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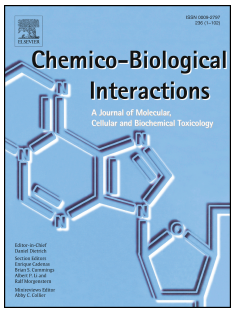

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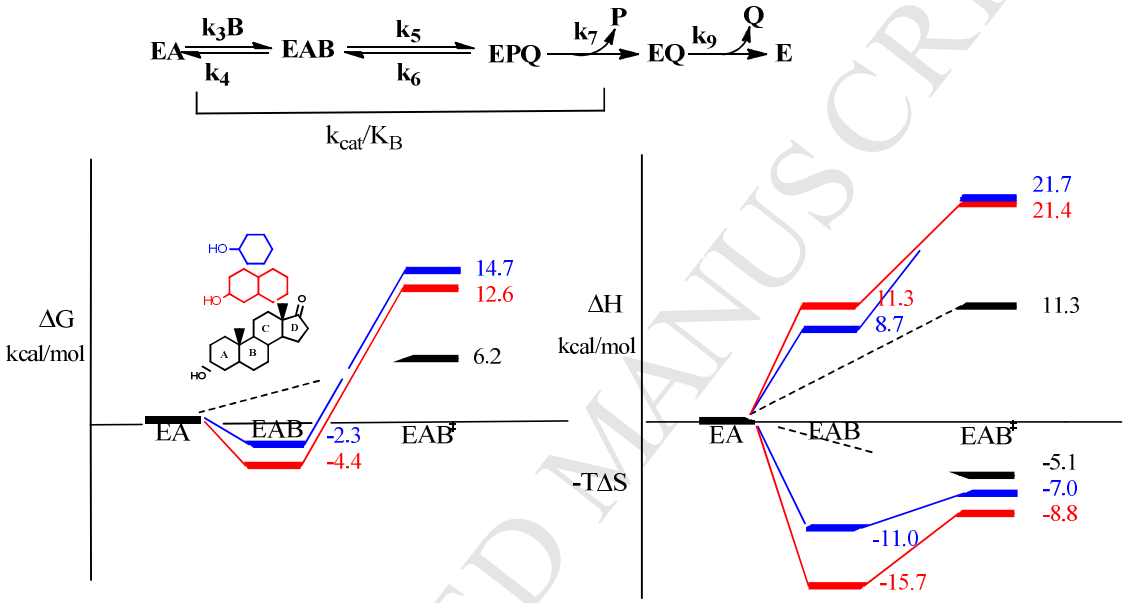
Graphical abstract- **Thermodynamic analysis of remote substrate binding interactions in 3α-hydroxysteroid dehydrogenase/carbonyl reductase catalysis**



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Thermodynamic analysis of remote substrate binding energy in 3α-hydroxysteroid dehydrogenase/carbonyl reductase catalysis

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Running Title: Enthalpic and entropic contributions from remote binding energy to enzyme catalysis

Abstract

The binding energy of enzyme and substrate is used to lower the activation energy for the catalytic reaction. 3α-HSD/CR uses remote binding interactions to accelerate the reaction of androsterone with NAD^+. Here, we examine the enthalpic and entropic components of the remote binding energy in the 3α-HSD/CR-catalyzed reaction of NAD^+ with androsterone versus the substrate analogs, 2-decalol and cyclohexanol, by analyzing the temperature-dependent kinetic parameters through steady-state kinetics. The effects of temperature on k_{cat}/K_m for 3α-HSD/CR acting on androsterone, 2-decalol, and cyclohexanol show the reactions are entropically favorable but enthalpically unfavorable. Thermodynamic analysis from the temperature-dependent values of K_m and k_{cat} shows the binding of the E-NAD^+ complex with either 2-decalol or cyclohexanol to form the ternary complex is endothermic and entropy-driven, and the subsequent conversion to the transition state is both enthalpically and entropically unfavorable. Hence, solvation entropy may play an important role in the binding process through both the desolvation of the solute molecules and the release of bound water molecules from the active site into bulk solvent. As compared to the thermodynamic parameters of 3α-HSD/CR acting on cyclohexanol, the hydrophobic interaction of the B-ring of steroids with the active site of 3α-HSD/CR contributes to catalysis by increasing exclusively the entropy of activation ($\Delta T\Delta S^{\ddagger}$ = 1.8 kcal/mol), while the BCD-ring of androsterone significantly lowers $\Delta\Delta H^{\ddagger}$ by 10.4 kcal/mol with a slight entropic penalty of -1.9 kcal/mol. Therefore, the remote non-reacting sites of androsterone may induce a conformational change of the substrate binding loop with an entropic cost for better interaction with the transition state to decrease the enthalpy of activation, significantly increasing catalytic efficiency.

Abbreviations: 3α-HSD/CR, 3α-hydroxysteroid dehydrogenase/carbonyl reductase; 2-decalol, decahydro-2-naphthanol; SDR, short chain dehydrogenase/reductase; ΔG, the Gibbs free energy change; $\Delta G^{\ddagger}$, the Gibbs free energy of activation; ΔH, the enthalpy change; $\Delta H^{\ddagger}$, the enthalpy change of activation; ΔS, the entropy change; $\Delta H^{\ddagger}$, the entropy change of activation;

Keywords: Enzyme catalysis, binding energy, steady-state kinetics, Gibbs free energy change, enthalpy and entropy

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1. Introduction

The mechanism of catalysis with high substrate specificity of enzymes attracts great interest of biochemists (1-3). Enzymes catalyze the reaction by binding the substrates to form an enzyme-substrate complex, interacting with the transition-state, and forming and releasing the product. The binding energy of enzyme and substrate is used for substrate destabilization and transition state stabilization to lower the activation energy and facilitate catalysis (4,5). Jencks proposed a Circe effect for enzyme catalysis in which the binding energy is used for the distortion, electrostatic interactions, desolvation, and entropy loss for bringing the substrates in the proper position for reaction and for better binding to the transition state (6).

