Catalytic activity of the D38A mutant of 3-oxo-Delta 5-steroid isomerase: recruitment of aspartate-99 as the base.

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Catalytic Activity of the D38A Mutant of 3-Oxo-Δ^5-steroid Isomerase: Recruitment of Aspartate-99 as the Base

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:
:

Technique(s) de communiation

Sections de l'article

Dans les base de données

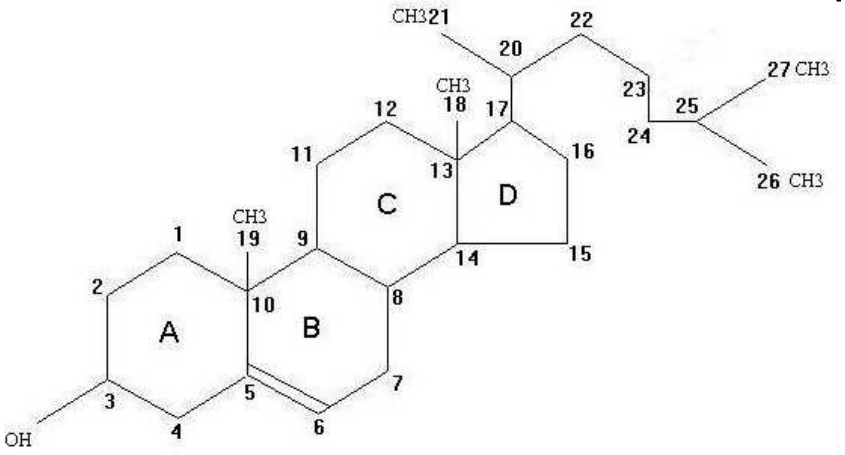
Titre, auteurs
Abstract (résumé)

Introduction
Materials and Methods
Results
Discussion
References (Biblio)

Présentation orale

Les sections sont fusionnées
On rajoute les partie qui sont mentionnées dans une autre article
Pas de biblio

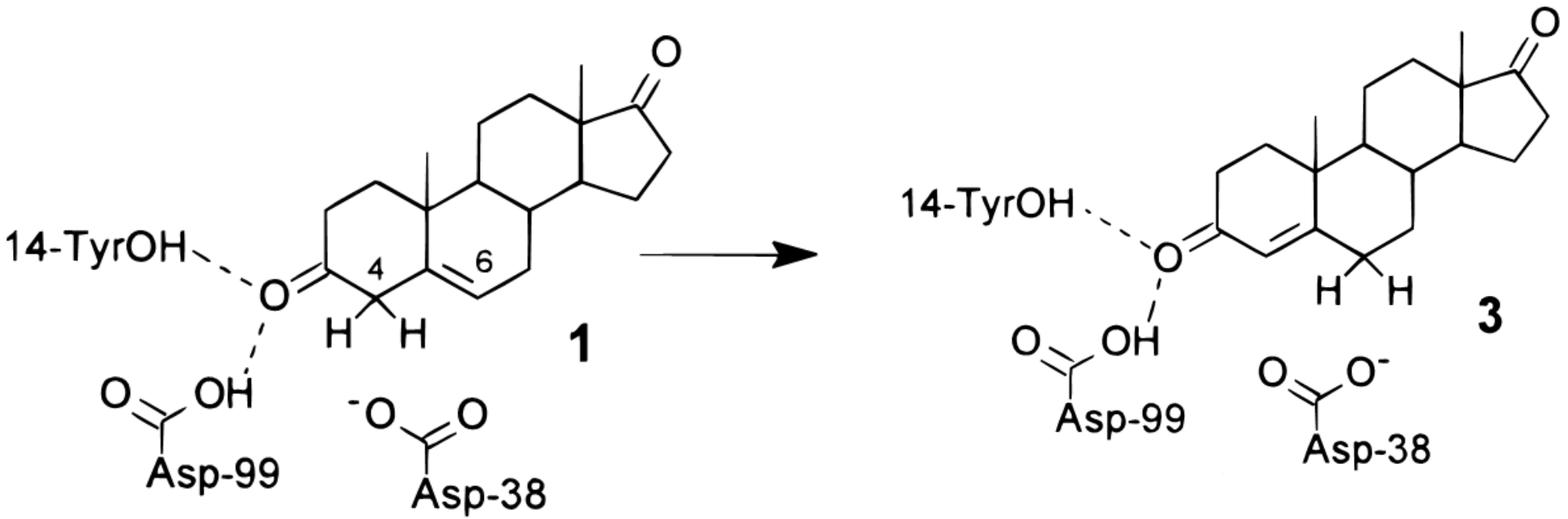
Numération des stérols



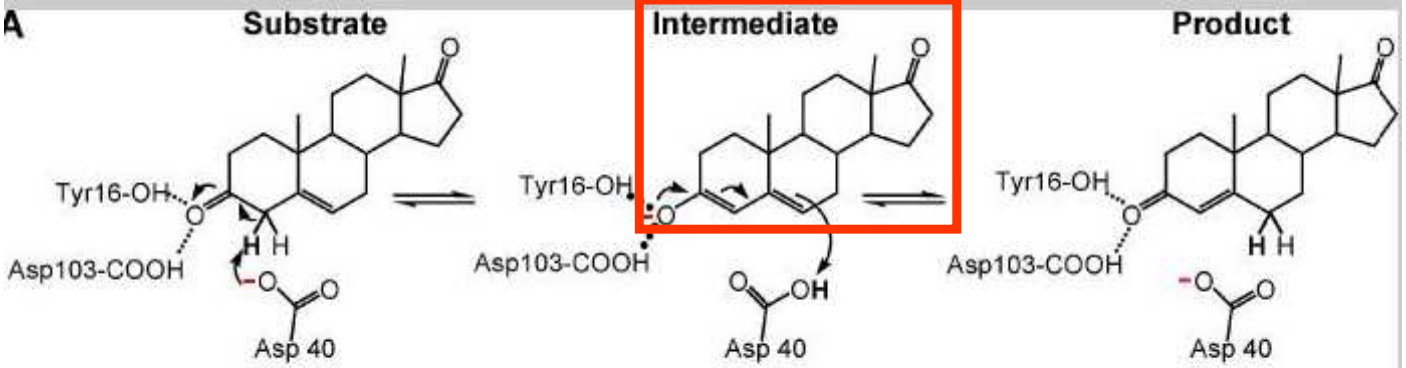
Cholesterol

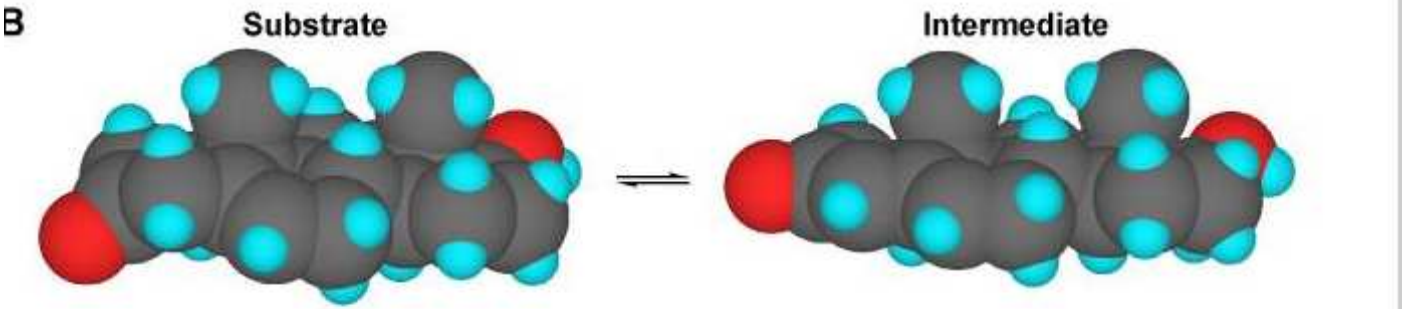
3-Oxo-Δ^5-steroid Isomerase (KSI)

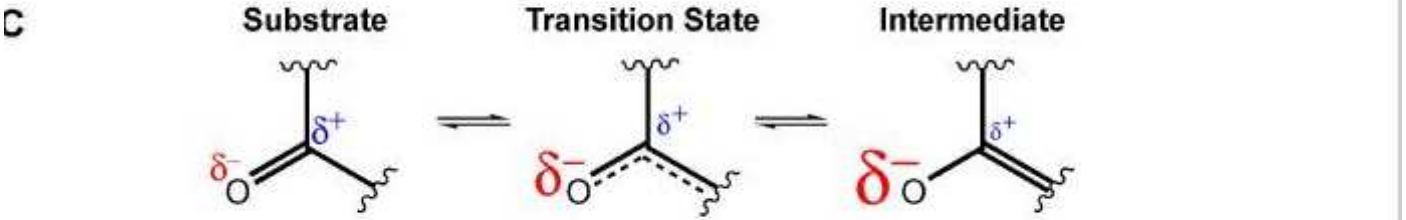
La réaction catalysée par la KSI



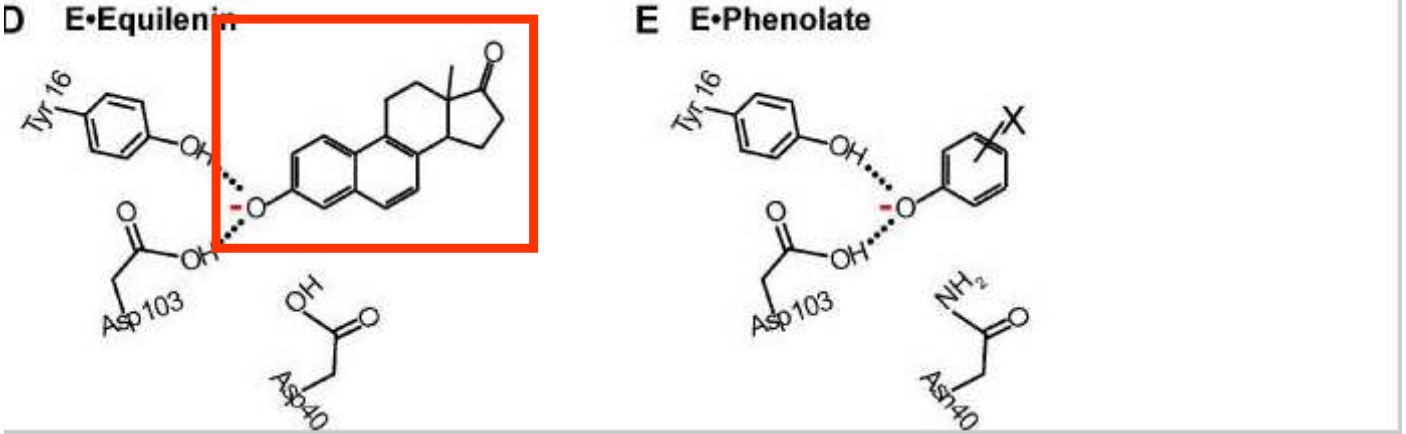
Chimie Q1: Keq; Q2: propriétés des H en position 4





