Conversion of a Flavoprotein Reductase to a Desaturase by Manipulation of the Flavin Redox Potential

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Recently there has been a surge of activity in exploring the ability of enzymes to effect chemical transformations with high selectivity.[1] The advent of recombinant DNA technology and site-directed mutagenesis and the use of nonconventional reaction media like organic solvents has resulted in the effective manipulation of enzymes and in the design of semisynthetic enzymes.[2] The fact that it is relatively easy to remove the flavin prosthetic group to obtain apoprotein and then reconstitute the apoprotein with chemically modified flavins provides flavoproteins with a means of manipulating their catalytic activity.[3] In the present communication we report successful conversion of a flavoenzyme NADPH-dependent reductase to an oxygen-dependent desaturase by this strategy.

The α,*β*-unsaturated ketone or enone functionality enjoys a unique position in organic chemistry as it is involved in a diverse array of reactions such as 1,2-additions or 1,4-conjugate additions, alkylations, or Diels−Alder reactions. It is also a part of numerous natural products.[4] However, the selective catalytic oxidation of simple carbonyl compounds directly to their corresponding α,*β*-unsaturated derivatives (enones) under mild conditions is one of the most difficult reactions to achieve using conventional synthetic methods.

Here we present a new general enzymatic catalyst for this transformation, developed by successful manipulation of an enzyme meant for reducing α,*β*-unsaturated carbonyl compounds to their corresponding saturated derivatives (flavoenzyme reductase) to catalyze exactly the opposite reaction of oxidizing saturated carbonyl compounds to their corresponding α,*β*-unsaturated enones (desaturase activity). Old yellow enzyme (OYE) is a mixture of homodimers and a heterodimer arising from two yeast genes, with each monomer binding a molecule of flavin mononucleotide (FMN). Although its exact physiological function is still unknown, there is considerable knowledge of structure−function aspects. The crystal structure is solved,[5] and several tight-binding ligands such as steroids have been identified, raising the possibility that this enzyme might be involved in the biosynthetic pathway of steroids.[6] It has been discovered in our laboratory that this enzyme catalyzes the reduction of the olefinic bond of α,*β*-unsaturated carbonyl compounds using NADPH and also catalyzes slow aromatization of cyclic enones such as cyclohexenone by a novel dismutation reaction.[7]

One of the important features of flavin catalysis is the oxidation−reduction potential, which controls the direction of electron flow during catalysis. Since the mechanistic requirements (in the case of OYE) for both oxidation and reduction are very similar, it was thought that, if the redox potential of the flavin is made significantly more positive, there is a chance to manipulate the redox chemistry of the enzyme. The oxidation−reduction

Table 1. Desaturase Activity of 8-CN-FMN-OYE

Substrate	Turnover number[a], Air saturation, min^{-1}	K_m, air saturation
Dihydrocinnamaldehyde	11.1[b]	9.6×10^{-5} M
3-Phenylbutanal	2.5	7.0×10^{-4} M
4-Phenylbutan-2-one	0.2	6.3×10^{-4} M
n-Propanal	4.0	1.33×10^{-3} M
n-Butanal	3.2	2.7×10^{-4} M
2-Methylbutanal	1.1	2.5×10^{-4} M
Butan-2-one	0.8	3.0×10^{-4} M
3-Methylbutanal	3.7[c]	1.7×10^{-4} M
Cyclopentanone	10.1[d]	8.4×10^{-4} M
2-Methylcyclopentanone	4.0	5.3×10^{-4} M
3-Methylcyclopentanone	2.7	4.3×10^{-4} M

[a] Turnover numbers were determined in air-saturated 0.02 M pyrophosphate buffer, pH 8.5, 25 °C, by following, spectrophotometrically, the formation of the unsaturated product at its absorbance maximum (from 220 nm with n-propanal to 292 nm for dihydrocinnamaldehyde). In all cases the reaction rates are faster in oxygen-saturated buffer. With three substrates, dihydrocinnamaldehyde, 3-methylbutanal, and cyclopentanone, complete kinetic evaluation was carried out by also varying systematically the oxygen concentration. Ping-pong kinetics were found, and true k_{cat} and K_m values are reported for these substrates. [b] $k_{cat} = 21$ min^{-1}; $K_m^{O_2} = 2.1 \times 10^{-4}$ M; $K_m^s = 1.8 \times 10^{-4}$ M. [c] $k_{cat} = 50$ min^{-1}; $K_m^{O_2} = 3.0 \times 10^{-3}$ M; $K_m^s = 1.1 \times 10^{-3}$ M. [d] Second order with substrate, $k = 1.33 \times 10^4$ M^{-1} min^{-1}; Second order with O_2 $k = 4 \times 10^4$ M^{-1} min^{-1}. (This value predicts a maximum turnover number of 10.3 min^{-1} for any substrate in air-saturated solution.)