The binding interactions between enzyme and substrate, both at the site of chemical transformation and with a non-reacting portion of the substrate, have been shown to contribute to catalysis (6-8). As compared to the catalysis with a truncated substrate analog, enzymes can use binding interactions with a non-reacting portion of the substrate to destabilize the ground state and stabilize the transition state (6), assist in binding and enhance the effective local concentration (1), provide substantial contributions to specific transition state stabilization (6,9-11), induce a conformational change, and promote significant changes in dynamics for residues located remotely from the binding site, thereby accelerating the chemical reactions (12-17). Remote binding interactions in 3-oxoacid CoA transferase, ketosteroid isomerase, triose phosphate isomerase, and orotidine 5-monophosphate decarboxylase have been demonstrated to provide substantial contributions to stabilize the transition state (9-11,18).

We have demonstrated the contributions of remote binding energy in the 3α-

HSD/CR-catalyzed reaction of NAD^+ with androsterone as compared to that for the truncated analog (Figure 1)(19). The steroid rings are involved in the 3α-HSD/CR catalysis. The B-ring of steroid improves catalysis (k_{cat}/K_B) without increasing k_{cat} by equally stabilizing both the transition state and ground state of the ternary complex by 2.2 kcal/mol. The remote BCD- and CD-rings of androsterone improve catalysis on both k_{cat} and k_{cat}/K_B through differential binding interactions with the active site of 3α-HSD/CR by contributing 8.5 and 6.4 kcal/mol to the stabilization of the transition state, respectively.

To obtain a clearer understanding of the factors involved in the energetics, the enthalpy and entropy changes that accompany the formation of an enzyme-substrate complex in both the ground state and transition state have been studied. Comparing the value of k_{cat} of the enzymatic reaction with the corresponding non-enzymatic reaction (k_{non}) shows that enzyme catalysis may arise mainly by reducing the enthalpy of activation (20,21). However, the actions of cytidine deaminase on 5,6-dihydrocytidine and GTP hydrolysis by EF-Tu on the ribosome have indicated that the reactions are entropy-driven (22-25). To better understand enthalpic and entropic contributions to enzyme catalysis, we compare an enzymatic reaction with a good substrate to the same reaction with its truncated analog, thereby enabling the determination of the function of remote binding interactions in catalysis. In this study, we determine the thermodynamic parameters for the reaction of androsterone with NAD^+ catalyzed by 3α-HSD/CR. Moreover, we compare these parameters to the thermodynamic parameters for the reaction with truncated analogs, 2-decalol and cyclohexanol. We analyze the kinetic parameters of 3α-HSD/CR-catalyzed reaction with varied temperatures, giving the thermodynamics parameters for the changes in enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) in the

transition state and the changes in enthalpy (ΔH) and entropy (ΔS) in the ground state for the binding interactions of enzyme and substrate. These energetic values provide valuable insight into how remote interactions of the active site with substrate contribute to catalysis.

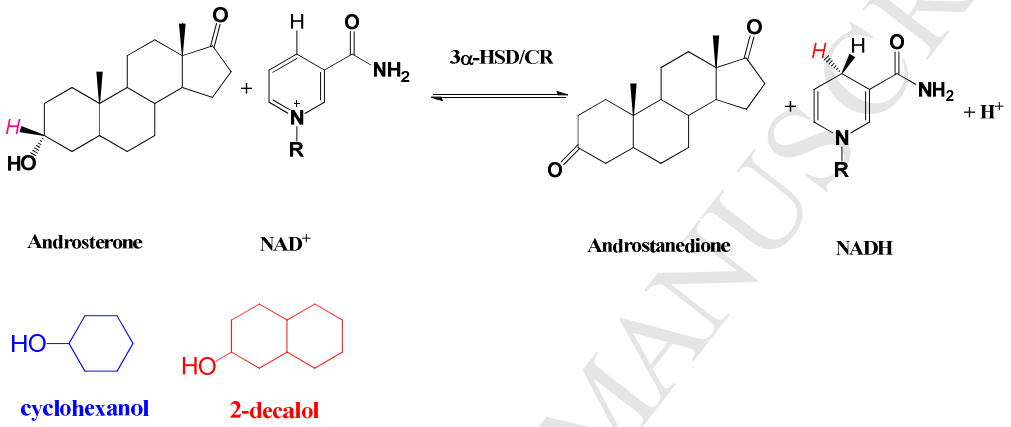

Figure 1. 3α-HSD/CR-catalyzed oxidoreduction of NAD^+ with androsterone and truncated analogs, cyclohexanol and 2-decalol.

2. Materials and methods

2.1. *Materials*

Androsterone came from Steraloids, while cyclohexanol, racemic 2-decalol, NAD^+, and equine alcohol dehydrogenase came from Sigma-Aldrich. All reagents were of the highest purity available.

2.2. *Overexpression and purification of 3α-HSD/CR*

3α-HSD/CR was expressed in *Escherichia coli* as described (19). In brief, the BL21 (DE3) cells were cultivated in 1 L LB medium at 37 °C. The recombinant 3α-HSD/CR was overexpressed by adding 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) when the culture reached an optical density at 600 nm of 0.6-1. After an additional 4 h for growth, the cells were harvested and lysed by sonication for 5 min with 10 s on and 10 s off cycles on ice. The cell debris was removed by centrifugation, and the supernatant was loaded onto a Ni^{2+}-NTA resin column (GE Healthcare Life Sciences). The enzyme was eluted via a stepwise increase in the concentration of imidazole from 10 mM to 500 mM to purify the protein. 3α-HSD/CR was purified to homogeneity, as determined using 12% SDS-PAGE. The protein concentration was determined by Bradford assay with bovine serum albumin as a standard.