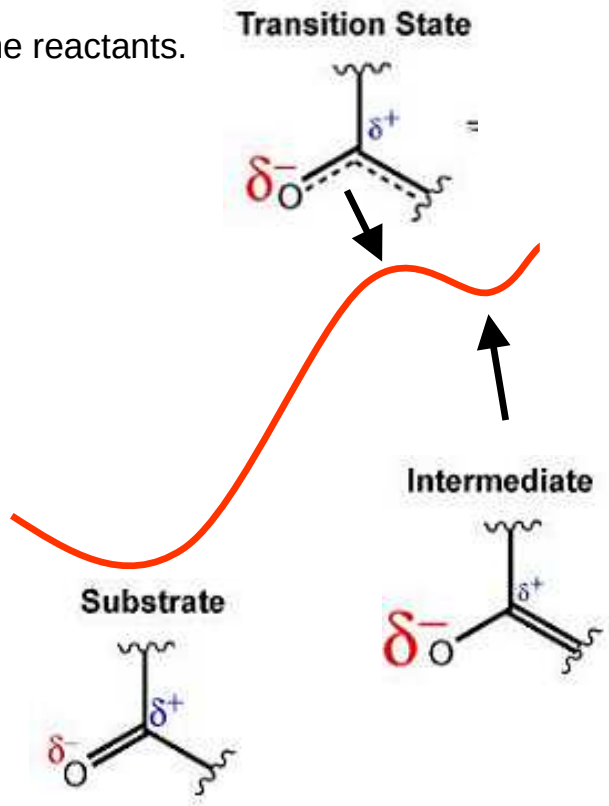


Analogue d'intermédiaire



A discussion of the transition state and of the free energy plots

- An important discussion on the transition state structure: The HAMMOND postulate (1955)
- Related species that are similar in energy are also similar in structure. The structure of a transition state resembles the structure of the closest stable species.
- Transition state structure for endothermic reactions resemble the product.
- Transition state structure for exothermic reactions resemble the reactants.



Séquence: http://www.ncbi.nlm.nih.gov/protein/?term=ksi

Propriétés de la KSI: http://expasy.org/tools/protparam.html

```
        10         20         30         40         50         60
MNLPTAQEVQ GLMARYIELV DVGDIEAIVQ MYADDATVEN PFGQPPIHGR EQIAAFYRQG

        70         80         90        100        110        120
LGGGKVRACL TGPVRASHNG CGAMPFRVEM VWNGQPCALD VIDVMRFDEH GRIQTMQAYW

       130
SEVNLSVREP Q
```

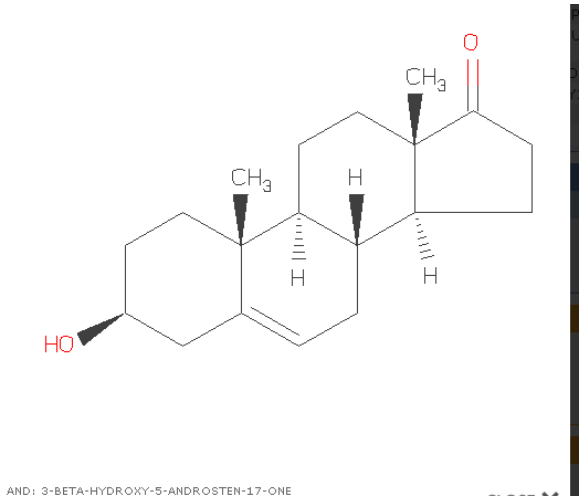
Ala (A)	12	9.2%
Arg (R)	9	6.9%
Asn (N)	5	3.8%
Asp (D)	7	5.3%
Cys (C)	3	2.3%
Gln (Q)	10	7.6%
Glu (E)	9	6.9%
Gly (G)	13	9.9%
His (H)	3	2.3%
Ile (I)	7	5.3%
Leu (L)	7	5.3%
Lys (K)	1	0.8%
Met (M)	7	5.3%
Phe (F)	4	3.1%
Pro (P)	8	6.1%
Ser (S)	3	2.3%
Thr (T)	4	3.1%
Trp (W)	2	1.5%
Tyr (Y)	4	3.1%
Val (V)	13	9.9%

Number of amino acids: 131

Molecular weight: 14534.5

Theoretical pI: 4.90

27 structures de la KSI: http://www.pdb.org/pdb/home/home.do



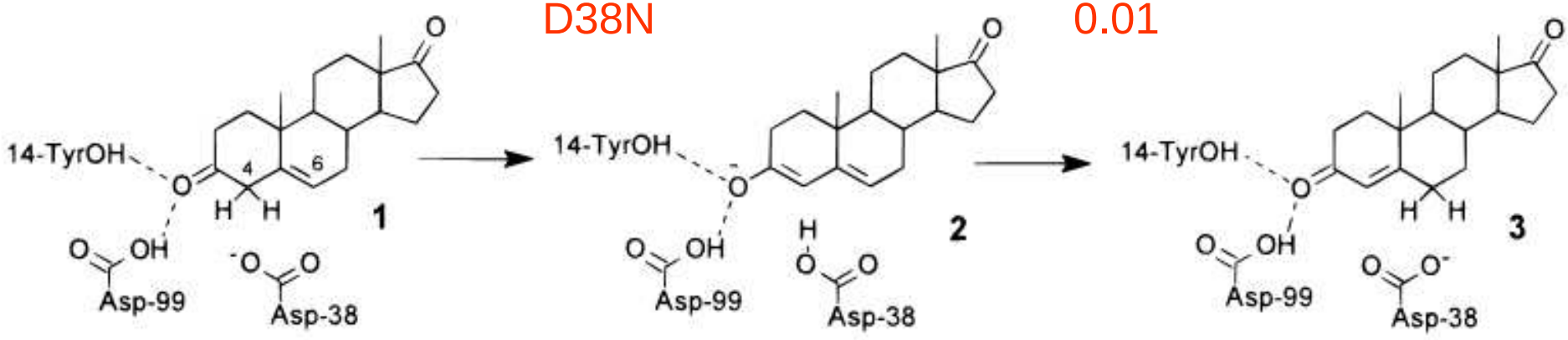
1E3R

Abstract

3-Oxo-Δ^5-steroid isomerase (KSI) from *Comamonas (Pseudomonas) testosteroni* catalyzes the isomerization of β,γ-unsaturated 3-oxosteroids to their conjugated isomers through an intermediate dienolate. Residue Asp-38 (pK_a 4.57) acts as a base to abstract a proton from C-4 of the substrate to form an intermediate dienolate, which is then reprotonated on C-6. Both Tyr-14 (pK_a 11.6) and Asp-99 (p$K_a \geq$ 9.5) function as hydrogen-bond donors to O-3 of the steroid, helping to stabilize the transition states. Mutation of the active-site base Asp-38 to the weakly basic Asn (D38N) has previously been shown to result in a >10^8-fold decrease of catalytic activity. In this work, we describe the preparation and kinetic analysis of the Ala-38 (D38A) mutant. Unexpectedly, D38A has a catalytic turnover number (k_{cat}) that is ca. 10^6-fold greater than the value for D38N and only about 140-fold less than that for wild type. Kinetic studies as a function of pH show that D38A-catalyzed isomerization involves two groups, with pK_a values of 4.2 and 10.4, respectively, in the free enzyme, which are assigned to Asp-99 and either Tyr-14 or Tyr-55. A mechanism for D38A is proposed in which Asp-99 is recruited as the catalytic base, with stabilization of the intermediate dienolate ion and the flanking transition states provided by hydrogen bonding from both Tyr-14 and Tyr-55. This mechanism is supported by the lack of detectable activity of the D38A/D99N, D38A/Y14F, and D38A/Y55F double mutants.

enzyme	K_M(μM)	k_{cat}(s^{-1})	k_{cat}/K_M($s^{-1}M^{-1}$)
WT(D38)	240	57000	2.3×10^8
D38A[e]	122	400	3.0×10^6
D38N		0.01	

Scheme 1



Abbreviations:

BSA, bovine serum albumin;

CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CD, circular dichroism;

CHES, 2-(cyclohexylamino)-1-ethanesulfonic acid;

D38X, mutant of 3-oxo-Δ^5-steroid isomerase with Asp-38 replaced by the residue X;

IPTG, isopropyl β-d-thiogalactopyranoside;

K_E, acid dissociation constant for the free enzyme;

