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Complexation of Calix[4]crown-5-Ether with α-Amino Acids and Their Molecular Recognition via UV/Vis Spectroscopy

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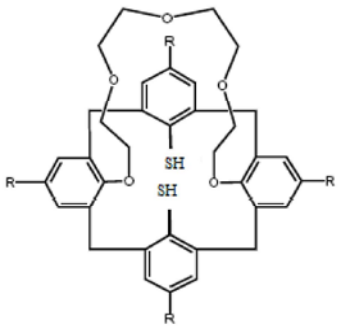
Abstract: The Association equilibrium constants of complexes between some α-amino acids (Alanine, Valine, Leucine, Arginine, Aspartic acid, Glutamic acid, Histidine and Lysine) with 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether in chloroform and methanol mixed solvent (50%: 50%, V:V) have been investigated by means of UV/Vis spectrophotometry titration. On the basis of complexation formation values, it has been shown that amino acid to ligand ratio is exclusively 1: 1. The stability constant values of the formed complexes in descending order are as: Ala > Val > Arg > Lys > Leu > Asp > Glu > His. The variations in stability constants occurred between a given amino acid and Calixcrown in terms of various interactions. The structures of three different optimized conformers have been optimized with semi-empirical and *ab initio* computations. The conformers' stabilities are in following order: cone > partial-cone >1, 3-alternate. Structures of complexes formed between the crown-5-ether moiety of Calix[4]arene and protonated Arginine and Lysine were determined through minimization of complex formation energies. It has been indicated that the complex stability depends on number of amine groups in the alkyl chain as well as the number of methylene groups between the amine groups.

Key words: Uv/Vis Spectrophotometry · Calixcrown · Amino acid · Hf · B3LYP · *Ab initio*

INTRODUCTION

Calix[n]arenes [1] are cyclic oligomers consisting of *n* phenol units bridged by methylene groups in *ortho*-position to the phenolic hydroxyl group [2]. Calixarenes are in the form of a cyclical oligomer having a "basket" shape, where the cavity can serve as a binding site for numerous guest species, including ions and molecules. Calixarenes are cup-shaped molecules which can form inclusion complexes with a wide range of guest species. Calixcrown has the capability of hydrogen bonding to guests and another fascinating aspect of Calixarenes is their conformational variability and formation of stable and separable conformers with different reactivity and binding capabilities [3, 4]. The polar and nonpolar features of cavities enable Calixarenes to interact with a wide range of guest species, depending on the binding groups substituted at each rim and the number of repeating units in the macrocycle. Calixarenes have been used in different aspects [5-13]. Compared to other applications, investigation of complex formation between proteins and also amino acids and Calixcrown derivatives is less common. Calixarene derivatives can even be used as synthetic receptors which mimic the role of hypervariable loops in antibody-combining regions [14]. The Calixcrown molecule applied in this research is (Fig. 1) 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether (ProLinker™), a bifunctional molecular linker which has a



R : OCH3

Fig. 1: Chemical drawing of (ProLinker™) 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether (Hydrogen atoms are not shown.)

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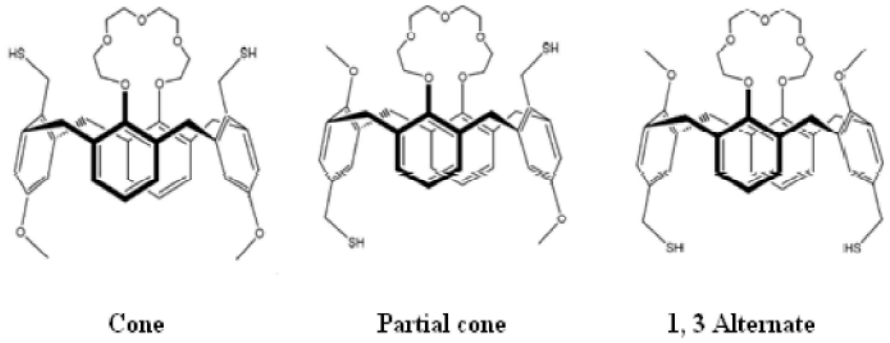