2.3. *Steady-state kinetics*

The oxidation of androsterone and its truncated analogs catalyzed by 3α-HSD/CRs was monitored by the formation of NADH spectrophotometrically at 340 nm (ε = 6220 M^{-1} cm^{-1}), using a Perkin Elmer Lambda 650 UV-vis spectrophotometer equipped with a temperature-controlled Peltier block multicell changer. Assays were performed across a suitable range of temperatures of 10-40 °C for 3α-HSD/CR-catalyzed reaction of NAD^+ with androsterone, 2-decalol and cyclohexanol, respectively. The initial velocity patterns were obtained by varying the concentration of androsterone or the truncated analog at several fixed concentrations of NAD^+ in 0.1 mg/mL bovine serum albumin (BSA), 0.1 M sodium 3-(cyclohexylamino)-1-propane sulfonate (Caps) buffer at

pH 10.5 at varied temperatures. The assay was performed at pH 10.5 for an optimum enzyme-catalyzed reaction (26). Due to the difference in activity for the truncated analogs, different amounts of 3α-HSD/CR were added for assays. Typically, the concentrations of 3α-HSD/CR for the assays of androsterone, 2-decalol, and cyclohexanol were 0.085 nM, 85 nM, and 34 nM, respectively. All reactions were initiated by adding enzyme. The initial velocity patterns were determined twice for 3α-HSD/CR acting on androsterone at each temperature, and representative data are shown. Other experiments were done once.

2.4. *Data fitting and calculation of the thermodynamic parameters*

Data from the initial rate measurements were fitted using Sigmaplot software with appropriate rate equations to obtain the kinetic parameters. Data for substrate saturation curves were fitted using Eq. 1. In Eq. 1, v and V represent the initial and maximum velocities and K_A the Michaelis constant for substrate A. Data for a sequential or a rapid equilibrium ordered mechanism were fitted to Eqs. 2 and 3, respectively, where A and B are the varied substrates, K_{iA} is the inhibition constant for A, and K_B is the Michaelis constants for substrate B, respectively. The standard errors associated with these kinetic parameters of V, K_{iA}, K_A, K_B, V/K_A, and V/K_B were calculated from the program. Double reciprocal plots of the families of lines obtained from the initial velocities against substrate concentrations were constructed for illustration.

Based on the transition state theory for chemical and enzyme-catalyzed reactions (27-29), the rate constant k for a reaction as a function of the temperature T is given by

Eq. 4, where R is the gas constant, T is the temperature in Kelvin, and $\Delta G^{\ddagger}$ is the Gibbs free energy of activation, *i.e.*, the difference in free energy between the transition state and ground state. The factor (k_BT/h) is a frequency factor for crossing the transition state, where k_B and h are the Boltzmann and Planck constants, respectively. The Gibbs free energy change, ΔG, is calculated from the binding constant, K_S, for the substrate using Eq. 5. The relationship between the Gibbs free energy change (ΔG) and the changes in enthalpy (ΔH) and entropy (ΔS) is shown in Eq. 6. Similarly, the Gibbs free energy of activation ($\Delta G^{\ddagger}$) is separated into the enthalpic and entropic terms and shown in Eq. 7. The kinetic analysis of the enzyme-catalyzed reaction with varied temperature giving the thermodynamics parameters of the Gibbs free energy of activation ($\Delta G^{\ddagger}$) is Eq. 4, which can be derived into Eq. 8. Therefore, the changes in enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) in transition state are calculated with the Eyring equation (Eq. 9) by plotting ln(k/T) versus (1/T). The changes in enthalpy (ΔH) and entropy (ΔS) for the interactions of enzyme and substrate in the ground state are obtained with the van't Hoff equation (Eq.10) by plotting lnK_s versus (1/T).

The kinetic scheme for the ordered bi bi kinetic mechanism with enzyme, E, saturated with A to form EA complex and the rate equations for kinetic parameters, k_{cat}, k_{cat}/K_B and K_B, are illustrated in Scheme 1. EA binds B with rate constant k_3 to form the EAB complex, which reacts to form the EPQ complex with rate constant k_5, followed by the release of the products P and Q with rate constants k_7 and k_9, respectively. k_4 and k_6 are the reverse rate constants, representing the dissociation of B from EAB, and the conversion of EPQ to EAB, respectively. These kinetic parameters are defined based on Cleland's nomenclature (30,31) with the derived microscopic rate constants for the

corresponding kinetic constants based on Scheme 1. The catalytic constant k_{cat} includes the steps from the chemical reaction, which converts the EAB to EPQ to the releases of products of P and Q, and reflects the rate-limiting step in overall reaction. The catalytic efficiency for B, k_{cat}/K_B, includes the steps from the binding of B with the EA complex to the first irreversible step, *i.e.*, the release of the first product P. When the rate limiting step is the chemical reaction, k_5, the kinetic parameters of k_{cat}, and k_{cat}/K_B are reduced to k_5, and k_3k_5/k_4, respectively, while K_B is reduced to k_4/k_3, the dissociation constant for B from EAB.

$$v = VA/(K_A + A) \quad (1)$$

$$v = VAB/(K_{iA}K_B + K_AB + K_BA + AB) \quad (2)$$

$$v = VAB/(K_{iA}K_B + K_BA + AB) \quad (3)$$

$$k = (k_BT/h)\exp(-\Delta G^{\ddagger}/RT) \quad (4)$$

$$\Delta G = -RT\ln(K_S) \quad (5)$$

$$\Delta G = \Delta H - T\Delta S \quad (6)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (7)$$

$$k = (k_BT/h)\exp(\Delta S^{\ddagger}/R)\exp(-\Delta H^{\ddagger}/RT) \quad (8)$$

$$\ln(k/T) = \ln(k_B/h) + \Delta S^{\ddagger}/R - (\Delta H^{\ddagger}/R)(1/T) \quad (9)$$

$$\ln K_s = \Delta S/R - (\Delta H/R)(1/T) \quad (10)$$

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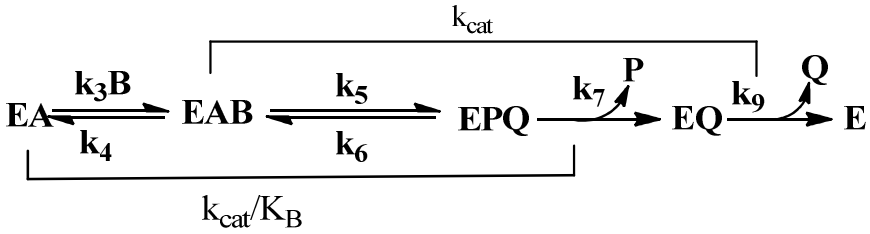

$$k_{cat} = \frac{k_5k_7k_9}{k_5k_7+k_5k_9+k_6k_9+k_7k_9}$$

$$k_{cat}/K_B = \frac{k_3k_5k_7}{k_4k_6+k_4k_7+k_5k_7}$$

$$K_B = \frac{k_9(k_4k_6+k_4k_7+k_5k_7)}{k_3(k_5k_7+k_5k_9+k_6k_9+k_7k_9)}$$

Scheme 1. The simplified ordered bireactant mechanism with enzyme saturated with substrate A and the rate equations for kinetic parameters, k_{cat}, k_{cat}/K_B and K_B.