K_{ES}, acidic dissociation constant for the enzyme−substrate complex;

KSI, 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1);

PAGE, polyacrylamide gel electrophoresis;

SDS, sodium dodecyl sulfate;

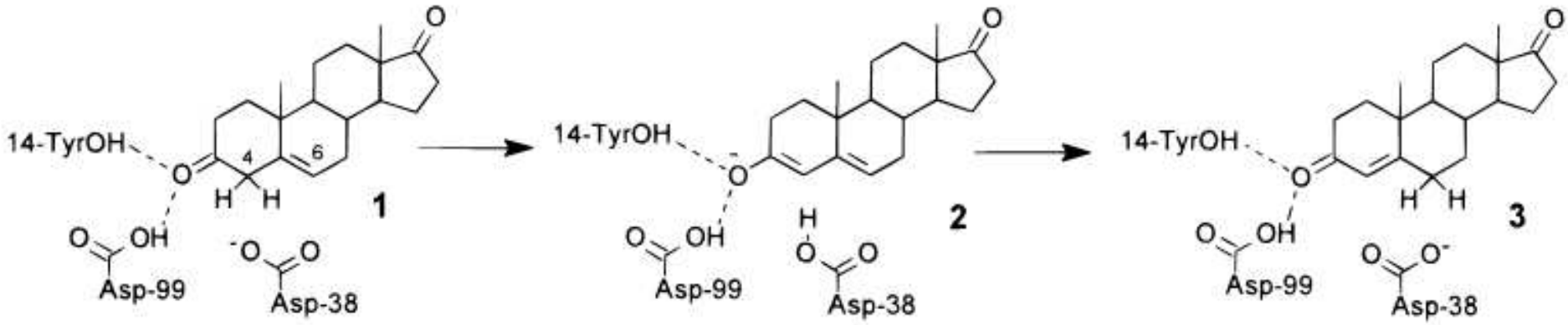
Tris, tris(hydroxymethyl)aminomethane;

WT, wild type.

Introduction

3-Oxo-Δ^5-steroid isomerase (Δ^5-3-ketosteroid isomerase, KSI,[1] EC 5.3.3.1) from *Comamonas testosteroni* (formerly known as *Pseudomonas testosteroni*) catalyzes the isomerization of β,γ-unsaturated 3-oxosteroids to their α,β-conjugated isomers at a rate that approaches the diffusion limit with specific substrates (*1*). The reaction proceeds through the formation of an intermediate dienol or dienolate ion by abstraction of a proton from C-4 by Asp-38 (*2*−*4*), with the majority of the evidence favoring a dienolate rather than a dienol (*5*−*7*). Although for a long time it was thought that electrophilic assistance is provided solely by Tyr-14 (*8, 9*), the recent determination of the solution structure revealed the existence of an additional polar group (Asp-99) at the active site (*10*). Mutation of this residue causes significant rate reductions in the D99A (ca. 3000-fold) and D99N (ca. 27-fold) mutants at pH 7.0 . These results can most simply be accounted for by the formation of hydrogen bonds directly to the dienolate oxygen by both Tyr-14 and Asp-99 (Scheme 1). Support for the mechanism of Scheme 1 is provided by the 2.26 Å X-ray structure of KSI with the intermediate analogue equilenin bound in the active site (*13*). This structure shows that the phenolic oxygen of Tyr-14 and one of the carboxyl group oxygens of Asp-99 are within hydrogen-bonding distance of O-3 of the steroid. In addition, as predicted by Scheme 1, the effects of mutations of Tyr-14 to Phe (Y14F) and Asp-99 to Ala (D99A) on both k_{cat} and k_{cat}/K_M are additive in the Y14F/D99A mutant

Scheme 1



Consistent with the involvement of Asp-38 as the base, mutation of this residue to the less basic asparagine produces an enzyme (D38N) that is 10^8–10^9-fold less active than the wild-type enzyme toward the specific substrate 5-androstene-3,17-dione (**1**) (*2, 15*).[2] For this mutant, the reaction involves formation of a tightly bound intermediate dienolate (**2**), which is then converted much more slowly to the product (**3**). We now report that replacement of Asp-38 by the nonbasic alanine (D38A) results in a much smaller decrease in activity than replacement by asparagine. Surprisingly, D38A is ca. 10^6-fold more active than D38N and only ca. 140-fold less active than wild type (WT), suggesting the involvement of a new mechanism that is unavailable to D38N. These results are interpreted in terms of a new binding mode of the steroid in the catalytic site, which allows Asp-99 to be recruited as the catalytic base.

enzyme	K_M(μM)	k_{cat}(s^{-1})	k_{cat}/K_M($s^{-1}M^{-1}$)
WT(D38)	240	57000	2.3×10^8
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D38N		0.01	

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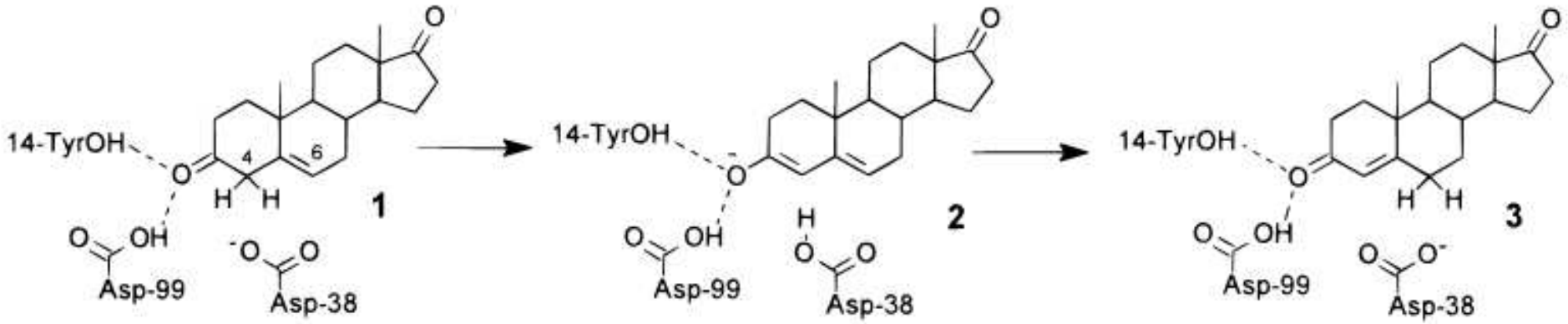


Mutagenesis and Expression Plasmids for D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N. The D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N mutants of KSI were prepared by use of the QuikChange site-directed mutagenesis kit (Stratagene) and a thermocycler (Powerbloc System, Ericomp). To create each expression plasmid, a double-strand plasmid vector, either $pKSI_{tac}$ (carrying the WT KSI gene, *17*), pLDT41 (carrying the D99N KSI gene, *11*), or $pKFH_{D38A}$ (carrying the D38A gene) was used as a template with a set of two primers designed to introduce the mutation in the KSI gene (Table 1). Recombinant plasmids were transformed into Epicurian Coli XL1-Blue supercompetent cells (Stratagene), and purified from the transformants by using the Wizard Plus miniprep DNA purification system (Promega). The complete sequences of the genes were determined by the Biopolymer Laboratory at the University of Maryland, Baltimore.

Experimental data were fit to equations with FigP (Biosoft), a least-squares program based on the Marquardt algorithm, with appropriate weighting.

Table 1: Plasmids and Primers Used in This Study for Mutagenesis

set of primers[a]	mutation(s) in KSI gene
5′GGAACCCACGGGGGCTTCCACCGTGG3′	D38A/D99N
5′CCACGGTGGAAGCCCCCGTGGGTTCC3′	
5′GGAACCCACGGGGGCTTCCACCGTGG3′	D38A
5′CCACGGTGGAAGCCCCCGTGGGTTCC3′	
5′GCCGTGGTACAACGCTTTGTGGCTGCGCG3′	D38A/Y14F
5′CGCGCAGCCACAAAGCGTTGTACCACGGC3′	
5′CGATTTCGTGAGTTTTTCGCCAACTCGCTC3′	D38A/Y55F
5′GAGCGAGTTGGCGAAAAACTCACGAATCG3′	

Protein Expression and Purification. The D38A and D38A/D99N proteins were expressed in Epicurian Coli XL1-Blue cells by inoculation of 6 L of 2× TY-IA medium (16 g of bactotryptone, 10 g of yeast extract, and 5 g of NaCl per liter, supplemented with 1 mM IPTG and 100 μg/mL ampicillin) with 300 mL (D38A) or 45 mL (D38A/D99N) of late-log-phase culture. Incubation was performed for 43 h at 30 °C (D38A) or 24 h at 37 °C (D38A/D99N) with constant shaking. The D38A/Y14F and D38A/Y55F proteins were expressed in Epicurian Coli XL1-Blue cells by inoculation of 2 L of 2× TY-IA medium with 10 mL of late-log-phase culture. Incubation was performed for 24 h at 37 °C. Cells were harvested by centrifugation for 30 min at 5000*g* (4 °C) and dissolved in ice-cold 50 mM Tris-HCl buffer, pH 7.5. The preparation of the cell extracts and the purification of the protein were performed according to published procedures (*2, 4*). One liter of stationary phase culture yielded 15 mg of the D38A protein, 9.6 mg of the D38A/Y14F protein, 16 mg of the D38A/Y55F protein, or 35 mg of the D38A/D99N protein. Polyacrylamide gel electrophoresis was carried out on a Bio-Rad Mini-Protean II apparatus, and the purity of the protein was determined by discontinuous SDS−PAGE on 15% gels with the buffer system of Laemmli (*18*).