Fig. 2: Structure of 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether (ProLinker™)conformers.

crown moiety for protein binding. Depending upon the relative orientation of para and phenolic sites, the 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether molecule can possess three possible conformations in the forms of cone (u-u-u-u), partial cone (u-u-u-d) and 1,3 alternate (d-u-d-u) conformers (Fig. 2). These conformers display selective properties of complexation with amino acids. Molecular recognition of bio-relevant amino acids by artificial receptors is an important problem in chemistry and biology [15]. Calix [4] arenes are of interest as cavity-shaped molecules forming host-guest complexes with amino acids [16]. Because of intermolecular interactions of thiol groups with the Crown ether's oxygen in upper rim, the structure of 1, 3-dimethoxy, 2, 4-dithiol- Calix[4]crown-5-ether has been supposed to be conformationally locked in cone conformer. Therefore in this study the Calixcrown consists of four benzene rings and a crown ring, which are arranged conically compared to thiol groups. It will also be theoretically proved in this research.

ProLinker™ has been utilized for preparing ProteoChip, which can be used for protein analysis via an SPR or QCM system without probing analyte proteins because of its capability to capture amino acid residues [17-19]. The Calixcrown-chip can serve as a powerful tool with a wide range of applications, including the study of protein-protein interaction, protein-DNA interaction, assaying enzyme activity and also for diagnostic applications with clinical samples from e.g. prostate cancer and HIV patients [5, 18-20]. The major problem in antibody- and antigen- microarray chips has been non-reproducibility of results which takes place because of random orientation of proteins on the chip [21]. Our last researches have shown that Calixcrown interacts with antibody and recombinant protein containing extra Arginine residues and makes them immobilized with right orientation [17]. The vertical orientation of protein molecule can induce a high-density attachment of protein which therefore leads to high specificity and reproducibility with minimal analytical error. We reported that Calixcrown (ProLinker™) on glass slide or Gold slide recognized the amine group of captured proteins through non-covalent interaction and was capable of immobilizing an antibody molecule with right orientation [17]. And, we also showed that the immobilization of yeast 9Arg-GAL4 mutant protein containing 9 extra Arginine residues at N-terminal was much greater than that of the control GAL4 protein on ProLinker™ -coated slide glass [17]. In order to confirm that the amine group of protein plays a key role in protein immobilization on Calixcrown-coated slide chip, Alanine (Ala), Valine (Val), Leucine (Leu), Arginine (Arg), Lysine (Lys), Glutamic acid (Glu), Aspartic acids (Asp) and Histidine (His) α-amino acids have been chosen among the positive charged and negative charged hydrophilic amino acids and also nonpolar amino acids. This study is designed to distinguish the reactions of ProLinker™ with simple single amino acids for interpretation of its reaction site with proteins. It could be supportive for synthesizing more specific and improved linkers.

MATERIALS AND METHODS

Experimental Section: 1, 3-dimethoxy, 2, 4- dithiol-Calix[4]crown-5-ether (purity: 93%, bp: 224°E, MW: 703.0 g/mol) was purchased from Proteogen Company (ProLinker™) . The α-aminoacids Alanine (Ala), Valine (Val), Leucine (Leu), Arginine (Arg), Lysine (Lys), Glutamic acid (Glu), Aspartic acid (Asp) and Histidine (His) were purchased from Sigma Aldrich Company and used without further purification. Association constants (K_A) of the complexation process were determined by titration experiments followed by UV/Vis spectrophotometry. The UV/Vis titrations were measured on an Uvikon 992 UV/Vis scanning spectrophotometer with a Pentium V computer using 10 mm quartz cells.

The complex's spectrum of ProLinker™ and amino acids were recorded in the range of 230-300 nm. The system was thermostated at 25°C by circulating water from an isothermal bath. In all cases, the procedure was repeated at least three times and the resulting average values and corresponding standard deviations are mentioned in the following text and tables.