3. Results

3.1. Temperature dependence of the kinetic parameters for the oxidation of androsterone catalyzed by 3α-HSD/CR

We studied the reaction of androsterone with NAD^+ catalyzed by 3α-HSD/CR at temperatures from 10 to 40 °C at pH 10.5. The pH-profile of the 3α-HSD/CR–catalyzed reactions is insensitive to changing pH at pH > 9 (26), reducing the likelihood of complications that might arise from differing temperature effects on proton dissociation constants. The extinction coefficient of NADH is not changed from 10-40 ° C. To ensure the enzyme activity is not lost during the assay time of 5 min, we incubated the enzyme at 40 °C and measured the enzyme activity at 25 °C in the presence of 1 mM NAD^+, 48.7

μM androsterone, 0.1 mg/mL BSA, 100 mM Caps, pH 10.5, and found that the enzyme maintained activity within error for up to 20 min at 40 °C.

Initial velocity patterns were determined by varying the concentration of androsterone at several fixed concentrations of NAD^+ at each temperature. Double reciprocal plots intersected to the left of the y-axis at each temperature and are presented in the supplementary Figure S1. The data were fitted to Eq. 2 to yield the kinetic parameters of k_{cat}, K_{iA}, K_A and K_B at each temperature (Supplementary Table S1). These kinetic values increase with increasing temperature. The values of k_{cat} and k_{cat}/K_B are (116 ± 9) s^{-1} and (6.8 ± 1.4) $\times 10^7$ $M^{-1}s^{-1}$ at 10 °C, and increase to (2.8 ± 0.3) $\times 10^3$ s^{-1} and (4.5 ± 0.7) $\times 10^8$ $M^{-1}s^{-1}$ at 40 °C, respectively. The values of K_{iA}, K_A and K_B also increase from 0.16 ± 0.10, 0.14 ± 0.03 and 0.0017 ± 0.0004 mM at 10 °C to 1.2 ± 0.4, 1.8 ± 0.4 and 0.0063 ± 0.0017 mM at 40 °C, respectively. The increases in the inhibition constant K_{iA} for NAD^+ with increasing temperature indicate the dissociation of 3α-HSD/CR-NAD^+ complex is an endothermic reaction.

Plots of the $\ln(k_{cat}/K_BT)$ and $\ln(k_{cat}/T)$ versus 1/T are linear for 3α-HSD/CR catalysis from 10 to 40 °C (Figure 2), and the data were fitted to Eq. 9. The slope corresponds to the enthalpy of activation ($\Delta H^{\ddagger}$), giving 11.3 ± 1.9 and 16.0 ± 2.1 kcal/mol, respectively, and the intercept determines the entropy of activation ($T\Delta S^{\ddagger}$), giving 5.1 ± 0.5 and 2.4 ± 0.3 kcal/mol at 298 K, respectively (Table 1).

(A) (B)

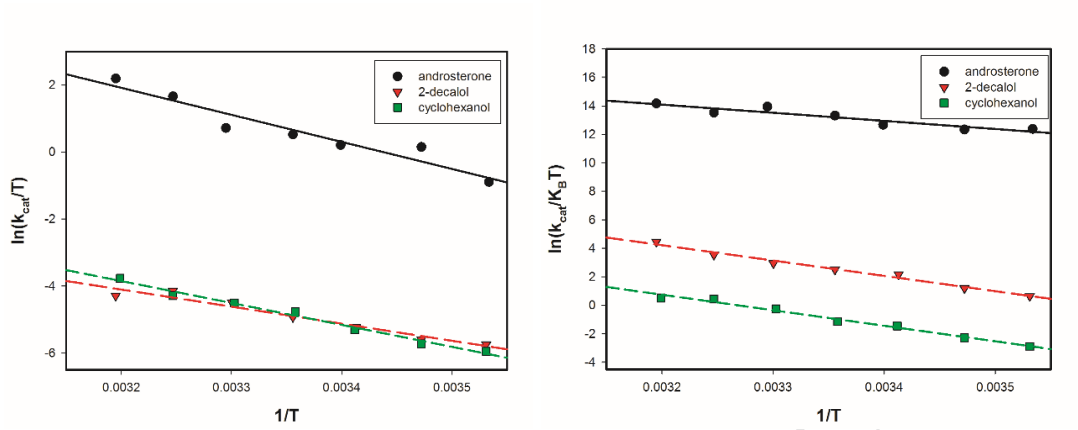

Figure 2. Eyring plots of the kinetic data for 3α-HSD/CR catalyzed reaction of NAD$^+$ with androsterone, 2-decalol and cyclohexanol. Plots of (A) ln(k_{cat}/T) vs 1/T, and (B) ln(k_{cat}/K_BT) vs 1/T are linear. The lines represent the fits of the data to Eq. 9, where "k" is either ln(k_{cat}/T) or ln(k_{cat}/K_BT), to obtain the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ recorded in Table 1.

Table 1. Thermodynamic activation parameters for the oxidation of androsterone and the truncated analogs with NAD catalyzed by 3α-HSD/CR[a,b,c].

