Interaction of Aldehyde dehydrogenase with acetaminophen as examined by spectroscopies and molecular docking

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ABSTRACT

The interaction of acetaminophen, a non-substrate anionic ligand, with Aldehyde Dehydrogenase was studied by fluorescence, UV–Vis absorption, and circular dichroism spectroscopies under simulated physiological conditions. The fluorescence spectra and data generated showed that acetaminophen binding to ALDH is purely dynamic quenching mechanism. The acetaminophen-ALDH is kinetically rapid reversible interaction with a binding constant, $K_a$, of $4.91 \times 10^3$ L mol$^{-1}$. There was an existence of second binding site of ALDH for acetaminophen at saturating acetaminophen concentration. The binding sites were non-cooperative. The thermodynamic parameters obtained suggest that Van der Waal force and hydrogen bonding played a major role in the binding of acetaminophen to ALDH. The interaction caused perturbation of the ALDH structures with an obvious reduction in the $\alpha$-helix. The binding distance of 4.43 nm was obtained between Acetaminophen and ALDH. Using Ficoll 400 as macro-viscosogen and glycerol as micro-viscosogen, Stoke-Einstein empirical plot demonstrated that acetaminophen-ALDH binding was diffusion controlled. Molecular docking showed the participation of some amino acids in the complex formation with $\sim 5.3$ kcal binding energy. With these, ALDH might not an excipient detoxifier of acetaminophen but could be involved in its pegylation/encapsulation.

1. Introduction

Aldehyde dehydrogenases (ALDH; EC 1.2.1.3) are short-chain dehydrogenases/reductases (SDR) superfamily containing NAD(P)$^+$-dependent enzymes that catalyse the irreversible dehydrogenation of a wide range of endogenous and exogenous aldehydes to their corresponding less toxic carboxylic acids [1–3]. ALDHs are widely distributed in prokaryotic and eukaryotic cells and play important roles in detoxification of toxic and reactive aliphatic and aromatic aldehydes formed during the metabolism of alcohols, amino acids, carbohydrates, lipids, biogenic amines, vitamins and steroids [4]. Currently, there are 19 known members of the ALDH superfamily [5,6]. ALDHs functional and physiological properties have been studied extensively and are involved in the maintenance of cellular homeostasis, modulate cell proliferation, differentiation, survival and cellular response to oxidative stress [1,7,8]. ALDHs play essential role in the metabolic pathways that are critical for cell development and response to environmental changes [9].

ALDHs are homo-biopolymers composed of two or four polypeptides of 50–55 kDa, and made up of N-terminal NAD$^+$-binding domain, a catalytic domain and an oligomerisation domain [10,11]. Aldehyde dehydrogenases kinetic mechanism is literarily an ordered sequential kinetic mechanism with NAD(P)$^+$ binding first, followed by the aldehyde [12–14]. In some cases, it is random kinetic mechanism with preference for initial binding of NAD(P)$^+$ [15]. The ternary complex forms thio-hemiacetal intermediate which is transformed to thioester by giving its hydride ion to NAD(P)$^+$. Eventually, the thioester is hydrolysed by a water molecule to carboxylic acid. The sequential dissociation of carboxylic acid and NADH, which is the rate-limiting step, ends the reaction [14,16].

ALDHs exhibit additional, non-enzymatic functions, the non-catalytic binding properties for endobiotics, some hormones and other small molecules [1,17]. It is 'housekeeping' functions linked with detoxification. This is associated with the ubiquitous, ample and constitutively expressed properties of the enzyme. These ligand binding properties might be connected to protective function through the sequestration of metabolites. They conceivably serve to prevent the accumulation or minimize potentially toxic free endobiotics and xenobiotics or involved in the uptake and transport of hydrophobic non-substrate prior to its detoxification. Catalytic and ligand complexing properties (ligandin) are important for detoxification mechanism [1] and there is connection in both [17]. Although ALDH catalytic mechanisms of detoxification have been investigated extensively, however, relatively little is known about its non-catalytic binding function.

Acetaminophen (N-acetyl-p-aminophenol, AAP) (Fig. 1) is a medically important, low cost, readily available and commonly used over
the counter analgesic and antipyretic drug [18,19]. Acetaminophen monotherapy is efficient and is safer than Aspirin and Ibuprofen [20]. The efficacy and tolerability in individual condition is warranted [18]. The mechanism of analgesic action of acetaminophen is complex and its action of medicament has not been completely understood [20]. At therapeutic doses, acetaminophen is safe drug but not devoid of side effects [18] and suggest the possibility of acetaminophen exerting other specific biological effects [21]. High dosages, in humans and experimental animals, lead to necrosis, nephrotoxicity, and extra hepatic lesions [22]. Nevertheless, it is grossly abused in Nigeria and it has been blamed for the rising cases of heart attacks, stroke and early death [23,24]. The negative effect of Acetaminophen on the antioxidant defense enzyme system has been documented [23]. The interaction of acetaminophen with Human Serum Albumin (HSA) was previously investigated [25]. The authors detailed the biochemical and biophysical data illustrating the relevance of HSA to the acetaminophen pharmacokinetics. However, in a pathogenic state of human serum albumin, lower albumin concentration and weaker drug–protein interaction can result in the increase of drug concentration in the blood and lead to toxicity [26,27]. More worrisome, is the use of acetaminophen with alcoholic beverages [21,28].