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Scheme 1

Scheme 2

Scheme 3. Reaction of 3-Me-cyclopentanone

potential for the native flavin was determined as −207 mV.[8] There is a good correlation between the redox potential of the flavin and the electron-withdrawing or donating properties of the substituent at the flavin 8-position, with electron-withdrawing substituents making the isoalloxazine ring highly electron deficient, which, in turn, raises the potential.[8,9] Accordingly, we have synthesized a flavin analogue with a powerful electron-withdrawing group at position 8, namely 8-CN-8-demethyl-riboflavin.[10] The redox potential was determined as −50 mV, about 160 mV more positive than that of the native flavin. The cyanoflavin was converted to FAD and FMN levels enzymatically. The apoprotein of OYE was prepared according to the established procedure and reconstituted with 8-CN-FMN.[10] The binding affinity of 8-CN-FMN to apoOYE was found to be similar to that of the native FMN, demonstrating that the subtle structural changes that were introduced into the synthetic analogue did not significantly alter the binding affinity of the flavin to protein.[10] The catalytic activity of the reconstituted enzyme toward several carbonyl compounds was investigated. OYE has a reductase activity toward enones such as cyclohexenone and cinnamaldehyde with k_{cat} values of 250 min^{-1} and 125 min^{-1}, respectively; however, the 8-CN-FMN enzyme has poor reductase activity, with a k_{cat} of 7.5 min^{-1}, and 3.5 min^{-1}, respectively, toward the same substrates. The reaction of native as well as 8-CN-FMN enzyme with dihydrocinnamaldehyde (HCA) was studied in detail. Native OYE showed no desaturase activity toward HCA. However, the 8-CN-FMN enzyme has a relatively efficient desaturase activity toward this compound, forming cinnamaldehyde (Table 1). The oxidized enzyme reacts with hydrocinnamaldehyde to give the reduced form, and the reduced enzyme is reoxidized by molecular oxygen, as shown in Scheme 1. It was found that the 8-CN enzyme reacts with a variety of structurally diverse carbonyl compounds (Table 1) to form their corresponding α,β-unsaturated derivatives, establishing its generality for catalyzing such a reaction. It must be emphasized that this new activity does not involve reaction of the enzyme with NADPH; instead, the saturated carbonyl compound acts as an electron donor and molecular oxygen as an acceptor. A potential limitation to the utility of the 8-CN-FMN enzyme is the capacity of the reduced form to be reoxidized by the unsaturated product, in competition with molecular oxygen, which could result in futile redox cycling. This would not be expected to be a serious issue at early stages of the reaction but could become one as oxidation product accumulates. However, GC/MS analysis of the product from aerobic reaction of dihydrocinnamaldehyde with 8-CN-FMN OYE showed complete conversion to cinnamaldehyde.

The stereochemistry of the oxidation reaction was determined as trans (Scheme 2). Trans dideutero-hydrocinnamaldehyde was synthesized with known stereochemistry by the reduction of cinnamaldehyde with the NADPD/D_2O/OYE system.[7] When the oxidation of this labeled hydrocinnamaldehyde was carried out with 8-CN-FMN-OYE, mass spectrum and NMR results showed the elimination of both deuterium atoms.

The oxidation was also found to be enantioselective. When the oxidation of racemic 3-methylcyclopentanone was carried out with 8-CN-FMN enzyme, it afforded 3-methyl-2-cyclopenten-1-one and 3-methyl-4-cyclopenten-1-one. Further studies with commercially available (*R*)-(+)-3-methylcyclopentanone resulted exclusively in the formation of 3-methyl-4-cyclopenten-1-one. When the oxidation of the corresponding *S* isomer, made by the reduction of 3-methyl-2-cyclopenten-1-one with NADPH and native OYE, was carried out with 8-CN-FMN-enzyme, it afforded, exclusively, 3-methyl-2-cyclopenten-1-one. It is known from the crystal structure that the enzyme has one binding site and the ligand is stacked above the *si* face of the flavin ring system with their planes approximately parallel. When the *S* enantiomer is bound to the protein, the hydrogen on the methyl-substituted carbon-3 is oriented in a favorable geometry for hydride transfer to occur at the flavin N-5. This results in the formation of the substituted olefin. However this is not the case with the *R* enantiomer, as the hydrogen on the same carbon would lie above the plane, making the hydride transfer unfeasible. As a result, the cyclopentane ring probably binds so that its opposite face is positioned above the flavin, making the hydride transfer from the C-4 geometrically favorable and forming an unsubstituted olefin. This concept is depicted in Scheme 3. The high enantioselectivity during the oxidation clearly demonstrates the possible applications of this new enzyme system for the resolution of carbonyl compounds.

The present studies accomplished the reversal of OYE redox chemistry, transforming the enzyme from a reductase to a desaturase, thereby providing an efficient biocatalytic system for an important chemical transformation. The present catalytic system carries out the oxidation without requiring any costly cofactors such as pyridine nucleotides, but simply using naturally abundant molecular oxygen as the oxidant.

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