Kinetic parameters	k_{cat}			k/K_B		
Substrate	cyclohexanol	2-decalol	androsterone	cyclohexanol	2-decalol	androsterone
$\Delta G^{\ddagger}$	17.0±0.8	16.9±1.4 (0.1)	13.6±2.1	14.7±1.3	12.6±1.3 (2.1)	6.2±2.0 (8.5)
$\Delta H^{\ddagger}$	13.0±0.7	10.1±1.0 (2.9)	16.0±2.1	21.7±1.2	21.4±1.2 (0.3)	11.3±1.9 (10.4)
$T\Delta S^{\ddagger}$	-4.0±0.3	-6.8±1.0 (2.8)	2.4±0.3	7.0±0.4	8.8±0.5 (-1.8)	5.1±0.5 (1.9)

[a]Values of $\Delta G^{\ddagger}$ are calculated from Eq. 7 with the propagated error calculated.
[b]The values in parenthesis are the differences in the thermodynamic activation parameters in the comparison with the value for cyclohexanol.
[c] Values given in kcal/mol at 298 K.

3.2. Temperature dependence of the kinetic parameters for the oxidation of truncated analogs catalyzed by 3α-HSD/CR

The reaction of 2-decalol and cyclohexanol with NAD^+ by 3α-HSD/CR was studied at temperatures from 10 to 40 °C. Double reciprocal plots of the initial velocity patterns for 2-decalol and cyclohexanol intersect on the y-axis at each temperature, indicating a rapid equilibrium ordered kinetic mechanism, as shown in the supplementary Figures S2 and S3 for 2-decalol and cyclohexanol, respectively. The data were fitted to Eq. 3 to yield the kinetic parameters of k_{cat}, K_{iA} and K_B at each temperature (Supplementary Tables S2 and S3). Similarly, the kinetic values of k_{cat} and k_{cat}/K_B increase with increasing temperature, while the K_B values decrease with increasing temperature. The values of k_{cat} for 2-decalol and cyclohexanol are 0.91 ± 0.11 s^{-1} and (0.74 ± 0.07) s^{-1} at 10 °C, and increase to 4.3 ± 0.3 s^{-1} and 7.2 ± 0.4 s^{-1} at 40 °C, respectively. The values of k_{cat}/K_B for 2-decalol and cyclohexanol are 540 ± 60 $M^{-1}s^{-1}$ and 15 ± 2 $M^{-1}s^{-1}$ at 10 °C, and increase to (2.7 ± 0.5) $\times 10^4$ s^{-1} and (5.2 ± 0.9) $\times 10^2$ $M^{-1}s^{-1}$ at 40 °C, respectively. The values of K_B for 2-decalol and cyclohexanol decrease from 1.7 ± 0.4 and 48 ± 9 mM at 10 °C to 0.16 ± 0.04 and 14 ± 3 mM at 40 °C, respectively.

Plots of $\ln(k_{cat}/K_BT)$ and $\ln(k_{cat}/T)$ versus 1/T are linear from 10 to 40 °C for 3α-HSD/CR acting on 2-decalol and cyclohexanol (Figure 2). Data were fitted to Eq. 9. The analysis of 3α-HSD/CR acting on 2-decalol and cyclohexanol shows a much greater sensitivity of k_{cat}/K_B to temperature than the reaction with androsterone, with a $\Delta H^{‡}$ of 21.4 ± 1.2 and 21.7 ± 1.2 kcal/mol, but less sensitivity of k_{cat} to temperature with a $\Delta H^{‡}$ of 10.1 ± 1.0 and 13.0 ± 0.7 kcal/mol, respectively. The entropies of activation ($T\Delta S^{‡}$) for

2-decalol and cyclohexanol at 298 K are 8.8 ± 0.5 and 7.0 ± 0.4 kcal/mol, respectively, obtained from the values of $k_{cat}/K_{B,}$ and -6.8 ± 1.0 and -4.0 ± 0.3 kcal/mol respectively, obtained from the values of k_{cat}. The thermodynamic activation parameters calculated from the observed effects of temperature on the enzymatic oxidation of androsterone, and truncated substrate analogs, 2-decalol and cyclohexanol, are summarized in Table 1.

The changes in enthalpy (ΔH) and entropy (ΔS) for the interactions of enzyme and substrate in the ground state are obtained with the van't Hoff equation (Eq.10) by plotting $\ln(1/K_B)$ vs (1/T) as shown in Figure 3 and Table 2. The binding of E-NAD^+ with 2-decalol or cyclohexanol ($1/K_B$) is endothermic with ΔH values of 11.3 ± 2.1 and 8.7 ± 1.8 kcal/mol, counterbalanced by an extraordinarily favorable entropy (TΔS) of 15.7 ± 2.1 and 11.0 ± 1.8 kcal/mol at 298 K, respectively.

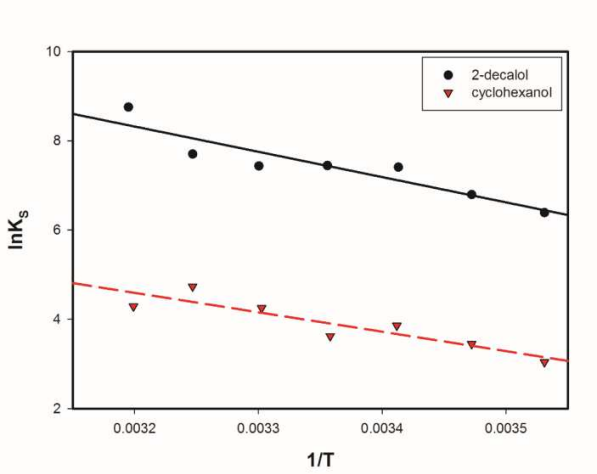

Figure 3. van't Hoff plots of kinetic data for 3α-HSD/CR-catalyzed reaction of NAD^+ with 2-decalol and cyclohexanol. The lines represent the fits of $\ln(K_s)$ to Eq. 10 for 2-decalol and cyclohexanol to obtain ΔH and ΔS given in Table 2. The kinetic parameter K_s is equal to $1/K_B$.

Table 2. Thermodynamic parameters for the binding of 3α-HSD/CR-NAD binary complex with cyclohexanol and 2-decalol to form the respective ternary complexes[a,b,c].

	cyclohexanol	2-decalol
ΔG	-2.3 ± 2.5	-4.4 ± 3.0 (2.1)
ΔH	8.7 ± 1.8	11.3 ± 2.1 (2.6)
TΔS	11.0 ± 1.8	15.7 ± 2.1 (4.7)

[a] Values of ΔG are calculated from Eq. 6 with the propagated error calculated.
[b]The values in parenthesis are the differences between cyclohexanol and 2-decalol in their thermodynamic parameters.
[c] Values given in kcal/mol at 298 K.