Purification de la KSI

The soluble fraction was mixed with ethanol to make a 50% ethanol solution and incubated on ice for 30 min. Precipitate was removed by centrifugation, and the supernatant solution was then adjusted to 1 mM EDTA, 80% ethanol, and 20 mM β-mercaptoethanol. The solution was allowed to stand at 4°C overnight, during which time a precipitate containing the isomerase formed and settled out. The precipitate of this solution was collected by centrifugation and extracted with a buffer containing 0.4 M KP_i (pH 7.0), 1 mM EDTA, and 20 mM β-mercaptoethanol. The resulting extract was directly applied onto an affinity column of deoxycholate-ethylenediamine-Sepharose CL-6B equilibrated with 0.4 M KP_i, pH 7.0. The affinity column was prepared by coupling deoxycholate to ethylenediamine-linked carbonyl diimidazole-activated Sepharose CL-6B as described previously (11, 12). The enzyme was eluted with a buffer containing 1 mM KP_i (pH 7.0), 25% ethanol, 1 mM EDTA, and 20 mM β-mercaptoethanol after the column was washed with more than 20 column volumes of 0.4 M and 1 mM KP_i, pH 7.0, sequentially. Fractions containing KSI as determined by assaying for KSI activity were pooled. Contamination by traces of higher-molecular-weight material was removed by eluting KSI with 1 mM KP_i, pH 7.0, on a Superose 12 column with a high-performance liquid chromatography system (Model System Prep; Pharmacia).

TABLE 1. Purification of KSI from *E. coli* BL21(DE3)/pKK-KSI

Procedure	Total amt of KSI (U)	Yield (%)	Protein concn (mg/ml)	Sp act (U/mg of protein)	Purification factor (fold)
Crude lysate	515,200	100	5.34	4,824	1
Ethanol precipitation	267,540	51.9	1.48	9,039	1.9
Affinity chromatography	212,892	41.3	1.54	34,560	7.2
Superose 12 chromatography	115,920	22.5	2.08	39,807	8.3

4. Zawrotny, M. E., Hawkinson, D. C., Blotny, G., and Pollack, R. M. (1996) *Biochemistry 35*, 6438−6442.

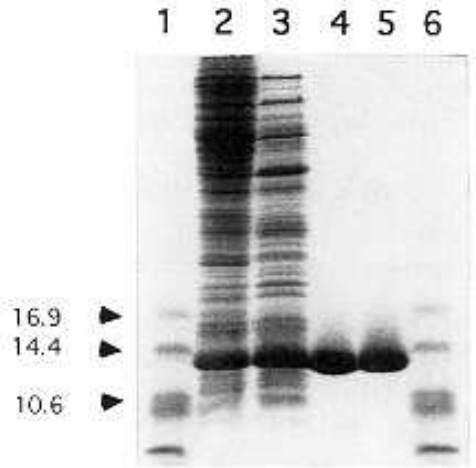
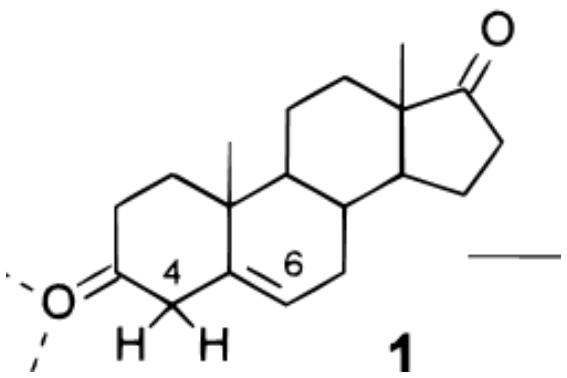


FIG. 4. Polyacrylamide gel electrophoresis of KSI during the purification procedure. The active fraction from each purification step was run on a continuous-gradient polyacrylamide gel (10 to 20%) containing 0.1% SDS and stained with Coomassie blue R250. Lanes: 1 and 6, molecular weight standards (10^3); 2, crude lysate; 3, ethanol precipitation; 4, affinity chromatography; 5, Superose 12 chromatography.

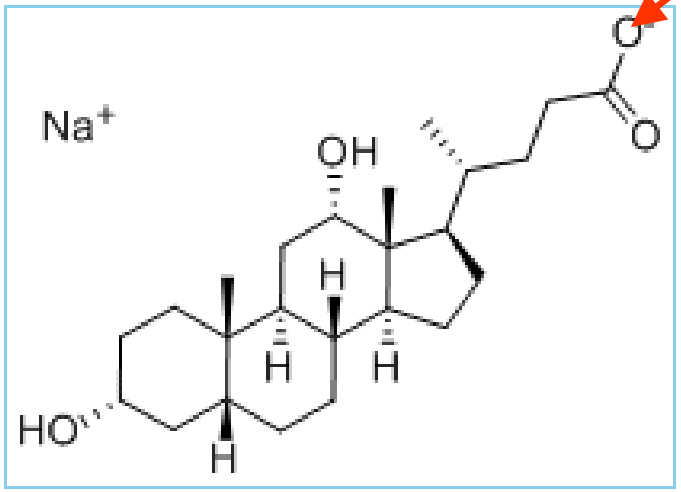
2 3 4 5

Purification par chromatographie d'affinité

Couplage à la résine



1



DOC

Q. Mesure de la concentration en protéine

Results

Mutagenesis and Protein Expression. The D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N mutants of KSI were prepared using the QuikChange site-directed mutagenesis kit. The entire D38A, D38A/Y55F, and D38A/D99N genes were sequenced and shown to contain only the desired mutations; the D38A/Y14F gene also contained an additional silent mutation in the codon for Gln-12 (CAA). The four proteins were expressed in *Escherichia coli* (XL1-Blue), and purified to homogeneity, as determined by SDS−PAGE stained with Coomassie Blue R-250. The UV spectra of both D38A and D38A/D99N show the characteristic “hand” shape observed for native KSI, with a principal maximum at 277 nm and a minimum at 250 nm (2), whereas the UV spectra of D38A/Y14F and D38A/Y55F are similar to those previously observed for Y14F and Y55F, respectively (2).

1.The **far-UV CD** spectra of D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N are identical to those of WT and D99N and show a minimum at 222 nm. *(structure secondaire)* **Il est important d’être sur que les mutants ayant une faible activité spécifique sont natifs**

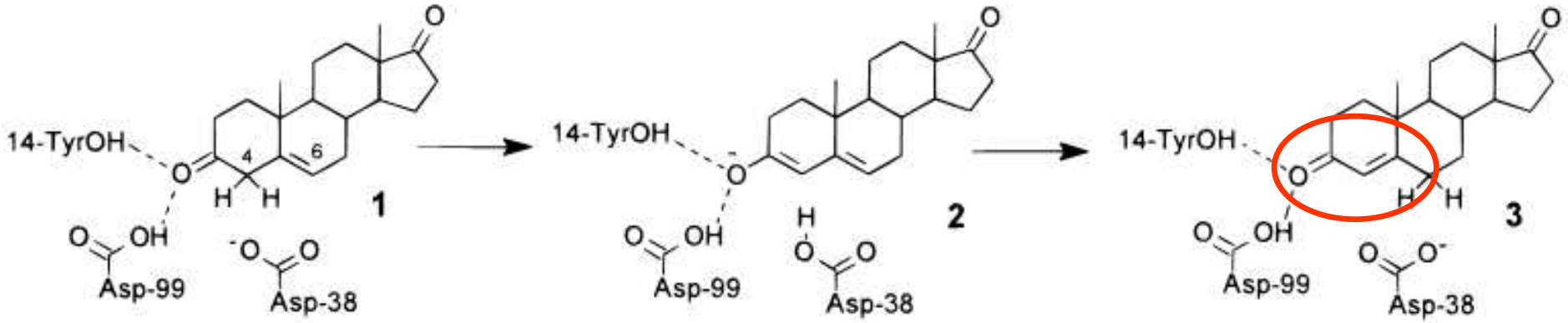
2. Etre sur qu’il n’y a pas de l’enzyme sauvage dans la préparation de mutant faiblement actif

Q: Autres analyses pour démontrer que les mutants sont natifs?

*Kinetics of the Isomerization of 5-Androstene-3,17-dione (**1**) and 5(10)-Estrene-3,17-dione (**4**).*

UV spectra and kinetic data were acquired on a Gilford Response I, Response II, or Cary 1 Bio spectrophotometer equipped with thermostated sample blocks. Solutions of **1** (10−120 μM) or **4** (10−180 μM) in 3.10 mL of 34 mM potassium phosphate (3.3% methanol, pH 7.0) were incubated at 25.0 °C ± 0.1. Stock solutions of KSI were prepared by diluting concentrated KSI into solutions of 0.25% BSA in 10 mM potassium phosphate buffer, pH 7.0, and isomerization was initiated by adding the appropriate diluted KSI solution. **Initial rates** for 5% conversion of **1** and **4** to their conjugated isomers were determined from the **change** of the absorbance at 248 nm (ε = 16300 M^{-1} cm^{-1}; *19*).

Scheme 1

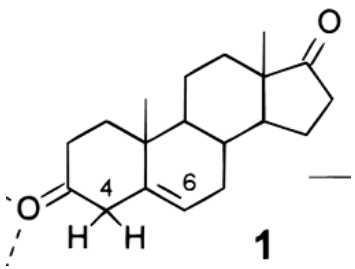


Q1. L'intermédiaire 2 participe-t-il à la Δabs?
Q2. Le substrat se fixe-t-il à la BSA?

Kinetic Parameters.

