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Efficient Factors in Protein Modification: Adenosine Deaminase Esterification by Woodward Reagent K

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Chemical modification of Adenosine Deaminase (ADA) with N-ethyl-5-phenyl isoxazoliom-3-sulfonate (Woodward's reagent K) (WR-K) was studied using experimental and theoretical techniques. Reaction concentration ranges were 0.8-6 mM WR-K at pH 7.8 and 27 °C. It was observed that the maximum number of moles of esterified residues per mol of enzyme ($\bar{\nu}$) in this concentration ranges is 4. However, esterification of ADA does not affect the activity of ADA, suggesting that the active site residues are not esterified. Similar results were obtained when the active site was blocked with 0.1 mM erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), followed by esterification, as measured by enol ester formation using absorbance at 340 nm. A theoretical approach was employed to study the modification process using molecular dynamic simulation, MM and QM/MM minimization. A full ASA empirical model and B3LYP method were used to evaluate the relative stability of some species which may arise from the reaction of ADA with WR-K. Theoretical results have shown that five residues (Glu 244, Glu 121, Glu 337, Asp 127, Asp 338) can be possible cases for modification in reaction 1:1 between ADA and WR-K at $\bar{\nu} = 1$. Glu 121 was possible initially modified in this process. Besides, it is specified that atomic accessible surface area cannot be an appropriate criterion in determination of primary sites which are modified by WR-K. Ultimately, it is clarified that among effective factors in modification of enzyme surface such as atomic accessible surface, stability of modified segment and local residues changes of ADA, latter factor plays a basic role in this process from kinetics and thermodynamics point of view.

Keywords: Adenosine deaminase, Woodward's reagent K, QM/MM minimization, Molecular dynamic, Free energy of hydration

INTRODUCTION

Adenosine deaminase (ADA, EC 3.5.4.4) is a major enzyme in purine metabolism. It is an essential enzyme for vital homeostasis in regulating the total amount of adenosine. It catalyzes the irreversible hydrolytic deamination of adenosine to inosine, or 2′-deaxyadenosine to 2′-deaxyinosine, and ammonia with a rate enhancement of approximately 2×10^{12}

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[1].

ADA has a (β/α)8 barrel structural motif. The active site has two lids, and is located in a deep oblong-shaped pocket at the COOH-terminal end of the β barrel, containing a zinc atom. The active site residues are His 15, His 17, His 214, His 238, Asp 295, Asp 296, Asp 19 and Glu 217. The zinc ion, which lies in the deepest part of the active site pocket, is coordinated by three NE2 atoms (His 15, His 17 and His 214), OD2 in Asp 295 and oxygen of water. Two acidic residues of active site (Asp 296 and Glu 217) are protonated at pH = 7 [2]. Then active site has only one acidic residues (Asp 19) with free and deprotonated oxygen in pH = 7 that can participate in chemical reactions. ADA has two distinct conformations, named the open form and the closed form [3].

The zinc acts as a powerful electrophile, activating water attacks to the substrate. An initial stereospecific hydroxyl group addition to the C6 position of the adenosine substrate forms the tetrahedral transition-state intermediate and a final ammonia elimination forms the inosine product [1].

Chemical modification can provide information about the structure of protein and active site residues [4-6]. Modification (or esterification) of different sites of the protein obtains different local residue changes that have different stabilities at both the modified protein and modified residue levels.

N-ethyl-5-phenyl isoxazoliom-3-sulfonate (Woodward's reagent K or WR-K) has been shown to be a suitable specific reagent for the activation of the carboxylic acid side chains of proteins [7]. WR-K is believed to be converted to its ketoketenimine at neutral pH, and then either breaks down to the amide or reacts with organic carboxylates (-COOH) to form the enol ester [8]. The activated carboxylic acid side chains, in the form of enol esters, are sufficiently stable to allow isolation of the protein, and a subsequent reaction with nucleophiles is then possible [9].

The free energy of hydration (ΔGh) is often correlated with the stability of the protein and the difference of hydration free energy between native and modified enzymes can reflect the change of an enzyme's stability to a large extent. In other words, if the value of ΔGh after modification becomes smaller, the value of ΔG of protein unfolding would be larger after modification, and so the stability would increase [10].

Hydration free energy can be divided into non-polar and electrostatic free energies contributions. Non-polar free energy can be represented by the accessible surface area (ASA) [11]. Also, electrostatic free energy contributions are treated using the Poisson-Boltzmann (PB) equation, as previously studied [12,13]. The combination of these two approximations forms the ASA/PB implicit solvent model that yields an approximation of the total hydration free energy, $\Delta Gh = \Delta G(nonpolar) + \Delta G(elec)$.

A different implicit solvent model, also using ASA, is based on the assumption that the hydration free energy of a solute can be expressed in terms of a linear sum of the atomic contribution weighted by partial exposed surface area: $\Delta Gh = \sum Wi \times Si(X)$. Here, Si(X) is the partial ASA of atom i, and Wi is an atomic free energy of hydration per unit area associated with atom i. These models are referred as full ASA. Because it is simple and inexpensive, this approach is widely used in computations on biomolecules [14,15]. Full ASA models have been used to investigate thermal denaturation of proteins [16], and to examine protein-protein associations [17].

In this study, a full ASA model is employed to obtain relative stability of modified ADA as well as transition states arising in the modification of ADA [18]. As stated elsewhere [18], atomic free energies of the latter model are determined by employing a large data set. In this model, atom types outnumber other ASA models [10,19] and thus have considerable flexibility in evaluation of hydration free energies. In this work, effective factors in the modification of enzyme surface are evaluated and the main factors in this process are specified using experimental and theoretical techniques. The modification of ADA with WR-K is studied as a typical example. Note that in this paper theoretical study is a complement for experimental works. In fact, experimental results as well as protonation of active site residues at pH = 7 have shown that active site remains definitely untouched in the modification process and theoretical study of active site modification is not essential. Thus computational techniques have been employed to determine the primary site situating on the ADA surface which can be modified and to specify main factors influencing the modification process.