Procedure: 1 cm^3 of solution with 1.5 mol /dm^3 concentration of 1, 3-dimethoxy, 2, 4- dithiol-Calix[4]crown-5-ether (ProLinker™) in (50%: 50%, V: V) methanol and chloroform solvent was titrated with 20 µL stepwise addition of α-amino acids. The pH did not change the titration. UV/Vis spectrum of the mixture undergoes small changes at 230-260 nm. However, the absorbance changes were sufficient to allow the treatment of the data by the computer program [22].

RESULTS AND DISCUSSION

The host-guest complexes ratio and the stability constant of the complexes were determined based on the changes in absorption spectrum of aromatic rings in the macrocyclic skeleton [23]. The values of the stability constants and thermodynamic parameters for the complexation of α-amino acids with ProLinker™ in solution are given in Table 1. It is well known that these amino acids are protonated at pH=2. To facilitate comparison, results given in the literature by different methods are included [24].

Assuming that the absorbance of the ligand would change upon complexation with amino acids, we performed spectrophotometric measurements. The complex M_pL_q is characterized by its stoichiometry, p and q, where M and L represent each amino acid and each ligand, respectively. To determine the formation constant of complexation, K_S, equation 1 is defined as:

$$pM + qL \rightleftharpoons M_pL_q \qquad K_S = [M_pL_q] / [M]^p[L]^q \qquad (1)$$

The method used to determine the formation constant has been described in a previous report [25]. The absorbance, A, was measured for the solutions, as described in experimental section. For calculating the formation constants, the spectrophotometric titration data were analyzed at a wavelength in UV range which is given by

Table 1: Average values of association constants (M^{-1}) for complexes of ProLinker™ with α-Amino acids in different wavelengths at 25°C (only for which the correlation coefficient are higher than 0.99 are included).

Amino Acid	Association constant (K_A)
Arg	1546±48
Asp	512±15
Glu	507±25
His	502±28
Ala	1662±26
Val	1583±37
Leu	1196±31
Lys	1356±47

Table 2: The 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether (ProLinker™) conformers calculated energies

Host	Method	Kcal/mol	Atomic Unit (a.u)
Cone	HF/6-31g	-1845435.7632	-2940.8913
	B3LYP/6-31+g(d, P)	-1842736.7328	-2936.5901
Partial Cone	HF/6-31g	-1836369.4942	-2926.4433
	B3LYP/6-31+g(d, P)	-1841825.9270	-2935.1386
1,3 Alternate	HF/6-31g	-1840367.5560	-2932.8146
	B3LYP/6-31+g(d, P)	-1842475.6210	-2936.1740

$$A = \epsilon_M[\text{amino acid}] + \epsilon_L[L] + \epsilon_C[\text{complex}] \qquad (2)$$

Where ϵ_M, ϵ_L and ϵ_C are the molar absorptivities of each amino acid, each ligand and the formed complex, respectively. For the mass balance

$$[\text{amino acid}] = C_M - [\text{complex}] \qquad (3)$$

$$[L] = C_L - [\text{complex}] \qquad (4)$$

Where C_M and C_L are the total concentration of each amino acid and each ligand, respectively. Substituting equations 1 and 3 - 4 into equation 2 and rearranging and eliminating the same terms at a wavelength that the amino acid has actually no absorbance yields:

$$A = C_l\epsilon_L - C_M\epsilon_L - C_L\epsilon_M + C_M\epsilon_M + C_L\epsilon_C + C_M\epsilon_C - \epsilon_L/K_S - \epsilon_M/K_S + \epsilon_C/K_S \pm \epsilon_L B \pm \epsilon_M B \pm \epsilon_C B)/2 \qquad (5)$$

Where B is equal to: $(1 + 2C_LK_S + 2C_MK_S + C_L^2K_S^2 - 2C_LC_MK_S^2 + C_M^2K_S^2) / K_S$. Using a suitable computer program [25] the data were fitted to equation 5 for estimating the formation constant of equation 1. We used the Gauss-Newton nonlinear least-squares method in the computer program to refine the absorbance through minimizing sum of squared errors resulted from equation 6,

