Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2663–2668

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

THE IMPORTANCE OF GLU^{361} POSITION IN THE REACTION CATALYZED BY CHOLESTEROL OXIDASE

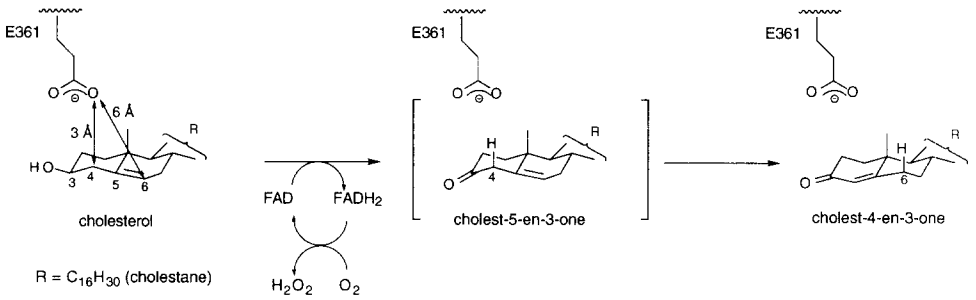
Ignatius J. Kass and Nicole S. Sampson*

Department of Chemistry, State University of New York, Stony Brook, NY 11794-3400, U.S.A.

Received 25 June 1998; accepted 3 August 1998

Abstract: Cholesterol oxidase stereospecifically isomerizes cholest-5-en-3-one to cholest-4-en-3-one. When the base catalyst for isomerization, Glu^{361}, is mutated to Asp, the rate of deprotonation of cholest-5-en-3-one is not affected, but protonation of the dienolic intermediate becomes rate-limiting. This may be a consequence of the large distance between the catalytic base and carbon-6 of the intermediate in the mutant enzyme.

Cholesterol oxidase from *Streptomyces* catalyzes the oxidation and isomerization of cholesterol to cholest-4-en-3-one (Scheme 1). It was first isolated for use in serum cholesterol assays and subsequently was found to have larvicidal properties against *Coeloptera*.[1,2] Cholesterol oxidase has also been used as a probe of membrane structure and cholesterol content.[3] A detailed understanding of its catalytic mechanism will guide engineering of the protein into a better tool for cell biology, and into a better insecticide.



Scheme 1

Both the oxidation and isomerization reactions occur in the same active site that is an 11 Å deep cavity sequestered from bulk solvent. Chemically reasonable mechanisms require a general base for removal of the proton on the 3-hydroxyl during oxidation, and a general acid to stabilize the formation of the dienolate that is formed by general base abstraction of the 4β-proton. Inspection of the X-ray crystal structure[4] of the homologous *B. sterolicum* cholesterol oxidase (58% amino acid identity[5]) suggested that His^{447} can act as the general base for oxidation,[6] and the imidazolium ion formed could act as a general acid for isomerization. Glu^{361} is positioned over the β-face of the steroid, and is close enough to abstract the 4β-proton. In a previous report, we described the consequences of mutating Glu^{361} to glutamine.[8] The E361Q mutation suppressed the isomerization reaction, however the oxidation reaction still occurred. Thus the product of the E361Q reaction was cholest-5-en-3-one, moreover it was produced catalytically. That is, the mutant enzyme released the intermediate of the wild-type reaction and was capable of multiple turnovers, although the mutant k_{cat} was 30-fold reduced from the wild-type k_{cat}. Those experiments clearly demonstrated that Glu^{361} is the catalytic base for isomerization. In this report, we

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00478-8

further investigate the role of Glu^{361} in the reaction catalyzed by cholesterol oxidase, and present the kinetic analyses of a second point mutant, E361D, that was prepared to determine the structural requirements for proton transfer in the isomerization reaction.