Specific activities for WT (55000 μmol min^{-1} mg^{-1}) and D38A (600 μmol min^{-1} mg^{-1}) were determined in 34 mM potassium phosphate buffer at pH 7.0, with 5-androstene-3,17-dione (**1**) as substrate. The D38A mutant possesses considerable activity toward **1** (ca. 1% of WT), and this activity is independent of the phosphate buffer concentration (10 or 34 mM). The double mutants D38A/Y55F, D38A/Y14F, and D38A/D99N have no detectable catalytic activity, even when 3 μM enzyme is used in the assay. From this result, an upper limit of 2.0×10^{-4} μmol min^{-1} mg^{-1} can be calculated for the specific activity of these mutants, ca. (3.0×10^{8})-fold less than WT. Moreover, under assay conditions with D38A/D99N concentrations comparable to those of the substrate **1**, there is no spectral evidence for the formation of an enzyme-bound dienol(ate) intermediates, whereas under the same conditions the D38N mutant catalyzes the conversion of **1** to a tightly bound dienolate.

As with WT and the other Asp-38 mutants (D38X), D38A is able to catalyze the isomerization of both **1** and **4**. Kinetic parameters for the isomerization of **1** and of **4** were determined in 34 mM phosphate buffer (pH 7.0) at 25.0 ± 0.1 °C. Linear kinetics were observed through the first 5−10% of the reactions, and the first 2−3% of the reactions were used to determine initial rates. The values of k_{cat} and k_{cat}/K_M for isomerization of **1** are 460 ± 50 s^{-1} and 2.8 (± 0.2) × 10^{6} M^{-1} s^{-1},

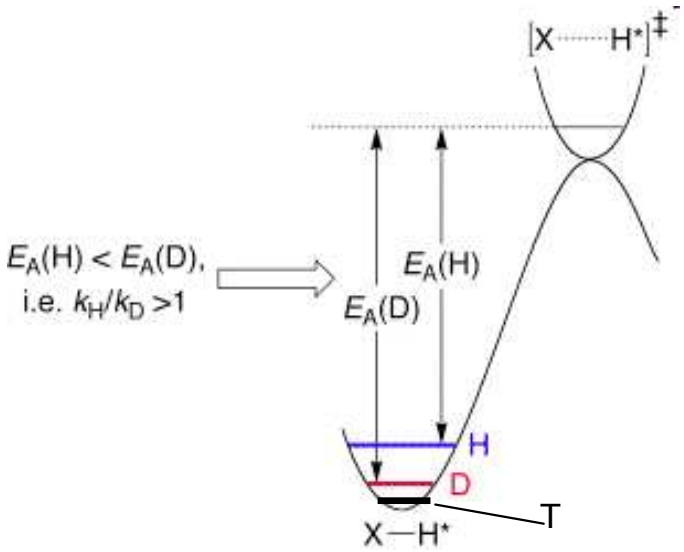


1

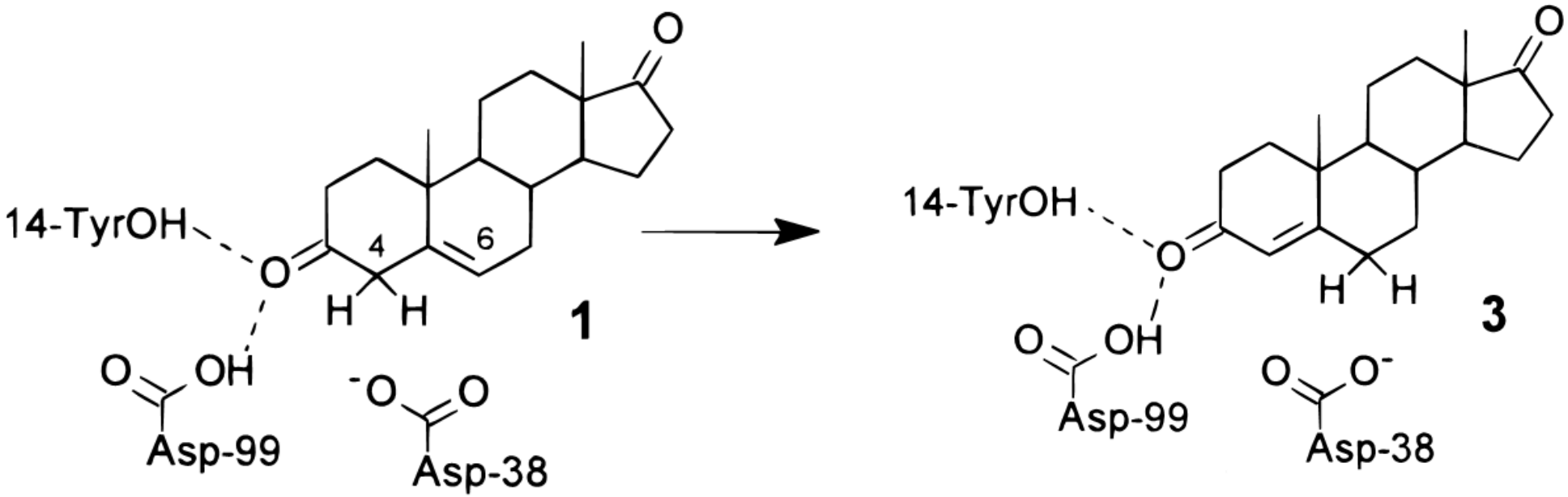
Effets isotopiques (pour information)

La liaison C-D est plus forte que C-H

La réaction est plus lente si la liaison C-D est coupée dans l'étape limitante de vitesse



Rates of isomerization of **1** by D38A were obtained in both H_2O and D_2O at pH (L = H or D) 6.5, 7.5, and 8.5 in 10 mM phosphate buffer at 25.0 ± 0.1 °C (Table 2). The solvent isotope effect on the kinetic parameters of D38A is independent of the pL between 6.5 and 8.5, with $^{H/D}(k_{cat}/K_M)$ = 1.3 ± 0.2, $^{H/D}(k_{cat})$ = 1.5 ± 0.1, and $^{H/D}(K_M)$ = 1.2 ± 0.2. These values are similar to those previously observed with WT at pH 7.5 (24).



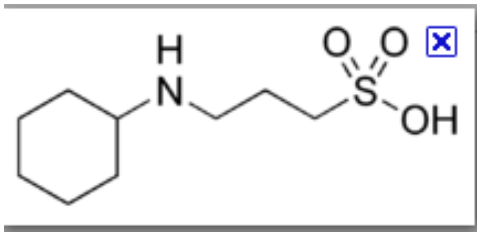
pH−Rate Profiles. The pH dependence of the kinetic constants for isomerization of **1** by D38A was determined at 25.0 ± 0.1 °C at constant ionic strength (μ = 0.1 M) adjusted with KCl, in

10 mM citrate (pH 3.5−5.0),

10 mM acetate (pH 4.5−6.0);

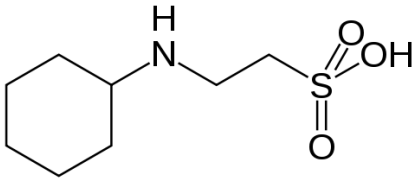
10 mM phosphate (pH 6.0−8.5),

5 mM CHES (pH 8.0−10.0), ***N*-cyclohexyl-3-aminopropanesulfonic acid**,



5 mM CAPS (pH 10.0−11.0) buffers.

***N*-Cyclohexyl-2-aminoethanesulfonic acid**,



Tampons de GOOD (voir http://en.wikipedia.org/wiki/Good%27s_buffers)

Q1: Pourquoi plusieurs tampons?

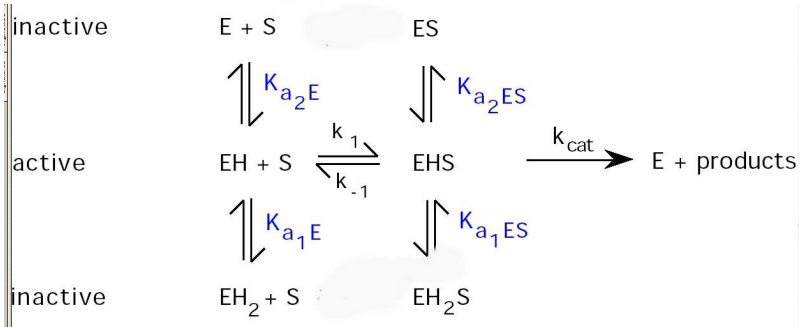
The stability of the D38A mutant was checked at all pH values by incubating the enzyme in the assay buffer at 25.0 ± 0.1 °C for 1 min and then initiating the reaction by addition of substrate. The observed rate was compared to the rate obtained with addition of substrate before enzyme. Specific activities were assayed under standard conditions (*21*) before and after the experiments, and in all cases at least 95% of the activity was retained.

The pH dependence of k_{cat} and k_{cat}/K_M for the isomerization of **1** by D38A was determined at 25.0 ± 0.1 °C and constant ionic strength (μ = 0.1 M) from weighted least-squares analysis of plots of 1/*v* vs 1/[**1**] at each pH. Over the pH range investigated (3.5 to 11.0), the enzyme is stable for the time required to make the rate measurements (<1 min). Since k_{cat}/K_M for D38A is at least an order of magnitude lower than k_{cat}/K_M for WT, which reacts at nearly the diffusion-controlled rate with **1**, it can be assumed that **1** is not a sticky substrate for D38A. Thus, plots of $(k_{cat}/K_M)^{obs}$ vs pH and $(k_{cat})^{obs}$ vs pH should provide pK_a values for the free enzyme (pK_E) and the enzyme−substrate complex (pK_{ES}), respectively (*25, 26*). The observed kinetic parameters were fit to eqs 3 and 4 (*27*) to give the kinetic constants and the pK_a values, which are given in Table 3, along with values determined previously for WT and other D38X mutants.

$$(k_{cat}/K_M)^{obs} = (k_{cat}/K_M)/\{1 + ([H^+]/K_{E1}) + (K_{E2}/[H^+])\} \quad (3)$$

$$(k_{cat})^{obs} = k_{cat}/(1 + [H^+]/K_{ES1}) \quad (4)$$

(voir le TD sur la Ribonucléase A)



Q: K_{ES2} n'apparaît pas dans l'expression de kcat. Votre hypothèse

The limiting values for D38A of k_{cat} (400 ± 20 s^{-1}) and k_{cat}/K_M [3.0 (± 0.2) × 10^6 M^{-1} s^{-1}] represent a decrease relative to WT by ca. 140-fold and 75-fold, respectively. The log (k_{cat}/K_M) vs pH profile (Figure 1) shows a limiting slope of +1 on the acidic side with an apparent pK_{E1} value of 4.2 ± 0.1, and a limiting slope of −1 on the basic side with an apparent pK_{E2} value of 10.4 ± 0.1. The log (k_{cat}) vs pH curve (Figure 1) exhibits dependence upon a single pK_a (pK_{ES1} 4.5 ± 0.1) at low pH.