EXPERIMENTAL

Materials

ADA (type IV, from calf intestinal mucosa), adenosine and WR-K were obtained from Sigma. Other related high-grade chemicals were obtained from Merck. The solutions were prepared in doubly distilled water.

Methods

Chemical modification. The modification of glutamate and aspartate residues of ADA was accomplished using 0.8, 2, 4, and 6 mM of WR-K. WR-K solutions were dissolved and stirred, and reacted with 5.78 µM ADA in a 50 mM Tris-HCl buffer solution with pH 7.8 (standard Tris buffer) at 27 °C. The reaction was completed within 60 min. The solution was then dialyzed against ten changes of 50 mM standard Tris buffer for three days to remove the excess reagent. The formation of the enol ester of acidic residues of ADA (by the reaction with WR-K) was determined by the increase in the absorbency at 340 nm on a Shimadzu-3100 spectrophotometer. The recorded absorbency was corrected using a standard Tris buffer as a blank over a wavelength range of 240-400 nm to obtain different spectra. The stoichiometry of the formation of the modified acidic residues, as a function of different concentrations of WR-K, was calculated from the molar extinction coefficient (7000 M^{-1} cm^{-1}) at 340 nm [8].

Reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) has been found to be a sensitive tool for the assay of thiol groups in proteins [20]. Between 250-450 nm wavelengths, different spectra of DTNB and ADA-DTNB complex register with the addition of DTNB (0.2 mM) to a reference contain a standard Tris buffer and sample cells containing a 5.78 mM ADA solution. During the reaction of DTNB and accessible ADA sulfhydryl groups, the nitromercaptobenzoate anion has an intense yellow color with a molar absorptivity of 13,600 M^{-1} cm^{-1} at 412 nm. The reaction of DTNB with ADA shows that there were no changes in absorbance at 412 nm that occur in the absence of free sulfhydryl groups in the ADA structure. This indicates that 340 nm absorbance was solely the result of carboxyl group modification.

The reaction of ADA with WR-K was followed by the removal of excess WR-K by dialysis, against a buffer which resulted in a covalently attached chromophore on its specific site, with an absorbency maximum of 340 nm and an extinction coefficient of 7000 M^{-1} cm^{-1}. This extinction coefficient and the absorbency maximum were used to quantify the reaction of WR-K with the carboxyl groups in proteins [8].

Circular dichroism (CD) measurements. The secondary structural changes of the captured systems were evaluated by far-UV CD spectroscopy. Measurements were recorded over 200-230 nm wavelengths using an AVIV spectropolarimeter, model 215 (USA), with a 0.1 cm path length sample cell. The calibration of the instrument was achieved using aqueous solutions of d-10-camphorsulfonic acid (ammonium salt) prior to use [21]. All CD measurements were carried out at 27 °C with the help of a thermostatically-controlled cell holder. Spectra were collected at 1 nm intervals, with a scan speed of 20 nm min^{-1}. Each spectrum was the average of two scans and noise in the data was smoothed using AVIV 215 software. The different concentrations of WR-K solutions (0.8-6 mM), as well as the protein solution (5.78 µM) in a 50 mM buffer solution of Tris-HCl, pH 7.8, for the far-UV-CD were freshly prepared.

Fluorescence spectroscopy. Fluorescence intensity was performed using a Hitachi fluorescence spectrophotometer, model MPE-4. The excitation wavelength was 290 nm and the emission was scanned from 300-400 nm every 1 nm. The measurements were made using a 1-cm path length fluorescence cuvette. Samples of 5.78 µM ADA were in a 50 mM standard tris buffer. All spectra were normalized for protein concentration.

Enzyme assays. The enzymatic activities were assayed using UV-Vis spectrophotometry. Using the Kaplan Method, the decrease in absorbency (265 nm) from the conversion of adenosine to inosine was followed using a Shimadzu-3100 spectrophotometer [22]. The standard assay solution (27 °C) contained a mixture of 1 ml of 50 mM Tris buffer (pH 7.8), 0.15 mM adenosine and 40 nM enzyme.

Theoretical methods. Molecular dynamic simulation and MM minimization were performed using a GROMACS 3.3.1 package under a Gromos force field (G43A1) [23,24]. The MD simulations were carried out at *NPT* ensemble and periodic boundary condition. The electrostatic interactions were calculated using the Particle-Mesh Ewald model with a 14 Å cut-off [25]. QM/MM calculations were carried out by coupling GROMACS 3.3.1 package with MOPAC 7 library [26,27]. Force field parameters for the ketoketenimine were

chosen from similar building blocks in the Gromos G43A1 force field. Force field parameters for zinc ion were extracted from other reference [28]. Point charges for the ketoketenimine bound to a Glu or Asp residues were obtained from a HF/6-31G* single point calculation in Gaussian 98 using the CHELPG fitting procedure [29]. Note that the limitation of this approach is that the polarization effect associated with the condensed phase environment is not explicitly included, although the tendency for the HF/6-31G* Quantum Mechanics (QM) level of theory to overestimate dipole moments has been suggested to account for this deficiency [30].

Accessible surfaces of atoms have been calculated based on the previous study [31]. A WASA model was employed to determine hydration free energies [18]. Gaussian 98 program revision A-9 [32] was used for energy calculation using the B3LYP/6-31G* method [33,34]. Note that other details about simulations have been mentioned in the following sections.