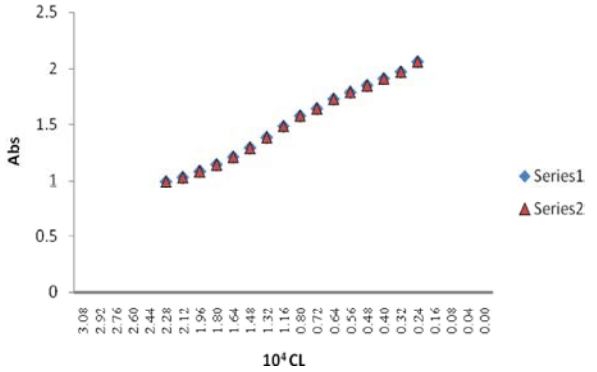

Fig. 3: The graphical fitting for Arginine and Calixcrown system at 25°C and 246 nm, (series 1) experimental corrected absorbance, (series 2) calculated absorbance from the computer program by the fitting method.

$$S = \sum(a_i - b_i)^2 \qquad (i = 1, 2, 3, \ldots) \qquad (6)$$

Where a_i is a quasi-experimental quantity and b_i is a calculated one. The computer program consisted of two different kinds of fittings, i.e. graphical and numerical. The final selection of species was accomplished based on both of these mentioned methods, considering in addition the various statistical criteria, i.e. sums of squared residuals, differences of C_M (experimental) and C_L (experimental) from those of calculated ones. Fig. 3 is illustrated as a typical example of a graphical fitting for the observed and calculated absorbance (from the computer program). A series of UV/Vis spectral titration experiments was undertaken to investigate the possible interactions between the host and each α-amino acid. In absence of the amino acid, 1, 3-dimethoxy, 2, 4- dithiol- Calix [4] crown-5-ether has its maximum absorption at 235 nm, which can be assigned to an intramolecular charge transfer (CT) absorption band. With addition of the amino acids to a solution of receptors in mixed solvent, the characteristic absorption peak of the host at 235 nm gradually decreased and a new absorption peak appeared approximately at 246 nm. It reveals that a complex has been formed. It was checked for other proposed species existing in significant concentrations over a reasonable range of data. Taking into account a binuclear complex alone or together with the mononuclear one does not improve the integrity of the fit and even leads to rejection of the model. The finally-chosen model, formed by ML, resulted in a satisfactory numerical and graphical fitting. The average values of the formation constants for the 1:1 complexes show a sharp breakpoint when the concentration of amino acid to ligand ratios reaches unity.

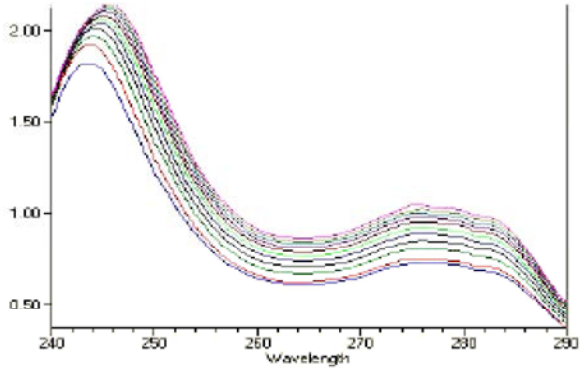

Fig. 4: UV/Vis absorption spectra of ProLinker™ in Chloroform & Methanol upon the addition of various amounts of the Lys equivalents. Nonlinear curve fitting for the change in absorbance at 243 nm with respect to the amount of Lysine added is shown in the inset. The correlation coefficient (*R*) of the nonlinear curve fitting is 0.9968.