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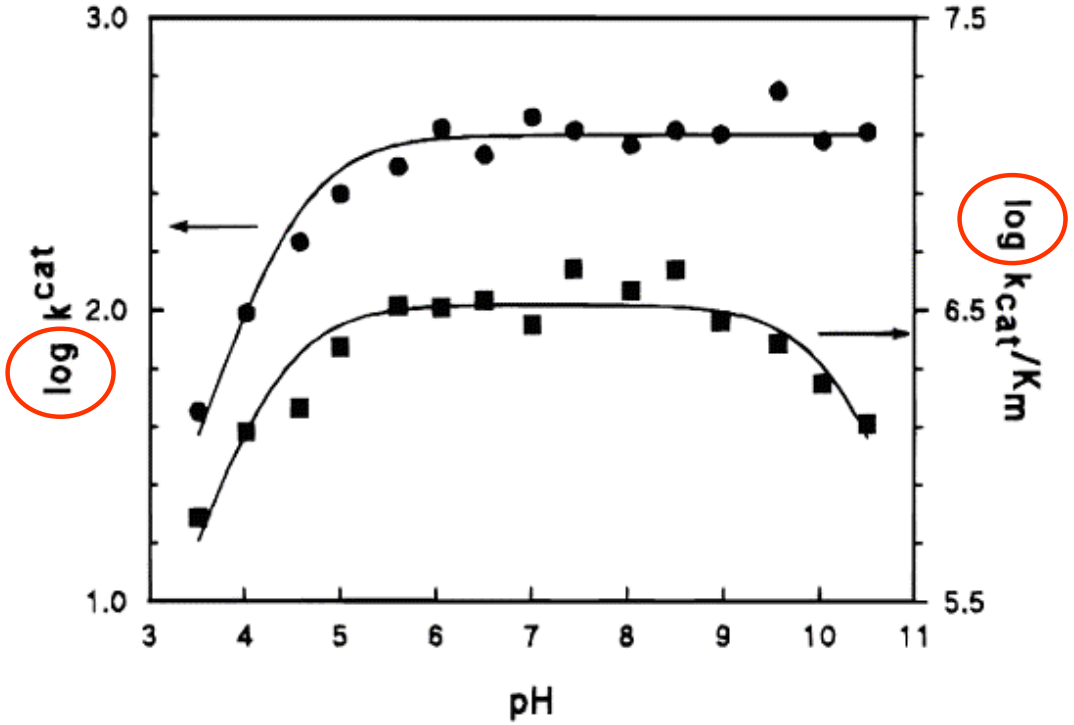
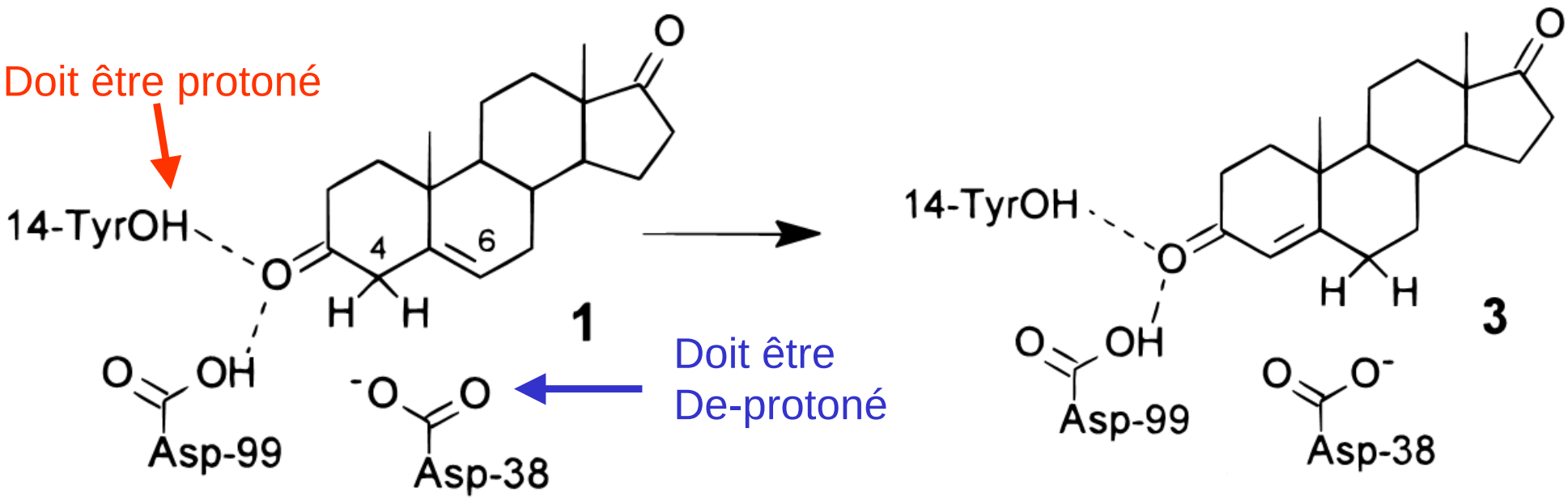
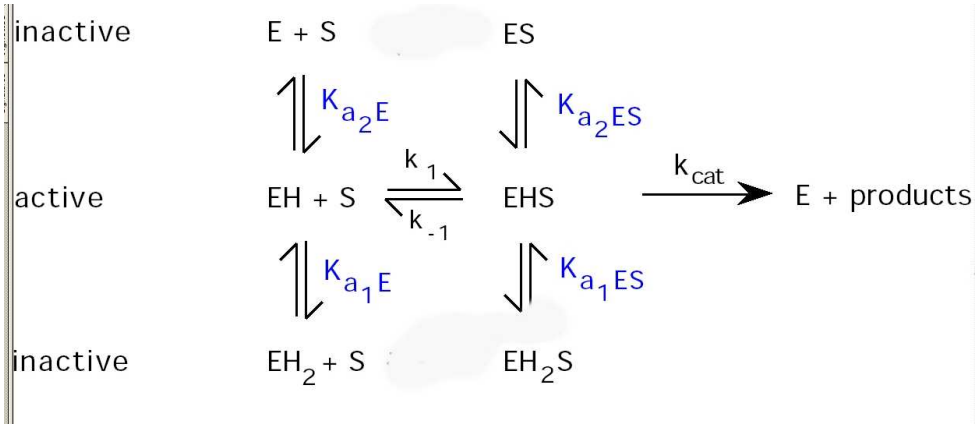


Figure 1 Plots of log $(k_{cat})^{obs}$ (·) and log $(k_{cat}/K_M)^{obs}$ (▪) for the isomerization of 5-androstene-3,17-dione (**1**) catalyzed by D38A as a function of pH at 25 °C. The curves are theoretical, based on eqs 3 and 4 and the parameters given in Table 3.



$$(k_{cat}/K_M)^{obs} = (k_{cat}/K_M)/\{1 + ([H^+]/K_{E1}) + (K_{E2}/[H^+])\} \quad (3)$$

$$(k_{cat})^{obs} = k_{cat}/(1 + [H^+]/K_{ES1}) \quad (4)$$



The pH dependence of k_{cat} and k_{cat}/K_M for the isomerization of **1** by D38A was determined at 25.0 ± 0.1 °C and constant ionic strength (μ = 0.1 M) from weighted least-squares analysis of plots of 1/*v* vs 1/[**1**] at each pH. Over the pH range investigated (3.5 to 11.0), the enzyme is stable for the time required to make the rate measurements (<1 min). Since k_{cat}/K_M for D38A is at least an order of magnitude lower than k_{cat}/K_M for WT, which reacts at nearly the diffusion-controlled rate with **1**, it can be assumed that **1** is not a sticky substrate for D38A. Thus, plots of $(k_{cat}/K_M)^{obs}$ vs pH and $(k_{cat})^{obs}$ vs pH should provide pK_a values for the free enzyme (pK_E) and the enzyme−substrate complex (pK_{ES}), respectively (*25, 26*). The observed kinetic parameters were fit to eqs 3 and 4 (*27*) to give the kinetic constants and the pK_a values, which are given in Table 3, along with values determined previously for WT and other D38X mutants.