The E361D mutant was prepared by PCR cassette mutagenesis,[9] and the mutant protein was purified as previously described.[8] The UV/vis and CD spectra were identical to wild type, indicating that the protein folded properly. The activity of the protein was determined in two ways. First, the oxidation activity was measured using cholesterol as a substrate and H_2O_2 production was followed with horseradish peroxidase as a coupling enzyme.[10] Second, the isomerization activity was measured using cholest-5-en-3-one as a substrate and the appearance of cholest-4-en-3-one was monitored at 240 nm.[10] The k_{cat} for oxidation was 11-fold reduced compared to wild type, the k_{cat} for isomerization was 320-fold reduced (Table 1). The K_m's were lowered about 5-fold. When the consumption of cholesterol was monitored by following the appearance of conjugated enone (i.e., oxidation plus isomerization), this 10-fold difference in oxidation and isomerization activity was confirmed. Analysis of the product mixture by HPLC[8] revealed that cholest-5-en-3-one was released from the enzyme, and then was slowly converted to cholest-4-en-3-one by E361D. The release of the intermediate, cholest-5-en-3-one, was anticipated because of our earlier work with the E361Q mutant.[8] This difference in activities allows the measurement of the rate of cholesterol conversion to cholest-5-en-3-one using the horseradish peroxidase-coupled assay without the later isomerization steps contributing to the steady-state rate.

Table 1: Steady-State Kinetic Parameters and Isotope (Solvent and Substrate) Effects on Wild-Type and E361D Mutant Cholesterol Oxidases

	cholesterol[a]			cholest-5-en-3-one[a]	
	wild type	E361D		wild type	E361D
k_{cat} (s^{-1})	44 ± 2	4.2 ± 0.2	k_{cat} (s^{-1})	64 ± 3	0.20 ± 0.01
K_m (μM)	3.0 ± 0.4	0.6 ± 0.2	K_m (μM)	6.2 ± 0.7	1.4 ± 0.3
$^{D}(V)_{H2O}$[b]	2.2 ± 0.1	1.1 ± 0.2	$^{D}(V)_{H2O}$[c]	0.86 ± 0.04[d]	1.00 ± 0.07[d]
$^{D}(V/K)$[b]	2.2 ± 0.1	1.2 ± 0.2	$^{D}(V/K)$[c]	0.86 ± 0.04[d]	1.1 ± 0.3[d]
$(V)_{H2O}/(V)_{D2O}$[e]	1.0 ± 0.1	0.9 ± 0.2	$(V)_{H2O}/(V)_{D2O}$[e]	0.9 ± 0.2	2.4 ± 0.1
$^{D}(V)_{D2O}$[b,e]	1.9 ± 0.2	1.0 ± 0.2	$^{D}(V)_{D2O}$[e]	1.04 ± 0.03[d]	1.1 ± 0.1[d]
			$(V/K)_{H2O}/(V/K)_{D2O}$[e]	1.4 ± 0.8	3.8 ± 1.6

[a]Assayed in 50 mM NaPi, 0.025% triton X-100, 3% *i*-propanol at 37 °C, pH or pD 7.0.[10] Errors are the standard deviation of the fit to three independent sets of data.

[b]Using 3α-[^{2}H]-cholesterol, and fitting the protonated and deuterated substrate initial velocity data simultaneously with Grafit (Erithacus, London, UK). The data were fit assuming isotope effects only on V, V/K, or V and V/K, and the fit with the lowest error was used.

[c]Using 4β-[^{2}H]-cholest-5-en-3-one, and fitting as described in note (b).

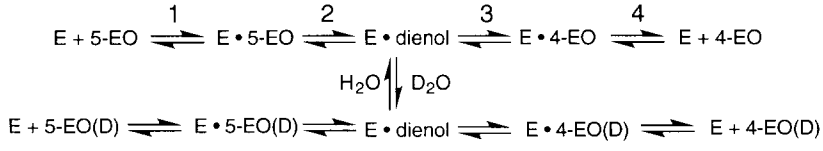
[d]These data were corrected for isotopic incorporation in substrate (0.88 mol D) as determined by ^{1}H NMR spectroscopy.

[e]Measured at a single substrate concentration that was either saturating (V) or 1 μM (V/K). pD was measured by adding 0.4 to the pH meter reading.[11]

The isotope effects on oxidation indicate that hydrogen transfer to FAD is no longer rate-limiting in the E361D-catalyzed reaction. The 11-fold reduction in k_{cat} may be accounted for by the fact that cholest-5-en-3-one is the product released from the enzyme rather than cholest-4-en-3-one as in the wild-type reaction. Thus, product release becomes partially rate-limiting and the primary isotope effects (^{D}V and $^{D}V/K$) on oxidation in the wild-type reaction are not observed in the E361D reaction. Furthermore, there were no solvent isotope effects on either wild-type or mutant-catalyzed oxidation. This conservative mutation essentially leaves the oxidation reaction unaltered. However, this mutation revealed the importance of chain length and hence, carboxylate positioning, for efficient isomerization.