RESULTS AND DISCUSSION

Experimental Results

Figure 1 shows the spectra of the effect of WR-K on ADA in a buffer solution in specific moles of WR-K per mol of ADA ($\bar{\nu}$). At WR-K concentrations of 0.8, 2, 4 and 6 mM, the effect on ADA and the corresponding $\bar{\nu}$ is equal to 1, 2, 3 and 4, respectively. Figures 2 and 3 show Km and Vm *vs* $\bar{\nu}$. These Figs. indicate that there was no change in activity and binding affinity for ADA upon modification by WR-K. The results indicate that the modification of sites ($\bar{\nu}$ up to 4) must not be in the active site of the enzyme and the acidic residual modification is on the ADA surface. For confirmation, the effect of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (Ki = 1.6 × 10^{-6} M) [1] on ADA before modification was also tested. No changes in the ADA spectra (not shown here) were observed. Therefore, the acidic residues of active site are not esterified by WR-K.

In addition to the experiments on the Cys of ADA, the ASA of ADA was calculated and no accessible sulfhydryl groups on the ADA surface were detected. Figure 4 shows the far-UV CD ellipticity of θ_{222} for the modification at different values of $\bar{\nu}$. No major changes in the secondary structure of ADA upon modification by WR-K were observed. Figure 5

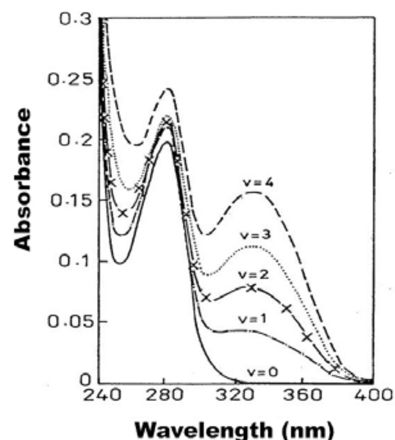


Fig. 1. Absorption spectra for the modification of ADA by WR-K. The enol ester has the maximum absorption at 340 nm in concentrations of WR-K (0, 0.6, 2, 4, 6 mM corresponding to $\bar{\nu}$ = 0, $\bar{\nu}$ = 1, $\bar{\nu}$ = 2, $\bar{\nu}$ = 3, $\bar{\nu}$ = 4, respectively).

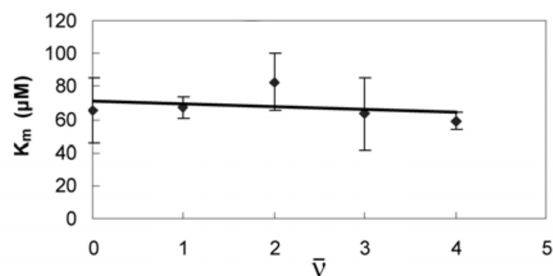


Fig. 2. Km of native and modified ADA plot at different values of $\bar{\nu}$.

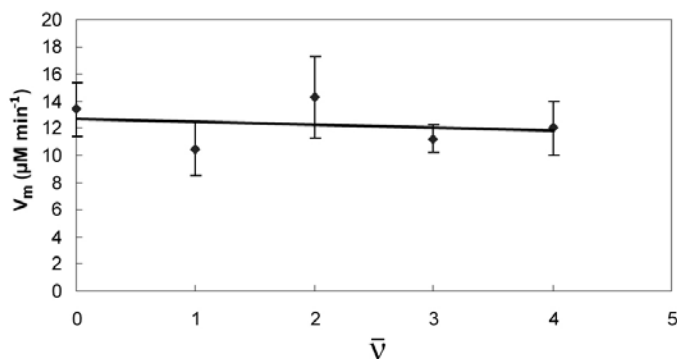


Fig. 3. Vm of native and modified ADA plot at different values of $\bar{\nu}$.

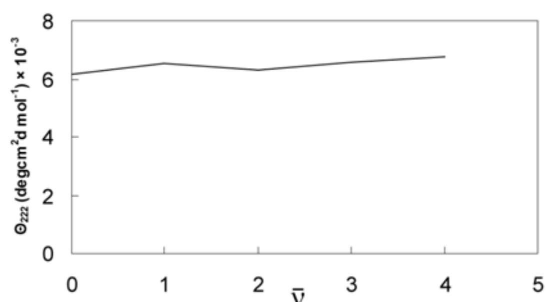


Fig 4. Ellipticity of θ222 at different values of $\bar{\nu}$.

between ADA and WR-K and to specify main factors in this process.

First, the equilibrium geometry of ADA was obtained using a molecular dynamics simulation and its atomic accessible surfaces area was calculated. This structure was used to design primary geometries of modified states of ADA. Notice that WR-K is initially converted to ketoketenimine and ADA is modified by a latter compound. These reactions are shown in Scheme 1 for a residue [8].

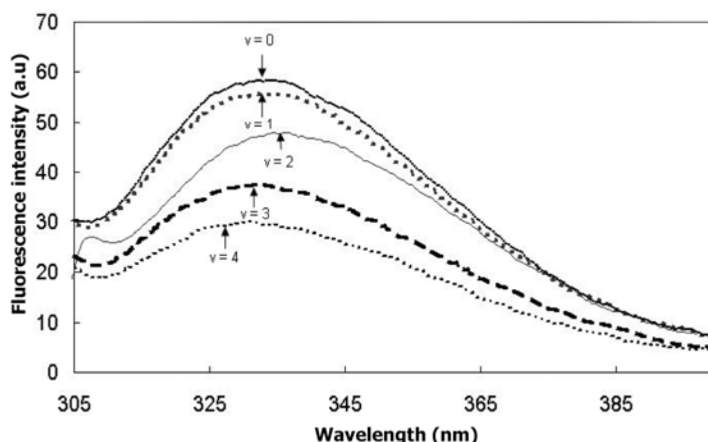


Fig. 5. Emission fluorescence spectra of native and modified ADA at different values of $\bar{\nu}$.