Table 3: Kinetic Constants for the Isomerization of 5-Androstene-3,17-dione (**1**) to 4-Androstene-3,17-dione (**3**) by WT and D38X Mutant KSIs

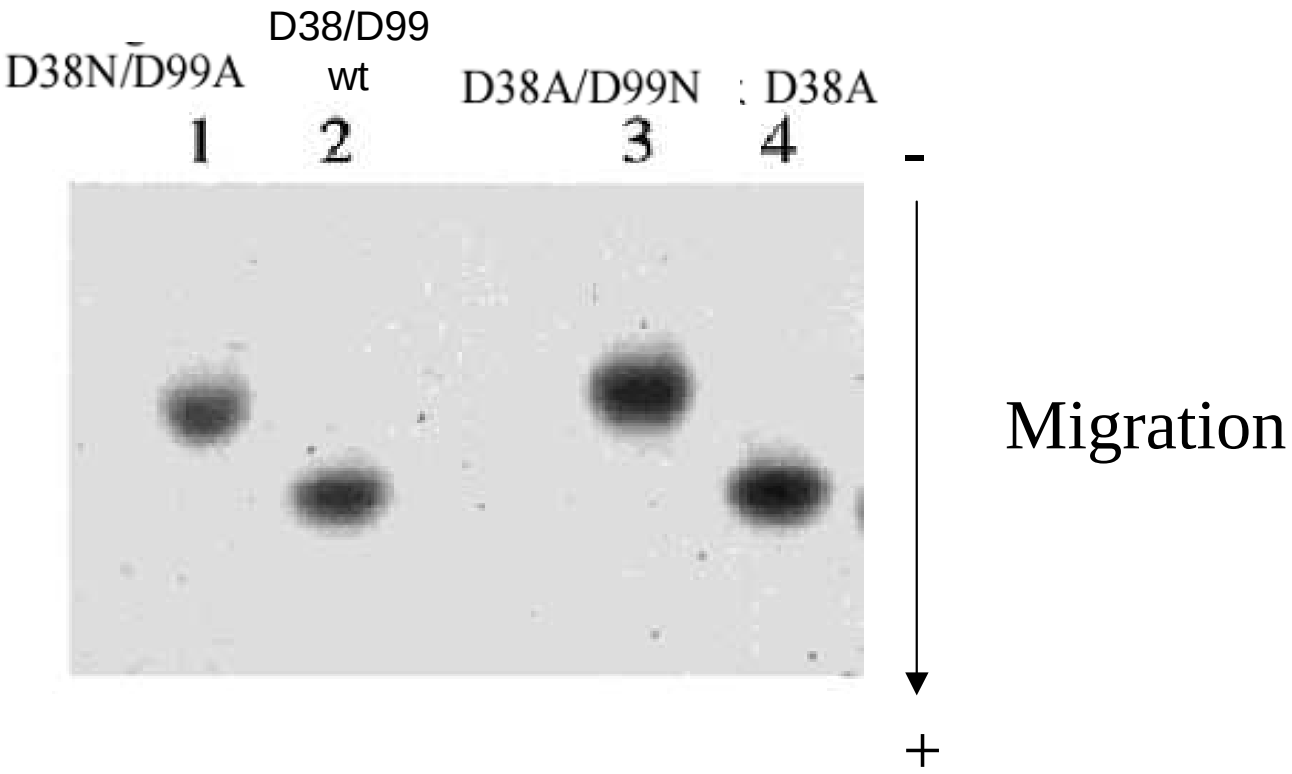
enzyme	K_M(μM)	k_{cat}(s^{-1})	k_{cat}/K_M($s^{-1}M^{-1}$)	pK_{E1}	pK_{E2}	pK_{ES1}	pK_{ES2}
WT(D38)	240	57000	2.3×10^8	4.57[b]	>9[c]	4.75[b]	9.3[c]
D38E[d]	73	210	2.4×10^6	4.65	≥9.5	6.13	8.83
D38A[e]	122	400	3.0×10^6	4.2±0.1	10.4	4.5±0.1	≥10.5
D38N		0.01					

Q1: Mutant D38E (activité faible, 1% du sauvage)

Q2: Mutant D38N (pas d'activité, <0.0001%)

Q3: Mutant D38A (activité 1% du sauvage)

Native Gel Electrophoresis. The relative charges of WT and mutants lacking Asp-38 and/or Asp-99 were determined by native gel electrophoresis at pH 7.0 (Figure 2) and 8.2. At both pH values, D38A migrates similarly to WT, whereas the D38A/D99N double mutant migrates with D38N/D99A.



La vitesse de migration est fonction de la taille et de la charge en électrophorèse en l'absence de SDS

UV Titration of D38A. UV spectra of D38A were recorded between 240 and 320 nm from pH 4.0 to 11.0, a pH range in which the protein is stable. An increased pH has two major effects on the spectrum of D38A, which are similar to those observed during the UV-monitored pH titration of WT and D38H (*23*). The absorbance at 240 nm increases strongly, and a red shift of 2 nm is observed for peaks between 240 and 320 nm (Figure 3). These changes in the spectra are reversible; after acidification of a solution of pH 10.0 to either pH 6.0 or 7.0, the spectrum does not differ from the spectrum at pH 7.0. The change of absorbance at 295 nm, indicative of tyrosine ionization (ε = 2540 M^{-1} cm^{-1}; *28*), shows sigmoidal behavior as a function of pH (Figure 4). A pK_a value of 10.0 ± 0.4 was obtained after the data were fitted to eq 1.

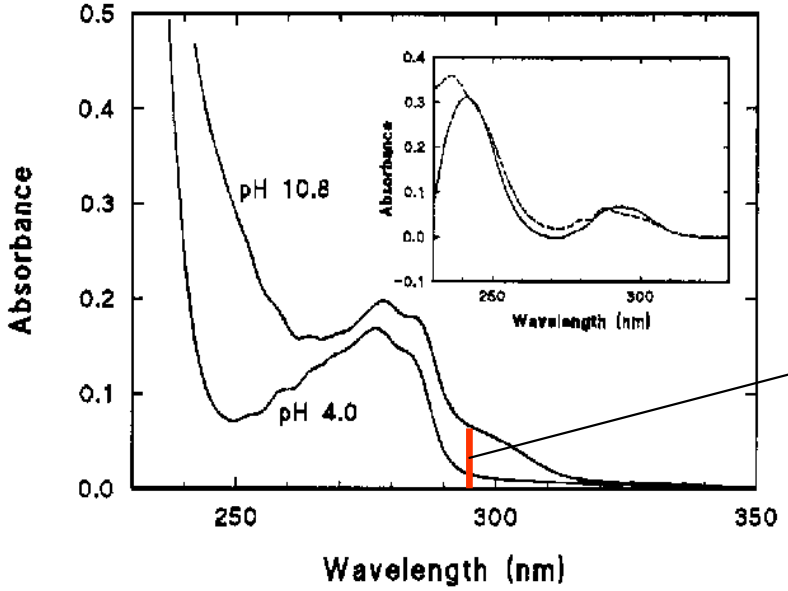


FIGURE 3: Absorbance spectra of D38A (38 μM) at pH 4.0 (10 mM citrate) and 10.8 (10 mM CAPS). Inset: Dashed line, difference spectra of D38A at pH 10.8 and pH 4.0); solid line, difference spectra of tyrosine at pH 10.8 and pH 4.0. Both concentrations are 38 μM.

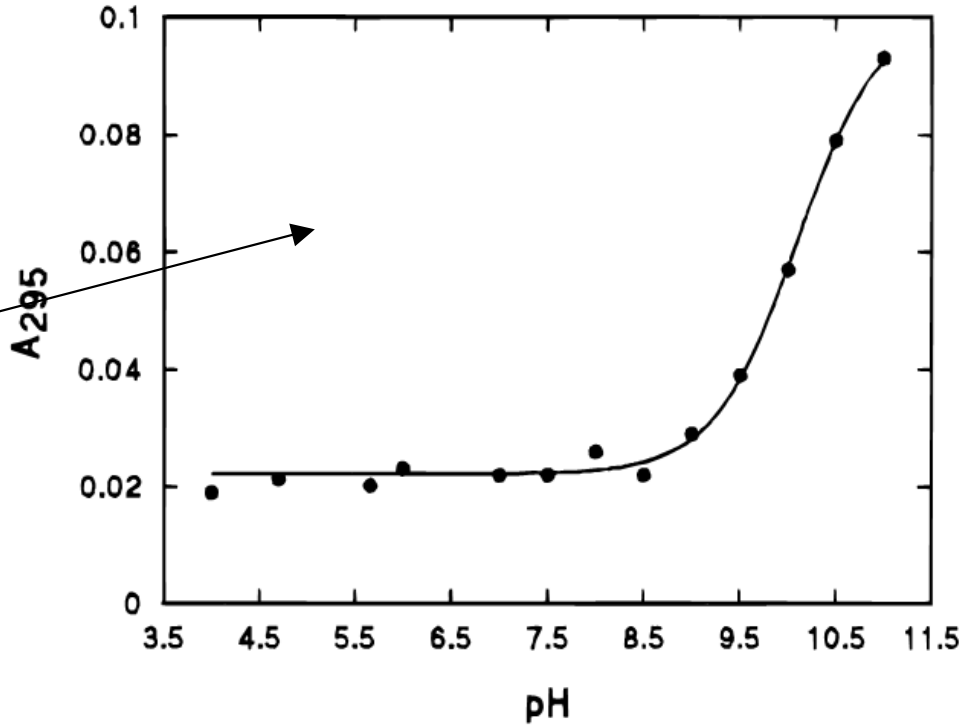


FIGURE 4: Absorbance of D38A (38.5 μM) at 295 nm as a function of pH. The data were fit to eq 1 and give a pK_E value of 10.0 ± 0.4.