It indicates the f ormation of stable complexes for α-amino acids, shown in Fig. 4. Nevertheless, the spectrophotometric titration curves of the ligand by other amino acids represent different absorbance with different affinities. The average values of the formation constants for the 1 : 1 complexes of 1, 3-dimethoxy, 2, 4-dithiol- Calix[4]crown-5-ether versus each α-amino acid at various wavelengths are listed in Table 1. Except Ala and Val due to spherical effects which make the respective complexes more stable than the other α-amino acids being studied in this research, Arg is the strongest amino acid among the hydrophilic charged amino acids and studies have indicated that Arg has the strongest complexation ability toward different Calixarene derivatives too. It means that, the factor which makes Arg complexes more stable is the fixed part in different derivatives of Calixarene, i.e. the phenyl aromatic groups. It has been clearly discussed in the theoretical study when comparing Arg and Lys structures in current research. The binding selectivity of ProLinker™ towards amino acids on the stability constant values of the formed complexes, is in this descending order as Ala > Val > Arg > Lys > Leu > Asp > Glu > His. The favorable electrostatic interactions between the positively charged amino acids and ProLinker™ lead to tight binding in the complexes of Arg and Lys as 1546 $M^{-1}$and 1356 M^{-1}. As expected in the case of Asp and Glu because of unfavorable electrostatic interactions. The lowest K_A 512 M^{-1} and 507 M^{-1} was observed the association constant as 502 M^{-1} is observed for His and 1196 M^{-1} for Leucine intermediate

between the positively charged amino acids Lys and Asp. Ala and Val show quite strong binding to ProLinker™ with K_A of 1662, 1583 M^{-1}.

Our previous studies have shown that ProLinker™ interacts with antibody and recombinant protein containing extra Arginine residues with modified oriented immobilization [17, 22] and it produces a high-density attachment of protein which leads to high specificity and reproducibility in Calixcrown-chip production. As Arg and Lys have similar structures in comparison with other hydrophilic charged amino acids, so it is expected that structure is a more effective parameter for stabilizing the complexes. Therefore, the theoretical study is designed with the purpose of distinguishing the complexation site and steric effects. The results suggest that Arg and Lys, more shielded than the other hydrophilic charged amino acids, fit better with the ligand.

Computational Section: In recent years, the progress in computational facilities provides the scientists the opportunity to study the relatively large and complicated supramolecular systems [26]. Numerous different theoretical researches have been carried out on Calixarene conformation study and also Calixarene derivatives. Also, metal complexes have been analyzed by HF, MP2 and DFT calculations [27-31]. Nonetheless, no much study has been performed on Calixcrown and amino acids.

The initial conformations of 1,3-dimethoxy 2,4- di thiol-Calix[4]crown-5-ether host firstly optimized by semi-empirical AM1 and PM3 energy minimization in Hyperchem software (version 8.0, Hyper Cube Inc.) was used for initial geometry optimization and calculation of the binding energies. Using conjugate gradient optimization method (Polad-Ribiere algorithm), the gradient was estimated to be 0.05 kcal. In order to reach the global minimum, some structural parameters of the initially optimized molecules were changed manually and the molecular structures were re-optimized. This procedure was repeated until the most stable structure was obtained [32]. In the next step, HF/6-31G optimizations of various conformers of host have been done by Gaussian 98 software [33]. It took more than 800 hours to reach a minimum value of error limit (less than 0.01 kcal/mol for each conformer) as shown in Fig. 5. When the front views are observed (Fig. 5 (b), (d) and (f)) of three different conformers of free ProLinker™, some of the oxygen atoms in the crown-5-ether derivative are not converged to the center of the crown-ether ring and are randomly pointing outward. After optimization of the conformers, B3LYP/6-311G (d, p) single point calculation

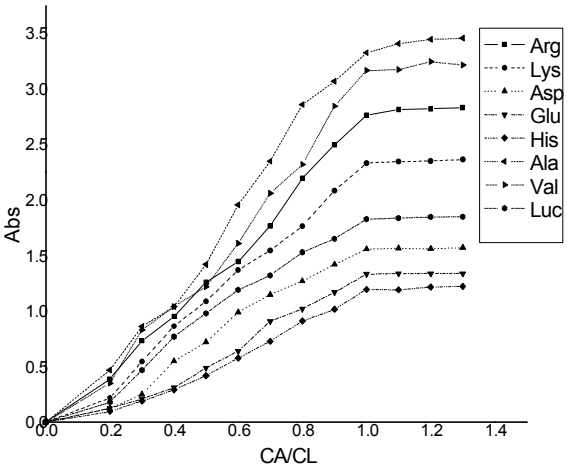

Fig. 5: Spectrophotometric titration plots of 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether toward protonated α-Amino acids at 25°C and 246 nm.