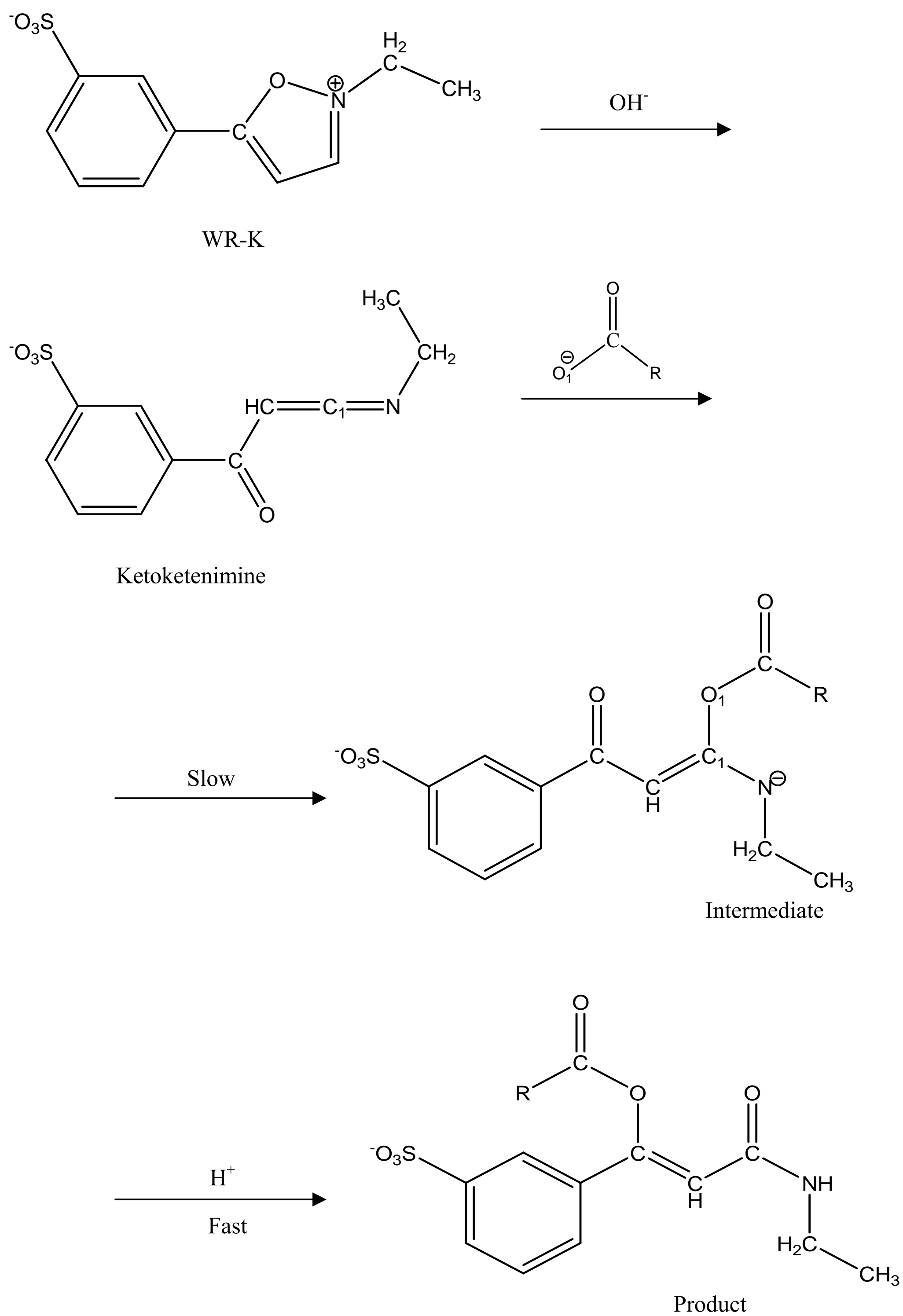
shows fluorescence spectra for the modification at different values of $\bar{\nu}$. This Fig. shows quenching of the spectra for ADA upon modification by WR-K and only slightly structural change occurred at $\bar{\nu} = 1$.

Theoretical Results

In accordance with previous reports, it was expected that the active site of ADA would be modified in reaction with WR-K [8]. However, as seen, glutamate or aspartate residues placed on the ADA surface are modified in the reaction of WR-K with ADA, in addition ASA calculation of ADA atoms shows that accessible surface area of Asp and Glu in active site are very small (utmost 8 $Å^2$ for OD1 ASP 19). Thus, a theoretical approach was considered in the present investigation as a complement for experimental works. Such approach has been employed to determine the primary site which can be modified on the surface in 1:1 ($\bar{\nu} = 1$) reaction

In each modified ADA structure, a glutamate or aspartate is modified as a stated product elsewhere. Equilibrium geometries of such modified structures at 300 K were obtained using a molecular dynamics simulation. Next, hydration free energies of latter geometries were calculated using a WASA model. Also, an optimized geometry for each modified structure was obtained by employing MM and QM/MM minimization methods on last equilibrium geometry at 300 K.