Previously, we had demonstrated that 30% of the deuterium in 4β-[^{2}H]-cholest-5-en-3-one is transferred to the 6β-position of cholest-4-en-3-one during isomerization.[10] The remainder of the label is 'washed-out'. That is, the deuterated conjugate acid, formed with the dienolic intermediate, exchanges its deuterium with hydrogen 7 times for every 3 times the dienolic intermediate is directly deuterated (eq 1). We expected that upon shortening the carbon chain of the base by one methylene (i.e., mutating Glu to Asp) that transfer of the proton might be more difficult, and as a consequence, the rate of dienolic intermediate protonation would decrease. This decrease in rate could lead to an increased loss of label in the transfer experiment, if the rate of exchange of the conjugate acid of Asp^{361} is not similarly decreased. When 4β-[^{2}H]-cholest-5-en-3-one was incubated with E361D, less than 2% of the deuterium label was transferred, and there was no deuterium remaining at the 4-position of the cholest-4-en-3-one product. Hence, the E361D enzyme still stereospecifically removes the 4β-hydrogen, and the substrate still binds in the correct orientation in the protein. However, the partition ratio between exchange of the conjugate acid and deuteration by the conjugate acid is increased to >49:1, suggesting that the mutation slowed down the rate of protonation.

In the wild-type reaction if cholesterol is used as a substrate in deuterated buffer, 30% of the product cholest-4-en-3-one contains deuterium at the 6β-position at the end of the reaction. This low level of deuterium incorporation is due to the approximately equivalent rates of dienolic intermediate protonation and exchange of the glutamic acid with solvent. In the mutant-catalyzed reaction, exchange of the conjugate acid with solvent is rapid compared to protonation of the dienolic intermediate, as evidenced by the loss of the 4β-deuterium from cholest-5-en-3-one to solvent during isomerization. This rapid 'wash-out' allowed us to perform a deuterium discrimination experiment. Use of protonated substrate in deuterated solvent, and analysis of the fraction of solvent deuterium incorporated into product, allowed us to determine whether protonation of dienolic intermediate (eq 1, step 3) or



(1)

cholest-4-en-3-one release from the enzyme (step 4) is rate-limiting. In the E361D-catalyzed reaction, the mass spectral and NMR analysis of the product showed that the deuterium content is 50% that of the solvent: there is a 2-fold discrimination against deuterium. The kinetic isotope effect in protonation of intermediate (step 3) results in hydrogen protonating the dienolic intermediate faster than deuterium, and the product formed contains less deuterium than the solvent. Product release from the enzyme is faster than deprotonation of product to reform the

dienolic intermediate. Hence, the deuterium content of the product isolated reflects the kinetic isotope effect on step 3. If product release were rate-limiting, step 3 would become an equilibrium step and the 6β-hydrogen of the product would equilibrate with the solvent via the dienolic intermediate. Consequently, the fraction deuterium in the product would be equal to the fraction deuterium in the solvent. The discrimination against deuterium incoporation observed strongly suggests that protonation of the dienolic intermediate (step 3) is slower than product release (step 4). It is sensible that transition state 3 becomes rate-limiting in the mutant, because for product release to become rate-limiting, the product would have to be released some 100-fold more slowly from the mutant enzyme than from the wild-type enzyme. This seems unreasonable considering the conservative mutation that has been made.

These experiments did not reveal, however, whether the mutation affected the rate of cholest-5-en-3-one deprotonation (step 2), as might be expected to occur upon Glu to Asp mutation. Therefore, primary kinetic isotope effects and solvent isotope effects were measured for both the wild-type and E361D catalyzed reactions of cholest-5-en-3-one. They are summarized in Table 1. The isomerization reaction is independent of pH between 6.0 and 10.0. Thus, changes in observed rates do not result from changes in pH dependence upon mutation or upon deuteration of solvent.

The isotope effects on isomerization are considered for the reaction mechanism outlined in eq 1. In the wild-type reaction, we did not observe a primary isotope effect on deprotonation (step 2) when 4β-[^{2}H]-cholest-5-en-3-one was used as a substrate. Because the majority of the isotope (70%) is lost upon formation of the dienolic intermediate, the relative rate of reaction of protonated and deuterated cholest-5-en-3-one is primarily determined by the flux of molecules to form the intermediate.[12,13] The lack of isotope effect on V/K when deuterated cholest-5-en-3-one is substrate suggests that a step prior to deprotonation controls the flux to the intermediate, e.g., substrate binding (step 1), in the wild-type reaction.