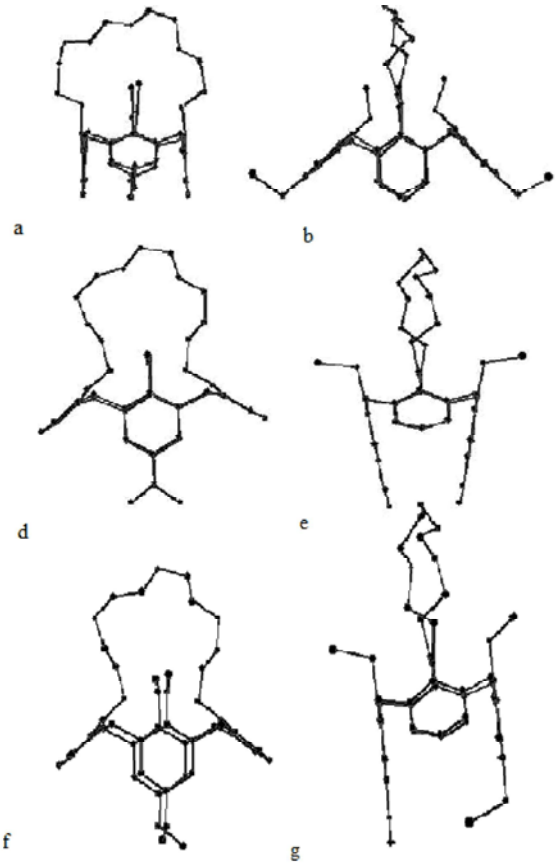

Fig. 6: Ab initio optimized structures of 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether (a) Side view and (b) front view of cone conformer, (c) side view and (d) front view of 1, 3-alternate conformer,(e) side view and (f) front view of partial cone conformer.

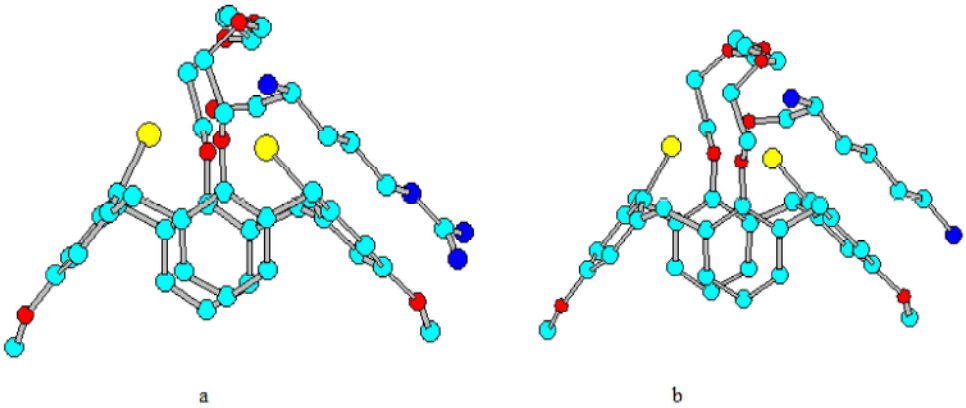

Fig. 7: Simulated configuration of Arginine-ProLinker™, Lysine-ProLinker™ optimized complexes. Cyan, red, blue and yellow balls represent carbon, oxygen, Nitrogen and sulfur atoms respectively.

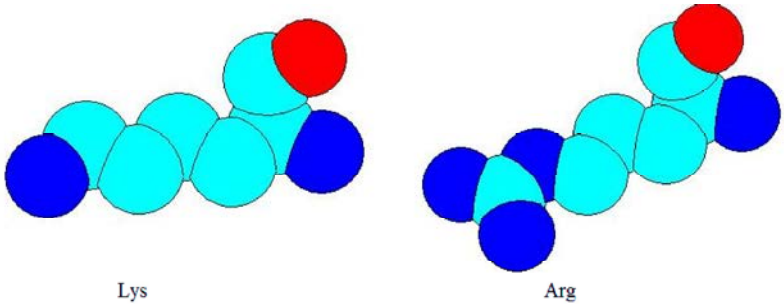

Fig. 8: Three-dimensional structures of Lysine and Arginine. There are three methylene carbons separate the primary ammonium ion from the secondary ion in protonated Arginine in compare to four methylene carbons separate the primary ammonium ions in protonated Lysine.