In a concurrent study, it was shown that an intermediate (Scheme 1) is formed in the rate determining step (not shown here). Because production of the intermediate is important from the kinetics point of view, primary geometry of this intermediate is designed for every residue that can be modified by ketoketenimine. Equilibrium and optimized geometries of these intermediates are obtained using the above-employed strategy for products. Then, transition state geometries owing to production of the intermediates that have been designed



Scheme 1. Mechanism of production of ketoketenimine and modification of a residue. Fast step includes two steps

based on equilibrium geometries relevant to these intermediates. Equilibrium and optimized geometries as well as hydration free energies of these transition states are obtained using MD simulations and QM/MM minimizations. Also, the energy of the modified segments of products and above transition states were evaluated using the B3LYP method. Finally, effective factors in the modification process are investigated.

Atomic accessible surfaces. The x-ray structure of ADA (PDB code: 1VFL) [3] was placed in a solvent box with about 20,000 spc216 of water molecules, 18 Å in length from the ADA to the box edges. Neutralization of the system required the addition of 11 Na^+ ions. First, the solvent alone with the Na^+ ions was subject to energy minimization with the solute kept fixed in its initial configuration. The solvent and Na^+ ions are then allowed to evolve using a molecular dynamics simulation for 20 ps with a step time of 1 fs, again keeping the structure of the solute molecule fixed. Next, the entire system is minimized using the steepest descent of 1000 steps (down to a gradient of < 100 kJ mol^{-1} nm^{-1}) followed by conjugate gradients of 9000 steps (down to a gradient of < 10 kJ mol^{-1} nm^{-1}).

In order to obtain equilibrium geometry at 300 K and 1 atm, the system was heated at a weak temperature coupling (τ = 0.1 ps) and pressure coupling (τ = 0.5 ps) by taking advantage of the Berendesen algorithms [35]. LINCS algorithm was used to constrain hydrogen bond lengths. Heating time for the molecular dynamics simulations at 100, 200 and 300 K was 100 ps. Then, equilibrium geometries of ADA were achieved using a molecular dynamics simulation for 800 ps at 300 K. Next, last equilibrium geometry was minimized using the steepest descent of 1000 steps followed by conjugate gradients of 9000 steps. This way, a minimum geometry is obtained for ADA. Note that the backbone root mean square deviation (RMSD) of ADA structure relative to its own starting structure was equal to 1.304 ± 0.076 Å. This low RMSD value indicates that the MD run was stable and ADA atoms did not significantly deviate from the starting structure during MD simulation. In addition, average temperature of last 100 ps of simulations at 300 K was equal to 300.002 ± 0.886 K. Therefore, the extracted equilibrium structure has been obtained under temperature stability conditions. Besides, the variance value of bond distances during simulation is much less than 0.02 Å, which is an indication that simulation is correct and there are no critical situation at bond distances in the simulation.

Table 1. ASA Values for OD1 or OD2 and OE1 or OE2 Atoms of ADA Surface Residues

	Residue		ASA (Å^2)
OE2	Glu	174	33.22
OE1	Glu	121	33.10
OD1	Asp	338	33.06
OE1	Glu	44	32.83
OE2	Glu	244	32.79
OE2	Glu	342	32.71
OE1	Glu	337	32.63
OE1	Glu	25	32.47
OE2	Glu	339	32.19
OD2	Asp	127	31.88
OE1	Glu	189	31.32
OE1	Glu	164	31.04

Ultimately, atomic accessible surfaces were calculated [31]. These results are summarized in Table 1. The data of Table 1 are the surface area values belonging to the carboxylate oxygen (OD1 or OD2 and OE1 or OE2) of twelve residues (Glu and Asp) that have more accessible surface area.

Geometries of product. The initial configurations of products were designed by modifying each of the residues as summarized in Table 1. As previously stated, modified segments of these structures are constructed based on what has been defined as product in Scheme 1. Thus twelve starting structures were designed for modified ADA. Initially, each of designed structures were minimized using the steepest descent of 1000 steps (down to a gradient of < 100 kJ mol^{-1} nm^{-1}) followed by conjugate gradients of 9000 steps (down to a gradient of < 10 kJ mol^{-1} nm^{-1}). Then, modified segments belonging to latter minimized geometries were dissected and their atomic charge were calculated using a HF/6-31G* single point calculation in Gaussian 98 using the CHELPG fitting procedure. Next, equilibrium and minima geometries of latter structures were obtained through molecular dynamics simulation and MM minimization. It is noteworthy that the

backbone root mean square deviations (RMSD) of each of these product structures relative to their own starting structures were about 1.142 ± 0.082 Å at simulations. These low RMSD values indicate that the MD runs were stable. In addition, average temperature of last 100 ps of simulations at 300 K was about 300.001 ± 0.856 K. Therefore, the extracted equilibrium structures have been obtained under temperature stability conditions. Besides, the variance values of bond distances during simulations are much less than 0.02 Å, which is an indication that simulations are correct and there are no critical situation at bond distances in simulations.

Hydration free energies of products. It is apparent that the evaluation of relative stability of the above species requires to take an entropic quantitative into account. As stated elsewhere, empirical WASA models can be used in hydration free energy calculations with reasonable precision and an error of less than 1 kcal mol^{-1} [18]. Nevertheless, these methods have a minor computational cost compared to free energy perturbation techniques. Thus, in this work, the hydration free energy of each of the modified ADA species is considered to be a criterion for its stability and this entropic quantitative is calculated using a WASA model. Initially, eleven equilibrium geometries for each mentioned structures and ADA are extracted from last 100 ps of molecular dynamics simulation at 300 K by 10 ps intervals. Next, hydration free energies for such extracted structures are calculated using a WASA model. Thus, an average hydration free energy has been obtained for each modified structure and ADA.