UV Titration of D38A. Ultraviolet spectra of D38A (38.5 μM) were acquired as a function of pH in 1.0 cm quartz cuvettes at 25.0 ± 0.1 °C from 240 to 320 nm, with a scan speed of 100 nm/min. The buffers (μ = 0.1 M, adjusted with KCl) were 10 mM citrate (pH 4.0−5.0); 10 mM acetate (pH 5.5−6.0); 10 mM phosphate (pH 7.0−8.5); 10 mM CHES (pH 9.0−10.0), and 10 mM CAPS (pH 10.5−11.0). The pK_a value was obtained by fitting the observed values of the absorbance at 295 nm (A) as a function of pH using eq 1, where a_H is the activity of hydronium ion, A_i is the absorbance of the solution at a_H = 0, A_f is the absorbance of the solution at a_H = infinity, and K_E is the acid dissociation constant.

$$A = (A_f K_E + A_i a_H)/(K_E + a_H)$$

$$A = A_i a_H)/(K_E + a_H)$$

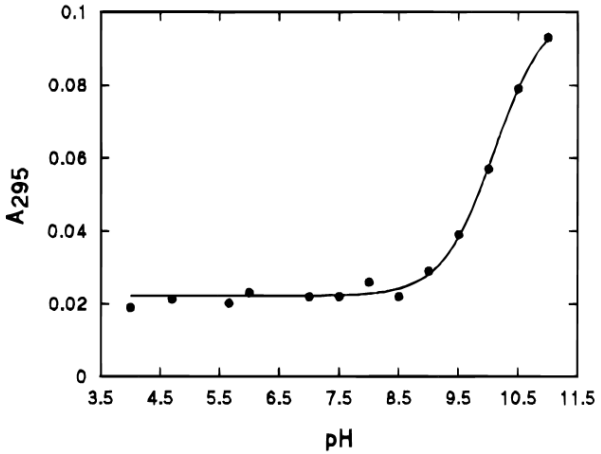


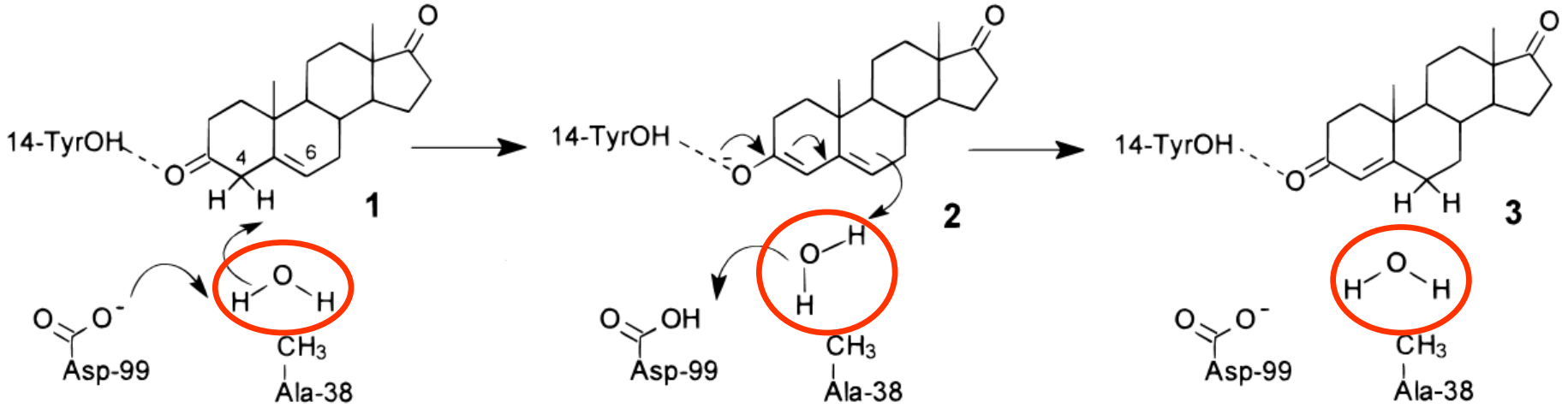
FIGURE 4: Absorbance of D38A (38.5 μM) at 295 nm as a function of pH. The data were fit to eq 1 and give a pK_E value of 10.0 ± 0.4.

L'équation est plus simple si on considère Af=0
(vous avez fait ça avec TP Kaleidagraph)

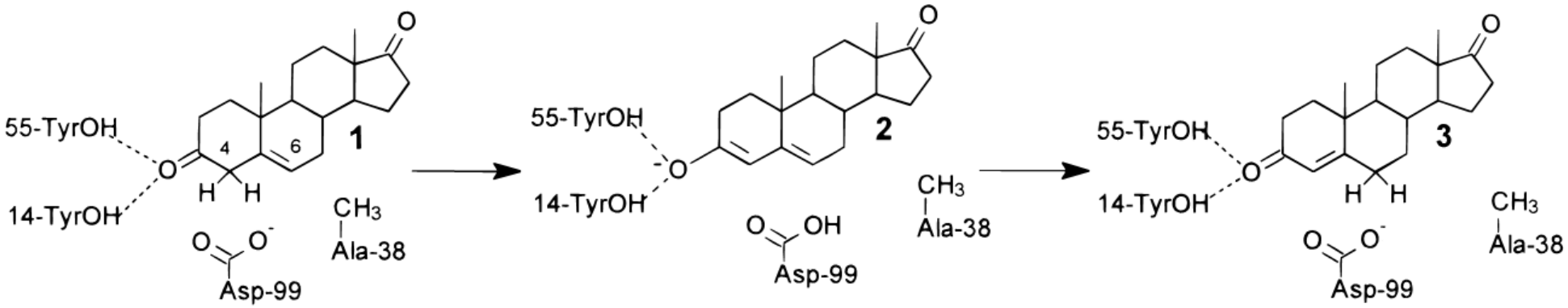
Conclusion:

L'Asp99 participe directement à la catalyse dans le mutant Asp38Ala, mais pas dans le mutant Asp38Asn

Scheme 2



Scheme 3



Intérêt général: flexibilité du site actif de la KSI; probablement une caractéristique générale des sites actifs des enzymes

The possible involvement of Asp-99 in catalysis was investigated by introducing a second mutation (Asn-99) into D38A that eliminates the carboxyl group of Asp-99 (D38A/D99N). Asn preserves the side chain volume of Asp but does not ionize at neutral pH. If Asp-99 is the catalytic base in D38A, then the D38A/D99N mutant should show little activity. However, if Asp-99 is not ionized in D38A and acts as a hydrogen-bond donor, as in the wild type, a much more modest decrease in k_{cat} should be observed. Thus, k_{cat} for D99N is decreased by a factor of about 25-fold relative to WT, reflecting the weaker ability of Asn to donate a hydrogen-bond compared to a carboxylic group. The lack of detectable activity for the D38A/D99N double mutant, corresponding to a decrease in k_{cat} of $\geq 10^6$-fold relative to D38A, is consistent with Asp-99 being the catalytic base in the D38A mutant, rather than a hydrogen-bond donor.

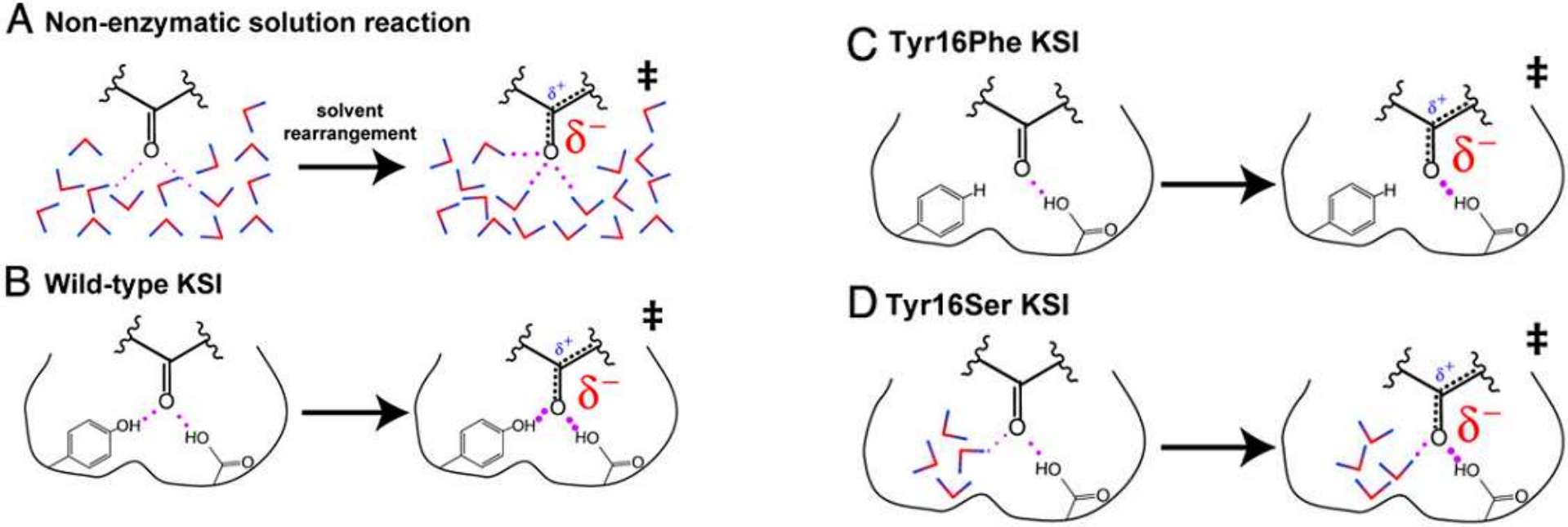


Fig. 5. Schematic models for the effects of Tyr16 mutations on KSI catalysis. Shorter hydrogen bonds are depicted with thicker dots. (*A*) Solution nonenzymatic reaction with water molecules shown as colored dipoles that reorient to solvate localized charge in the dienolate-like transition state. (*B*) The wild-type oxyanion hole has two positioned enzyme groups that donate hydrogen bonds to the reacting substrate that may strengthen in the transition state. (*C*) The Tyr16Phe mutation ablates one of the oxyanion hole hydrogen bonds but does not permit water entry, resulting in a hydrophobic surface that desolvates the localized negative charge in the dienolate-like transition state. (*D*) The Tyr16Ser mutation replaces Tyr16 with a water-filled cavity that provides aqueous-like solvation of the reacting substrate.