4. Discussion

4.1. Principles of enthalpy and entropy contributions in enzyme catalysis

The transition state theory provides a framework for understanding chemical reactions and enzyme catalyzed reactions (27-29). The rate constant for a reaction as a function of the temperature T is given by Eq. 4, where $\Delta G^{\ddagger}$, the Gibbs free energy of activation, is the difference in free energy between the transition state and ground state. Gibbs free energy is separated into enthalpic and entropic terms. The enthalpy change (ΔH) reflects the energy change of the system as a result of forming and disrupting many individual interactions when the substrate binds to the enzyme. Breaking of bonds with water, buffer, and/or ion molecules causes an unfavorable ΔH, while the formation of new bonds with amino acids at the active site leads to a favorable ΔH (32). Entropy is a measure of the disorder or randomness in a system. The entropy change (ΔS) can be attributed to changes in solvation, in translational and rotational freedom, or to changes in conformation of enzyme and substrate (32,33).

Enzymes can destabilize the enzyme-substrate complex by paying for the energy to bring the reactants together in an appropriate orientation and induce conformational changes for better interactions in the transition state, and to carry out general acid-base, metal ion, and electrostatic catalysis to stabilize the transition state through the functional groups. The chemical step involved in bond making and breaking in the transition state costs a large enthalpy change, which can be overcome by favorable ionic and hydrogen bonding interactions with enzymes that are not available in solution (20). Binding of substrate with enzyme to form the enzyme-substrate binary complex results in the loss of translational and rotational degrees of freedom as two molecules become one, and the loss of conformational degrees of freedom from the substrate and from some residues in the enzyme (16,34). The release of ordered water molecules that surround hydrophobic and charged substrates can be associated with a gain of entropy (35,36); the release of a water molecule from an active site can increase the entropy 2-3 kcal/mol (37).

To understand the origins of enzyme catalysis, we have investigated how the binding interactions of the enzyme and a non-reacting portion of the substrate contribute to 3α-HSD/CR catalysis (19). Truncation of androsterone to 2-decalol and cyclohexanol was shown to increase the K_m value and significantly reduce the k_{cat}/K_m value. To get insight into the enthalpic and entropic components of these contributions, we determined the effects of temperature on the kinetic parameters of k_{cat}, k_{cat}/K_m and K_m for the wild-type enzyme acting on androsterone, 2-decalol and cyclohexanol.

4.2. Enthalpic and entropic contributions for 3α-HSD/CR acting on androsterone

We analyzed the kinetic parameters, k_{cat} and k_{cat}/K_B, of the 3α-HSD/CR-catalyzed the reaction of androsterone with NAD^+ with varied temperature, yielding the thermodynamic activation parameters of $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$, respectively. However, the kinetic complexity of the enzyme reaction complicates the interpretations of these values. 3α-HSD/CR shows an ordered bi bi kinetic mechanism with the NAD^+ binding first and the NADH released last. The kinetic mechanism for enzyme saturated with NAD^+ is shown in Scheme 1, where A and Q refer to NAD^+ and NADH, respectively. B can be androsterone, 2-decalol or cyclohexanol, and P is its corresponding oxidized carbonyl products. In previous studies, we demonstrated that the rate limiting step for 3α-HSD/CR catalyzed reaction of androsterone with NAD^+ is the release of NADH in the overall reaction, but it is hydride transfer at low, limiting concentrations of androsterone (38). The kinetic parameter k_{cat} reflects the release of NADH from the E-NADH binary complex for the 3α-HSD/CR acting on androsterone (*i.e.*, k_9 in Scheme 1). The kinetic parameter k_{cat}/K_B reflects the binding of substrate, the hydride transfer and release of product for 3α-HSD/CR acting on androsterone, steps k_3 to k_7. (However, when k_7 is fast and k_5 is slow, the expression reduces to k_3k_5/k_4.) For this substrate, the expression of the kinetic parameter K_B is a complicated rate equation (Scheme 1) and cannot be simplified to the dissociation constant for androsterone.

Hence the enthalpy change of activation ($\Delta H^{\ddagger}$) and entropy change of activation ($T\Delta S^{\ddagger}$) obtained from k_{cat}/K_B indicate the formation of the transition state of $EAB^{\ddagger}$ from the reaction of EA with B. The free energy of activation for the reaction of EA with androsterone is 6.2 kcal/mol (Table 1). The corresponding unfavorable enthalpy change of activation is 11.3 kcal/mol. This large positive enthalpy of activation can be attributed

to the formation of the transition state for the hydride transfer from substrate to nicotinamide ring of NAD^+ in the active site. The corresponding positive entropy change of activation is 5.1 kcal/mol at 298K indicating a favorable entropy contribution.

The observed unfavorable enthalpy change of activation ($\Delta H^{\ddagger}$) of 16 kcal/mol and favorable entropy change of activation ($T\Delta S^{\ddagger}$) of 2.4 kcal/mol obtained from the analysis of k_{cat} are attributed to the dissociation of NADH from the 3α-HSD/CR-NADH binary complex as a result of the interruptions of the interactions between 3α-HSD/CR and NADH within the binary complex and the increasing motion in the active site for the release of NADH, respectively. A similar thermodynamic activation value for the dissociate rate of NADH from E-NADH binary complex has been reported for beef heart lactate dehydrogenase with $\Delta H^{\ddagger}$ of 17.5 kcal/mol and $T\Delta S^{\ddagger}$ of 2.3 kcal/mol at 298K (39).