Such average values as well as their standard deviation are summarized in Table 2. It should be noted that native ADA and ketoketenimine are stated as reactants elsewhere. It is obvious that hydration free energy is a more appropriate criterion than atomic accessible surface to judge and specify sites on the enzyme surface that can be modified. Also, differences between the values in Table 2 are more than the error value of the WASA method, as reported elsewhere [18]. From Tables 1 and 2, it is clear that the order of Table 1 for residues is not in agreement with Table 2. Thus, the atomic accessible surface area is not a significant criterion to determine the primary residue which is modified in the reaction of ADA with ketoketenimine. Latter factor can be only employed to single out some sites for modification from other sites.

A comparison in Table 2 of the hydration free energy value for each modified structure with those of reactants (ADA and ketoketenimine) shows that Asp 127 can be initially modified in reaction 1:1 between ADA and ketoketenimine, thermodynamically. Because the hydration free energy value for the modified structure of the esterification of Asp 127 is less than total ΔGh value for reactants (native ADA and ketoketenimine). Also, considering standard deviation values of Table 2, it becomes clear that Glu 244, Asp 338, Glu 121 and Glu 337 supposedly can be modified in the modification process. This is because the standard deviation of hydration free energy values for the modified structures of the esterification of the corresponding residues is more than the difference in ΔGh values between each one of these species with the ΔGh value summed for reactants.

Geometries of intermediates and transition states. According to aforementioned discussion, Asp 127 is probably as a candidate site for modification in reaction 1:1 between ADA and WR-K. To evaluate competition between residues kinetically, the relative stability of transition states belonging to production of intermediates in modification of these residues must be considered, because these intermediates are formed in the rate-determining step in the modification reaction. First, the initial configurations of intermediates are designed by modifying each residue that can be modified by ketoketenimine. In these structures, modified segments are designed based on what has been introduced as intermediate in Scheme 1. Initially, these intermediates were minimized and atomic charges belonging to modified segments were calculated. Finally, equilibrium and minima geometries of five designed intermediates were obtained using molecular dynamics simulation and MM minimization. Note that neutralization of the system required the addition of 12 Na^{+} ions. Note that the backbone root mean square deviation (RMSD) of each of intermediate structures relative to their own starting structure was about 0.92 ± 0.04 Å. Again these low RMSD values indicate that the MD runs were stable. In addition, average temperatures of last 100 ps of simulations at 300 K were about 300.003 ± 0.84 K. Therefore, the extracted equilibrium structures have been obtained under temperature stability conditions. Besides, the variance values of bond distances during simulations are much less than 0.02 Å, which is an indication that simulations are correct and there are no

Table 2. Hydration Free Energy Values for Modified Structures and Unmodified Segments of Products and Transition States that Arise from Modification of ADA, and Native ADA (AVE = Average, STD = Standard Deviation)

Residue	$\Delta G_{hydration}$ (kJ mol^{-1})							
	Products				Transition states			
	Modified structure		Unmodified segment		Modified structure		Unmodified segment	
	AVE	STD	AVE	STD	AVE	STD	AVE	STD
Asp 127	-34884.81	1271	-34775.27	1272	-32925.15	912	-32819	912
Glu 244	-33342.30	573	-33213.22	573	-32240.02	715	-32094.29	715
Asp 338	-33296.94	1041	-33169.83	1038	-31131.81	1247	-30999.47	1259
Glu 121	-33181.84	1037	-33007.74	1029	-33123.26	715	-32973.06	720
Glu 337	-33125.40	999	-32987.74	996	-31296.19	1372	-31200.88	1385
Glu 164	-32532.57	953	-32442.11	954				
Glu 339	-32434.33	899	-32320.73	900				
Glu 25	-32255.21	891	-32175.50	887				
Glu 189	-32178.64	723	-32045.76	724				
Glu 44	-32134.63	1188	-31985.93	1188				
Glu 342	-31293.73	912	-31155.99	908				
Glu 174	-31224.98	543	-31098.42	544				
Native	-33604.38	861						
Ketketoenimine + native ADA	-33756.51	861						

critical situation at bond distances in simulations. Next, transition states geometries were designed based on equilibrium geometries of intermediates.

The most appropriate approach to locate a transition state with MM (force field) is to use an algorithm that optimizes the input structure to a true saddle point that is a geometry characterized by a Hessian matrix with one and only one negative eigenvalue. To do this the MM program must be able not only to calculate second derivatives, but must also be parameterized for the partial bonds in transition states, which is a feature lacking in standard MM force fields. In fact, since locating transition states in reaction of a macromolecule with a ligand is very difficult and so expensive and also MM programs are usually not able to optimize an input geometry toward a saddle point, one normally optimizes to a minimum subject to constraint expected for the transition state. Such constraint is usually obtained from considering a model system. Such system is a small molecule containing the same functional groups as those taking part in the actual reaction of protein with ligand [36]. In this study, aforementioned constraint is chosen based on a reaction coordinate which is specified in reaction of isolated glutamate (A dissected glutamate that hydrogen atoms have been added to its C terminal and N terminal) with ketoketenimine. Latter reaction has been investigated in a concurrent theoretical study and it has been shown that C1-O1 bond (see Scheme 1) is critical mode for conversion of reactants to intermediate. This C1-O1 bond is equal to 2 Å in transition state. This way, the equilibrium structures of intermediates at 300 K are employed to predict the geometries of the transition states between the reactants (ketoketenimine and ADA) and intermediates. In the each of the transition states, C1-O1 bond length is constrained to 2 Å (see Scheme 1). First, latter geometries were minimized and atomic charges belonging to modified segments were

calculated. Afterward, a molecular dynamics simulation was done for 300 ps for each of the suggested geometries followed by a structural minimization calculation.