Mutation of Glu^{361} to Asp slows down the reaction 320-fold and 100% of the label is lost upon formation of the dienolic intermediate. Therefore, the relative rates of reaction of protonated and deuterated cholest-5-en-3-one are determined only by the flux into the intermediate (steps 1 and 2). Yet we do not observe a primary kinetic isotope effect on carbon-4β-H bond breaking in the mutant-catalyzed reaction. This suggests that upon mutation, deprotonation (step 2) does not become slower and is not the rate-limiting step in forming the dienolic intermediate. Alternatively, there may be no kinetic isotope effect on deprotonation. This is very improbable. For the nearly identical reaction catalyzed by ketosteroid isomerase, the isotope effect on deprotonation is 5.3.[14] Even if the transition state for proton transfer is very asymmetric or nonlinear, we would expect to see a small isotope effect on the order of 1.5–3.[15–19] For example, in the 1,2 isomerization reaction catalyzed by triosephosphate isomerase, Glu^{165} is the active-site base that abstracts the proton. Upon mutation to Asp, the reaction is some 1000-fold slower. X-ray structural analysis showed that this rate reduction is a consequence of the increased distance between the carboxylate oxygen and the intermediate, and use of the anti orbital of the carboxylate oxygen.[20] However, intrinsic tritium kinetic isotope effects of 2 and 6 were observed for the proton transfer steps, despite the apparent decrease in basicity and increase in transition state asymmetry. For the E361D cholesterol oxidase mutant, we are left with the possibilities that the mutation slows down the rate of substrate binding or protonation of the dienolic intermediate. Although it is improbable that the Glu to Asp mutation would slow down substrate binding 300-fold, solvent isotope effects were examined to eliminate this possibility.

For the wild-type reaction, no solvent isotope effects were observed. Either substrate binding or product release is most-likely rate-limiting. The second-order rate constant k_{cat}/K_m is 6 x 10^8 M^{-1} min^{-1}, a rate that

approaches the diffusion limit. This rate is clearly not affected by the deuterated solvent. This observation is consistent with the lack of solvent isotope effect for the E361D-catalyzed oxidation reaction, for which it seems reasonable that cholest-5-en-3-one release is rate-limiting. The lack of solvent isotope effects on the wild-type reaction are important for consideration of the E361D solvent isotope effects. When deuterated solvent is used in the E361D catalyzed reaction, we observed a ^{D}V of 2.4 and a $^{D}V/K$ of 3.8. (The large error on $^{D}V/K$ is due to the low substrate concentrations required for the measurement. However, the kinetic isotope effect is significant despite the large error.) This reveals that protonation (step 3) is rate-limiting in the E361D mutant-catalyzed reaction. That is, the solvent isotope effect is due to the rapid exchange of the protonated base with solvent deuterium and deuteration of the dienolic intermediate. It is unlikely that this isotope effect is on substrate binding or product release because of the lack of solvent isotope effects observed with wild type on both cholesterol oxidation and cholest-5-en-3-one isomerization, and with E361D on cholesterol oxidation. The solvent isotope effect is consistent with the deuterium discrimination of 2-fold that was measured by product analysis. These data considered together with the increased exchange vs. protonation ratios measured, and lack of primary kinetic isotope effect on deprotonation described above, indicate that the E361D mutation slows down protonation (step 3), but not deprotonation (step 2) in the isomerization reaction catalyzed by cholesterol oxidase.

This selective change in rate-limiting step, in which the deprotonation rate is not affected, but the protonation rate is decreased, was unexpected. Protonation represents the chemically facile process of proton transfer from a relatively strong acid to a strong base, whereas deprotonation occurs via a weaker carboxylate base abstracting a proton from a weakly acidic ketone. Inspection of the X-ray crystal structure with substrate bound suggests that this phenomenon is a consequence of the large distance (6 Å) between Glu^{361} and carbon-6, that is presumably increased by its mutation to Asp.[21] In contrast, Glu^{361} is only 3 Å away from carbon-4, and mutation to Asp may not significantly increase the carboxylate to carbon-4 distance. Although the electron density suggests that Glu^{361} is mobile, significant repositioning of the substrate would be required to orient Glu^{361} equidistant between carbon-4 and carbon-6.[21] Repositioning would disrupt hydrogen bonds to the general acid/base catalyst His^{447} needed for both oxidation and isomerization. Our data imply that Asp^{361} (and perhaps also Glu^{361}) is positioned asymmetrically between carbon-4 and carbon-6 in the transition states for proton transfer.