4.3. Enthalpic and entropic contributions in catalysis by 3α-HSD/CR acting on the truncated substrates

We then analyzed the kinetic parameters, k_{cat} and k_{cat}/K_B, of 3α-HSD/CR acting on 2-decalol and cyclohexanol with varied temperature to obtain the thermodynamic activation parameters of $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$, respectively. Since the rate limiting step is hydride transfer for 3α-HSD/CR acting on cyclohexanol and 2-decalol, the K_B for 2-decalol and cyclohexanol is then presumably similar to the true dissociation constant K_d (*i.e.*, k_4/k_3). The kinetic parameter k_{cat} reflects the hydride transfer for cyclohexanol and 2-decalol (*i.e.*, k_5), and k_{cat}/K_B reflects the steps from the binding of substrate to the hydride transfer for the 3α-HSD/CR acting on 2-decalol and cyclohexanol (*i.e.*, k_3k_5/k_4).

The free energies of activation for the reaction of EA with 2-decalol and cyclohexanol are 12.6 and 14.7 kcal/mol, respectively (Table 1). These reactions have favorable, positive entropy changes of activation of 8.8 and 7.0 kcal/mol at 298K, and the corresponding unfavorable enthalpy changes of activation are 21.4 and 21.7 kcal/mol. The enthalpies of activation are similar for 3α-HSD/CR acting on 2-decalol and cyclohexanol, but are significantly smaller for androsterone.

The formation of $EAB^{\ddagger}$ from EAB for 2-decalol and cyclohexanol are both enthalpically and entropically unfavorable, with the same free energy of activation of 17 kcal/mol. However, the enthalpic and entropic components contribute differently to enzyme catalysis, so that 2-decalol oxidation has values of both $\Delta\Delta H^{\ddagger}$ and $\Delta T\Delta S^{\ddagger}$ that are lower by about 3 kcal/mol relative to cyclohexanol. Hence, the B-ring of the steroid participates in favorable binding interactions in the transition state in the active site of enzyme that are compensated for by the same entropic cost.

The binding of 2-decalol and cyclohexanol with the $E\text{-}NAD^{+}$ complex is entropy-driven with $T\Delta S$ of 15.7 and 11.0 kcal/mol, but strongly endothermic with ΔH of 11.3 and 8.7 kcal/mol, respectively (Table 2). Binding of substrate with a positive entropy change has been indicated for nonpolar compounds. A hydrophobic molecule causes local water molecules to rearrange around the nonpolar molecule, thereby increasing the local order and decreasing the entropy of the system. Since the configurational entropy of 2-decalol and cyclohexanol can be expected to decrease on binding to the $E\text{-}NAD^{+}$ complex, these results indicate the importance of hydrophobic effects in the binding process, that is, water molecules surrounding 2-decalol and cyclohexanol can increase their entropy by binding to the active site of the $E\text{-}NAD^{+}$ complex, and ordered water

molecules in the active site also increase their entropy as they transfer to the bulk solvent on ligand binding. Meanwhile, breaking hydrogen-bonding interactions within the ordered water molecules causes the observed unfavorable ΔH.

4.4. Enthalpic and entropic contributions from remote portion of substrate in the 3α-HSD/CR-catalyzed reaction

The thermodynamic activation parameters calculated from the observed effects of temperature on the kinetic parameters, k_{cat}, k_{cat}/K_B, and $1/K_B$ for the 3α-HSD/CR catalyzed reaction of NAD^+ with androsterone, and its truncated analogs, 2-decalol and cyclohexanol, are summarized in Tables 1 and 2. Energy diagrams are shown in Figure 4 for assessing the remote binding energy and its enthalpic and entropic contributions to 3α-HSD/CR catalysis by comparing androsterone with its truncated analogs, 2-decalol and cyclohexanol. The energy state for EAB is not reported for androsterone because K_B does not provide a dissociation constant for B. We have shown both uniform binding interactions and differential binding interactions from the non-reacting portion of androsterone with the active site in 3α-HSD/CR catalysis (19). In this study, we show that the B-ring of steroid contributes equally to the stabilization of both the transition state and ground state of the ternary complex by 2.1 kcal/mol, whereas the remote BCD-ring and CD-ring of androsterone improves catalysis through differential binding interactions with the active site of 3α-HSD/CR by contributing 8.5 and 6.4 kcal/mol to the stabilization of the transition state, respectively (Figure 4A). We dissect the free

energy change into their enthalpic and entropic components. The enthalpy (ΔH) and entropy (-TΔS) changes that occur from the binding of EA with androsterone, 2-decalol, and cyclohexanol to form $EAB^{‡}$, respectively, are illustrated in Figure 4B.

As compared to cyclohexanol, the k_{cat}/K_B value for 3α-HSD/CR acting on 2-decalol increases 37-fold by decreasing the free energy of activation of 2.1 kcal/mol at 25 °C. The forces involved in the hydrophobic interactions of the B-ring of steroid with the active site of 3α-HSD/CR are mainly due to an increase in the entropy of activation without a change in enthalpy of activation in forming the transition state in the EAB ternary complex. Meanwhile, enthalpy–entropy compensation from the hydrophobic interactions of the B-ring of steroid with the active site of 3α-HSD/CR in proceeding from the ternary complex to the transition state is observed, leading to equal free energy contributions on the stabilization of the ground state and transition state of the ternary complex EAB; thereby the k_{cat} value is the same for 3α-HSD/CR acting on cyclohexanol and 2-decalol. Comparing the entropy of activation for reaching the transition state from the EAB ternary complex with 2-decalol and cyclohexanol, deletion of a ring of 2-decalol to form cyclohexanol becomes more favorable ($\Delta T\Delta S^{‡}$ of 2.8 kcal/mol at 298K), consistent with the possibility that the increase in the substrate's range of motion within the active site may interfere with catalysis. In contrast, an additional B-ring provides better interaction in the transition state with the active site ($\Delta\Delta H^{‡}$ of 2.9 kcal/mol). Therefore, the increased binding interactions by the B-ring in a binding process result in more negative $\Delta H^{‡}$ at the expense of increased order, leading to a more negative $T\Delta S^{‡}$.

