under the same conditions, the metabolite was little oxidized, suggesting that the equilibrium of these reactions is shifted towards the reductive direction.

On the basis of the product inhibition patterns, the enzymatic reaction of BF reductase from rabbit liver is considered to follow an ordered Bi Bi mechanism with BF as a substrate, in which NADPH binds to the free enzyme and NADPH* leaves lastly. The results obtained from the binding studies, which showed that both NADPH and NADPH* bind to the free enzyme, also support this mechanism. This type of mechanism has been observed with some other carbonyl reductases. For examples, carbonyl reductases from human brain and mouse liver followed the ordered Bi Bi mechanism. However, the kinetic mechanism of carbonyl reductases from guinea-pig lung and dog liver is known to be a di-iso ordered Bi Bi mechanism with coenzyme-induced isomerization.

Cibacron blue F3GA, a nucleotide analogue, is known to bind to many enzymes such as carbonyl reductases and aldehyde reductases, and is used as a dead-end type inhibitor. If the enzymatic reaction of BF reductase follows the ordered Bi Bi mechanism, the inhibition by Cibacron blue F3GA would be competitive with respect to NADPH and noncompetitive with respect to BF. As expected, Cibacron blue F3GA inhibited BF reductase in such a manner. From the fluorescence studies, Cibacron blue F3GA was found to bind to free BF reductase. The effect of Cibacron blue F3GA on the fluorescence of BF reductase was similar to that of NADPH, and the effect of the inhibitor on the fluorescence of the enzyme–NADPH complex was similar to that of NADPH*. These findings strongly support the idea that the enzymatic reaction of BF reductase follows the ordered Bi Bi mechanism.

In the present study, we have demonstrated that the reduction of BF catalyzed by BF reductase proceeds through an ordered Bi Bi mechanism. This is the first report of the kinetic mechanism for the reduction of a ketone-containing drug catalyzed by carbonyl reductase.

References