Acknowledgment. This work was supported by an American Heart Grant-in-Aid, and in part by NIH Grant HL-53306, and a Camille and Henry Dreyfus New Faculty Award (N.S.S.), and a DOE/GAANN fellowship (I.J.K.). The NMR Spectroscopy facility at SUNY Stony Brook is supported by a grant from the NSF (CHE 9413510). The Spex fluorimeter was purchased with a grant from the NSF (CHE 9709164). We thank Prof. Vernon Anderson for assistance with the isotope effect curve fitting.

References and Notes

(1) Purcell, J. P.; Greenplate, J. T.; Jennings, M. G.; Ryerse, J. S.; Pershing, J. C.; Sims, S. R.; Prinsen, M. J.; Corbin, D. R.; Tran, M.; Sammons, R. D.; Stonard, R. J. *Biochem. Biophys. Res. Commun.* **1993**, *196*, 1406.

(2) Greenplate, J. T.; Duck, N. B.; Pershing, J. C.; Purcell, J. P. *Entomol. Exp. Appl.* **1995**, *74*, 253.

(3) Lange, Y. *J. Lipid Res.* **1992**, *33*, 315.

(4) Vrielink, A.; Lloyd, L. F.; Blow, D. M. *J. Mol. Biol.* **1991**, *219*, 533.

(5) Ohta, T.; Fujishiro, K.; Yamaguchi, K.; Tamura, Y.; Aisaka, K.; Uwajima, T.; Hasegawa, M. *Gene* **1991**, *103*, 93.

(6) This numbering refers to the X-ray crystal structure system for numbering amino acid residues.[4] Numbering begins at the N-terminus of the processed Brevibacterium enzyme. His^{447} is encoded by codon 484 in the Streptomyces gene[7] and codon 492 in the Brevibacterium gene;[5] Glu^{361} is encoded by codons 398 and 406, respectively.

(7) Ishizaki, T.; Hirayama, N.; Shinkawa, H.; Nimi, O.; Murooka, Y. *J. Bacteriol.* **1989**, *171*, 596.

(8) Sampson, N. S.; Kass, I. J. *J. Am. Chem. Soc.* **1997**, *119*, 855.

(9) PCR cassette mutagenesis to construct pCO214 was performed as described in Sampson et al.[10] for pCO202 using the MluI and HindIII sites. The primer corresponding to the coding strand contained the E361D mutation: 5'-CgCAgACgCgTgggACAACAgCgACTCCTCggTCTTCgCggACATCgCCCCCATgCCggCC-ggCC-3'.

(10) Sampson, N. S.; Kass, I. J.; Ghoshroy, K. B. *Biochemistry* **1998**, *37*, 5770.

(11) Schowen, K. B.; Schowen, R. L. *Methods Enzymol.* **1982**, *87*, 551.

(12) Leadlay, P. F.; Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5617.

(13) Knowles, J. R.; Albery, W. J. *Acc. Chem. Res.* **1977**, *10*, 105.

(14) Xue, C.; Talalay, P.; Mildvan, A. S. *Biochemistry* **1990**, *29*, 7491.

(15) Collins, C. J.; Rainey, W. T.; Smith, W. B.; Kaye, I. A. *J. Am. Chem. Soc.* **1959**, *81*, 460.

(16) Hawthorne, M. F.; Lewis, E. S. *J. Am. Chem. Soc.* **1958**, *80*, 4296.

(17) Raines, R. T.; Sutton, E. L.; Straus, D. R.; Gilbert, W.; Knowles, J. R. *Biochemistry* **1986**, *25*, 7142.

(18) Westheimer, F. H. *Chem. Rev.* **1961**, *61*, 265.

(19) Winstein, S.; Takahashi, J. *Tetrahedron* **1958**, *2*, 316.

(20) Joseph-McCarthy, D.; Rost, L. E.; Komives, E. A.; Petsko, G. A. *Biochemistry* **1994**, *33*, 2824.

(21) Li, J.; Vrielink, A.; Brick, P.; Blow, D. M. *Biochemistry* **1993**, *32*, 11507.