Hydration free energies of transition states. Hydration free energies and their standard deviation for the the transition states were calculated. The results are summarized in Table 2. Table 2 shows that, taking into account the hydration free energy of reactants, it is clear that transition states are formed in endothermic processes. Because the hydration free energy value for each of these structures is more than the total Gh values for ketoketenimine and native ADA. In other words, the hydration free energy value of the Glu121 transition state is more negative than those of transition states. Thus, Glu121 can be possible case for modification in reaction 1:1 between ADA and WR-K from the standpoint of kinetics.

Protein local residues changes effect. As stated elsewhere [37] in contrast to studies of enzyme catalysis, the thermodynamic and kinetic properties of the reaction of modifying agents with proteins can be compared with the corresponding properties of the reaction of the same modifying agent with small molecules containing the same functional groups as those with which they react in the intact protein. To evaluate this, the B3LYP method and the WASA model were used to evaluate the role of protein local residue changes in the modification process. Transition states and products were chosen that arise from the modification of residues Glu 121, Glu 244, Glu 337, Asp 127 and Asp 338. Then, equilibrium and MM optimiz ed geometries of these species were considered.

It should be noted that modified segment of each modified structure is a modified residue (glutamate or aspartate residue and ketoketenimine) and unmodified segment is remainder of modified structure. At first, the hydration free energy of the unmodified segment was calculated for each compound. The results obtained are summarized in Table 2. Then, in order to obtain a better structure for the modified segment of these transition states and products, a combination of the G43A1 and the PM3 semi-empirical methods were employed. Note that among semiempirical methods, PM3 and AM1 are best candidates and the former can be preferred to the latter. Also, it is reported that optimized geometry of HF/6-31G* are only moderately better than those of AM1 and PM3 [38]. The modified segments of the structures were treated as the QM portion for minimization and the remainder of the atoms as the MM portion. Link atoms are employed in these calculations, so that each link atom is a hydrogen atom in QM region and a dummy atom in MM region. QM/MM minimization was performed by a steep descent of 1000 steps (down to a gradient of < 10 kJ mol^{-1} nm^{-1}) followed by a BFGS minimization of 1000 steps (down to a gradient of < 1 kJ mol^{-1} nm^{-1}). Ultimately, the modified segment of the minimized structures was dissected and their energies were calculated using the B3LYP method.

B3LYP results are presented in Table 3. The results of this table show that, among glutamate residues, Glu 337 forms the most stable transition state and product in the modification process. Thus, the latter residue must be modified in reaction 1:1 between ADA and WR-K, from the kinetics and thermodynamic points of view, if only the modified segments are taken into account. This is clear from the E0 and G values in Table 3. The E0 and G values of the modified segments of transition state and product are the least when Glu 337 is modified. Data of this Table shows that, among aspartate residues, Asp 127 is initially modified kinetically, if the stability of modified segments of transition states is considered. This is also evident from E0 and G values of the transition states in Table 3, where the modified segment of the transition state of the modification of Asp 127 is more stable than those of the transition state of Asp 338. In addition, G values for the modified segments of products in Table 3 show that, among aspartate residues, Asp 338 forms a product more stable than that of Asp 127. Thus, Asp 127 and Asp 338 are modified in reaction 1:1 between ADA and ketoketenimine kinetically and thermodynamically, respectively, when only modified segments of modified ADA are considered.

However, a closer look at Table 2 it is clarify that the unmodified segment of the transition state due to modification of Glu 121 is also more stable than those of other transition states. Nevertheless, if the stability of unmodified segments of products is considered, Asp 127 in initially modified thermodynamically. This is evident from the comparison of the hydration free energy values for the unmodified segments of products, as presented in Table 2. Thus, local residues changes of ADA on a pathway reaction support modification of glutamate 121 residue and aspartate 127 more than that of other residues kinetically and thermodynamically,

Table 3. Electronic Energy (E_0) and Absolute Free Energy (G) Values (kJ mol^{-1}) for Modified Segments of Product and Transition States which Arise from Modification of ADA

Residue	Product		Transition state	
	E_0	G	E_0	G
Glu 244	-4344003.77	-4345044.49	-4343096.44	-4343232.8
Glu 121	-4344458.28	-4344578.27	-4343070.17	-4343208.75
Glu 337	-4344904.57	-4345049.64	-4343211.6	-4343360.7
Asp 338	-4241739	-4241878.33	-4239181.40	-4239309.43
Asp 127	-4241686.67	-4241821	-4239905.59	-4240037.66

respectively.

It is apparent that among residues that are supposed to be modified, a possible residue is initially modified which generates more stable transition state rather than forming more stable product. Ultimately, it can be concluded that Glu 121 can be modified in reaction 1:1 between ADA and ketoketenimine.

Above discussion demonstrates that local residue changes of protein during modification plays a basic role in the process both kinetically and thermodynamically. Because the modification process is not a catalytic reaction, the results indicate that local residue changes in enzyme structure are an effective factor in non-catalytic processes similar to catalytic reaction in enzymes. Thus, it is completely obvious that the protein structure and local residue changes in protein modification cannot be ignored.

CONCLUSIONS

This work endeavors to specify the main factors in protein modification using experimental and theoretical techniques. Possible sites of ADA that are modified by WR-K were determined as a typical case. Experimental results indicate that active site of ADA is not modified. Theoretical results show that five residues of ADA surface are possible cases for modification by WR-K in reaction 1:1 ($\bar{\nu} = 1$). These residues are Glu 121, Glu 244, Glu 337, Asp 127 and Asp 338. Among these residues, Glu 121 can be possible case for modification in reaction 1:1 between ADA and ketoketenimine. It is also demonstrated that the atomic accessible surface area cannot classify the exact site for protein surface modification, but that the free energy of hydration of products and transition states is a good candidate for this. Ultimately, it is clarified that among effective factors in modification of enzyme surface such as atomic accessible surface, stability of modified segment and local residues changes of ADA, latter factor is the most important from kinetics and thermodynamics point of view.