As compared to cyclohexanol and 2-decalol, the k_{cat}/K_B value for 3α-HSD/CR acting on androsterone increases 1.8×10^{6}- and 4.9×10^{5}-fold by decreasing the free energy

of activation by 8.5 and 6.4 kcal/mol at 25 °C, respectively. The remote BCD-ring and CD-ring of androsterone contribute to the enthalpic stabilization of transition state by 10.4 and 10.1 kcal/mol, with entropic costs of 1.9 and 3.7 kcal/mol in an overall energetically more favorable enzymatic reaction, respectively. The crystal structures of apo- and NAD^+-bound 3α-HSD/CR have been determined and exhibit an incomplete and unresolved substrate binding loop (40). The flexible substrate binding loop of 3α-HSD/CR is involved in binding the nucleotide cofactor and androsterone to participate in catalysis (38,41). A significant induced conformational change from a disordered substrate binding loop to a closed helix-turn-helix is demonstrated in the binding of NADH with *Pseudomonas sp.* 3α-HSD (42). Therefore, the BCD-ring and CD-ring of androsterone may be involved in the conformational change of the substrate binding loop for better binding in the transition state than in the ground state of the EAB complex, resulting in the entropy cost and enthalpy gain. Loop closure upon substrate binding is important for enzyme catalysis. The lyase activity of isochorismate-pyruvate lyase is enthalpically driven with a very large entropic penalty of -24.3 cal/(mol K) for the ordering of the loop and substrate for the conversion of the ES complex to the transition state (33). The binding energy from the remote interaction of phosphite dianion with a flexible phosphate gripper loop is used by triosephosphate isomerase, glycerol 3-phosphate dehydrogenase, and orotidine 5'-monophosphate decarboxylase to facilitate catalysis (43,44). The increase in phosphate gripper loop size in orotidine 5'-monophosphate decarboxylases can contribute to greater enthalpic transition state stabilization through more extensive loop-substrate interactions with a larger entropic penalty for immobilization of the larger loop (45).

In summary, we used the truncated analogues to explore the function of a non-reacting portion of androsterone in 3α-HSD/CR catalysis by analyzing the enthalpy and entropy changes during the reaction. The truncation in the analogues of cyclohexanol and 2-decalol significantly decreases catalytic efficiency (k_{cat}/K_B) for the 3α-HSD/CR-catalyzed reaction as compared to that for androsterone. Both the conformational change of the substrate binding loop of 3α-HSD/CR and the hydrophobic effect of the steroid ring contribute to enzyme catalysis. Thermodynamic analysis shows that the binding of the E-NAD^+ complex with either 2-decalol or cyclohexanol to form the ternary complexes is endothermic and entropy-driven, indicating that desolvation plays an important role in the binding process and that the hydrophobic interactions of the B-ring of the steroids with the active site of 3α-HSD/CR can contribute to catalysis by mainly increasing the entropy of activation. Although the enthalpy of activation is unfavorable in forming the transition state for the 3α-HSD/CR-catalyzed reaction, the remote BCD-ring of androsterone may induce a conformational change of the substrate binding loop of 3α-HSD/CR for better interactions in the transition state by lowering the enthalpy of activation by 10 kcal/mol. However, the B-ring of the steroid probably cannot induce the conformational change of this loop and makes no further contribution to the enthalpic stabilization of the transition state. These results are consistent with the induced fit model (46) proposed by Koshland in that the changes in the enzyme structure caused by the substrate will bring the catalytic groups into the proper alignment for enzyme action, whereas poor-substrates will not. Our results show why 3α-HSD/CR catalyzes a very fast reaction with the optimal substrate androsterone and a very slow reaction with the poor substrates 2-decalol and cyclohexanol.

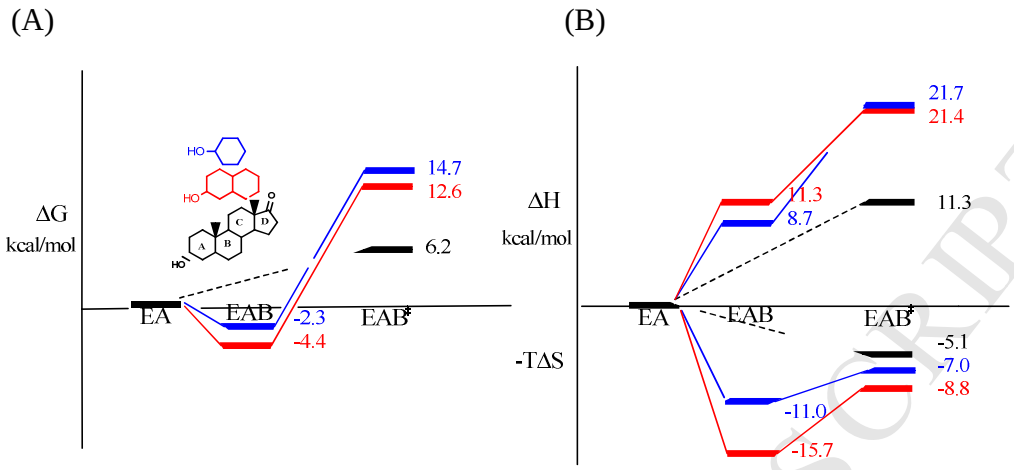

Figure 4. Energy diagram for the 3α-HSD/CR-catalyzed reaction. (A) Gibbs free energy diagram, and (B) enthalpy and entropy diagram. The energy profile for 3α-HSD/CR acting on androsterone (black trace) is compared to that with 2-decalol (red trace) and cyclohexanol (green trace). The x-axis corresponds to the reaction coordinate for the 3α-HSD/CR-catalyzed reaction. Entropy units are given as -TΔS for the direct comparison as an increase in -TΔS is unfavorable and a decrease in -TΔS is favorable. The listed values are from the Tables 1 and 2, and the values of ΔG and -TΔS are presented at 298K.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Highlights

- 3α-HSD/CR catalyzed reaction is entropy-driven.
- Binding of E-NAD binary complex with 2-decalol or cyclohexanol is endothermic and entropy-driven.
- Formation of the transition state from ternary complex is both enthalpically and entropically unfavorable.
- The hydrophobic interactions of B-ring of steroids contribute to catalysis by increasing $T\Delta S^{\ddagger}$.
- The BCD-ring of androsterone lowering $\Delta H^{\ddagger}$ results in the significant increases in kcat/Km.

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