(w100 h) of the final structure was performed in order to include the effect of electron correlation and adding the polarization function as well as to reduce the basis sets superposition error (BSSE) [34]. To verify that the resultant structure was not located in local minimum point, the normal mode frequency calculation was carried out for the optimized host using HF/6-31G method. The calculation was achieved by a computer system which contained 4 parallel-processing CPUs. It was possible to determine the vibrational frequencies of the optimized structure after two weeks of non-stop calculation. Each vibrational spectrum contained no negative frequencies; therefore, it was confirmed that the optimized structure is really in minimum point.

Also, the relative binding affinity study of cone-shaped 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether toward Lysine and Arginine protonated imine tatomer [35-40] was carried out. Focusing on the binding site of crown ether moiety of the host molecule, we employed B3LYP/6-31G (d) //HF/6-31G calculation method [41]. The calculations have shown (Fig. 6) that the primary amine ion which interacts with the crown ether makes a more stable complex compared to other probable complexes and it is the same between all studied α-amino acids in this research, but differences raised from π electrons of the aromatic rings which are significant enough to make Arg and Lys complexes more stable than other amino acids. The complexes are more stable when three methylene carbons are between the amine groups (Fig. 6). Distance from the centroid symmetry plane that contains the crown ether function to the phenyl π electrons is 3.92342 Å and the distance between amine groups in Arginine is 3.92361 Å. Therefore, this matching makes Arginine complexes more stable than Lysine ones with 5.10196 Å distance between the amine groups. The interaction of protonated secondary amine groups with π electrons of the aromatic rings increases Arginine stability energy, so it is more stable than Lysine. Since there exist four aromatic rings versus one crown ether in each host molecules, so the complexation reaction of Arginine is more probable and stable than Lysine.

CONCLUSION

The energy of complexation between the ProLinker™ molecule and protonated amino acids was shown by molecular modeling and UV/Vis spectroscopy study in order to recognize the complexation site. The binding selectivity of 1, 3-dimethoxy, 2, 4- dithiol- Calix [4] crown-5-ether towards amino acids based on the stability constant values of the formed complexes in chloroform and methanol mixed solvent (50%: 50%, V: V) by means of UV/Vis spectrophotometry titration has been investigated. In present study, it has been demonstrated that the stability constants are in this descending order as: Ala > Val > Arg > Lys > Leu > Asp > Glu > His. Comparison of the association constants yields that spherical effects make Ala and Val complexes stronger among these series of α-amino acids studied in this research. Favorable electrostatic interactions make Arg and Lys complexes very stable. The association constants of Glu, Asp and His were almost the same with one-third value of that of Arg. Leu association constant is intermediate between respective values of Lys and Asp. The positive effect of the interaction between protonated amino group and π electrons of the aromatic rings in complexation stability has been shown as well. Furthermore, the negative effect of unfavorable electrostatic interactions in complexation was studied which changes the stability of Asp and Glu complexes. The structures of three different conformers have been optimized via *ab initio* (HF/6-31G) calculations and the geometry optimization of the final structures was provided by single point calculation B3LYP/6-31G (d, p). *Ab initio* B3LYP/6-31 G(d, p) calculations indicates that the comparative stability of different conformers of 1, 3-dimethoxy, 2, 4- dithiol- Calix[4]crown-5-ether is in following order: cone > partial-cone > 1,3-alternate conformer.

Given that using 1, 3-dimethoxy, 2, 4- dithiol-Calix[4]crown-5-ether (ProLinker™) as biolinker in our previous study showed that the recombinant protein containing extra Arginine residues was better immobilized in Calixcrown-chip, hence for reaction site investigation the Arginine and Lysine complexes were computationally searched via ProLinker™. The difference between Arginine and Lysine complexes arises from the distance between protonated amine ions. When three methylene carbons separate the protonated amine ions, the stabilization energy becomes slightly greater due to somewhat inefficient overlap of the protonated secondary amine ions with π electrons of the aromatic rings.

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