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REFERENCES

[1] G. Cristalli, S. Costanzi, C. Lambertucci, G. Lupidi, S. Vittori, R. Volpini, E. Camaioni, Med. Res. Rev. 21 (2001) 105.

[2] D.K. Wilson, F.A. Quiocho, Biochemistry 32 (1993) 1689.

[3] T. Kinoshita, I. Nakanishi, T. Terasaka, M. Kuno, N. Seki, M. Warizaya, Biochemistry 44 (2005) 10562.

[4] S. Hashemnia, A.A. Moosavi-Movahedi, H. Ghourchian, F. Ahmad, G.H. Hakimelahi, A.A. Saboury, Int. J. Biol. Macromol. 40 (2006) 47.

[5] S. Safarian, A.A. Moosavi-Movahedi, S. Hosseinkhani, Z.G. Xia., M. Habibi-Rezei, G. Hosseini, C. Sorenson,

N. Sheibani, J. Protein Chem. 22 (2003) 643.
[6] P.H. Petra, Biochemistry 10 (1971) 3163.
[7] B.M. Dunn, C.B. Anfinsen, J. Biol. Chem. 249 (1974) 3717.
[8] U. Sinha, J.M. Brewer, Anal. Biochem. 151 (1985) 327.
[9] P. Bodlaender, G. Feinstein, E. Shaw, Biochemistry 8 (1969) 4941.
[10] Z. He, Z. Zhang, J. Protein Chem. 18 (1999) 557.
[11] B. Lee, F.M. Richards, J. Mol. Biol. 55 (1971) 379.
[12] J.G. Kirkwood, J. Chem. Phys. 2 (1934) 351.
[13] P. Debye, E. Hückel, Physik. Zeit. 24 (1924) 305.
[14] F. Fraternali, W.F. Van Gunsteren, J. Mol. Biol. 256 (1996) 939.
[15] D. Eisenberg, A. Mcclachlan, Nature 319 (1986) 199.
[16] T. Lazaridis, M. Karplus, Science 278 (1997) 1928.
[17] M.D. Cuming, T.N. Hart, R.J. Read, Protein Sci. 4 (1995) 2087.
[18] J. Wang, W. Wang, S. Huo, M. Lee, P.A. Kollman, J. Phys. Chem. B. 105 (2001) 5055.
[19] T. Ooi, M. Oobatake, G. Nemethy, H.A. Scheraga, Proc. Natl. Acad. Sci. USA 84 (1987) 3086.
[20] A.F.S.A. Habeeb, Methods Enzymol. 25 (1972) 457.
[21] T. Takakuwa, T. Konno, H. Meguro, Anal. Sci. 1 (1985) 215.
[22] N. Kaplan, Methods Enzymol. 2 (1955) 473.
[23] E. Lindahl, B. Hess, D. van der Spoel, J. Mol. Mod. 7 (2001) 306.
[24] W.F. Van Gunsteren, S.R. Billeter, A.A. Eising, P.A. Hünenberger, P. Krüger, A.E. Mark, W.R.P. Scott, I.G. Tironi, Biomolecular simulation, the GROMOS96 manual and user guide. Hochschulverlag AG der ETH, Zürich, 1996.
[25] D.M. York, A.L. Wlodawer, G. Pedersen, T.A. Darden, Proc. Natl. Acad. Sci. USA 91 (1994) 8715.
[26] G. Groenhof, M.F. Lensink, H.J.C. Berendsen, J.G. Snijders, A.E. Mark, Proteins: Structure, Function, and Genetics 48 (2002) 202.
[27] G. Groenhof, M.F. Lensink, H.J.C. Berendsen, A.E. Mark, Proteins: Structure, Function, and Genetics 48 (2002) 212.
[28] A. Coi, M. Tonelli, M.L. Ganadu, A.M. Bianucci, Bioorg. Med. Chem.14 (2005) 2636.
[29] C.M. Breneman, K.B. Wiberg, J. Comput. Chem. 11 (1990) 361.
[30] O.M. Becker, A.D. MacKerell, Jr., B. Roux, M. Watanabe, Comput. Biochem. Biophys., Marcel Dekker Inc., New York, 2001, p. 21.
[31] H. Naderimanesh, M. Sadeghi, S. Arab, A.A. Moosavi-Movahedi. Proteins: Structure, Function and Genetics. 42 (2001) 452.
[32] M.J Frisch, A.B. Nielsen, A. Frisch, GAUSSIAN 98, Version A.9, Gaussian Inc., P.A. Pittsburgh, 2000.
[33] A.D. Becke, J. Chem. Phys. 98 (1993) 5648.
[34] G.A. Peterson, M.A.A. Al-Laham, J. Chem. Phys. 94 (1991) 6081.
[35] H.J.C. Berendsen, J.P.M. Postma, W.F. van Gunsteren, A. Dinola, J.R. Haak, J. Chem. Phys. 81 (1984) 3684.
[36] E. Lewars, Computational Chemistry, Introduction to the Theory and Applications of Molecular and Quantum Mechanics, Kluwer Academic Publishers, New York, 2003, Chapter 2.
[37] E.T. Rakitzis, Biochem. J. 217(1984) 341.
[38] J.J.P. Stewart, J. Comput. Chem. 10 (1989) 209.