The link between Aldehyde dehydrogenase and Acetaminophen metabolism is becoming increasingly imaginable [7,21]. ALDH has been identified as a major acetaminophen-binding protein [28]; and was down regulated in mouse liver exposed to high dosage of acetaminophen [29]. However, the affinity and interaction mechanism of acetaminophen to ALDH still remain uncharted. The effect of the complexation on ALDH structure and conformation is yet to be elucidated.

Several spectroscopic techniques, as powerful tools, have been used to study the interaction between drugs and proteins. They allow non-intrusive measurements of substances in low concentration under physiological conditions [30]. Fluorescence technique is the simplest method to study the interaction of drugs/ligands and bio-macromolecules because it has the advantage of high sensitivity, rapidity and ease of implementation [31,32]. It is an important method to sense changes in the local microenvironment of fluorescent chromophore [33] and help understand the biopolymer’s binding mechanisms to drugs and provide clues to the nature of the binding phenomenon [34,35].

The information on the acetaminophen-ALDH binding mode, the binding constant and the effects of acetaminophen complexation on the protein structure is obscured. In the present work, the binding of Acetaminophen to ALDH was studied under physiological conditions by spectroscopic techniques. The quenching mechanism between Acetaminophen and ALDH with regards stoichiometric and thermodynamic of ligand binding and consequently the effect on the protein conformation were investigated at molecular level. In addition, the effects of pH and viscosity of Acetaminophen -ALDH complex were also examined. All these were complimented by in silico analysis and molecular docking.
2.2.3. Effect of viscosity

Efforts to probe the effects of solution viscosity upon ALDH-acetaminophen association constant, $K_a$, were explored using glycerol as microviscosogen and Ficoll 400 as macro-viscosogen. Viscosities were determined relative to a solution containing only buffer (25 mM potassium phosphate buffer, pH 7.4) using all glass Ostwald viscometer at 25 °C. The resulting data were fitted into Stoke-Einstein empirical relationship of \[ (K_a)^n/K_a = \left(\frac{n_p}{n_f}\right)^m \]. Superscript o indicate absence of viscosogen. Exponent of 1, according to the formula is maximum diffusion-limited binding.

2.2.4. Acetaminophen-ALDH molecular modeling and docking

The docking analysis of acetaminophen molecule with yeast aldehyde dehydrogenase docking was carried out using AutoDock Tools (ADT v1.4.2) and AutoDock Vina. Bakers yeast aldehyde dehydrogenase “Fasta” file (accession ID = AAA34419.1) was retrieved from www.pubmed.org and used to model the starting structure of Bakers yeast aldehyde dehydrogenase. Homology modeling was done using SwissModel Server (http://swissmodel.expasy.org). The coordinate file of template from protein data bank (PDB ID: 1BXS.1) with 31.89% sequence identity was used to model the 3D structure of yeast aldehyde dehydrogenase. The quality of protein model was done using ERRAT. Acetaminophen structure was retrieved from Pubchem databases, (CID 1983). In (SDP) format and then converted to Protein Data Bank (PDB) coordinates using the Open Babel (http://openbabel.org). Ligand binding site calculation was performed on BSP-SLIM server (http://zhanglab.ccb.med.umich.edu/BSP-SLIM/). The modeled structure of aldehyde dehydrogenase molecule and acetaminophen were loaded on BSP-SLIM server to identify the binding pocket and pose of the ligand. The best pose with docking score of 2.501 was selected. BSP-SLIM is known as a blind docking method, which primarily uses the structural template match to identify putative ligand binding sites, followed by fine-tuning and ranking of ligand conformations in the binding sites through the SLIM-based shape and chemical feature comparisons. The consistency of the docking results was first checked prior to docking of acetaminophen by comparing the best docking poses retrieved from BSP-SLIM server. This was done by removing the ligand from the binding site and subjecting again to re-docking into the binding pocket in the conformation found in the structure retrieved from BSP-SLIM server. Thus, a RMSD of 0.819 Å was obtained signifying that the docking procedure could be relied upon to predict the binding mode of our compounds.

2.3. Statistical analysis

All kinetic, statistical and graphical analysis for ALDH-Acetaminophen characterization was performed using KaleidaGraph 4.5 software (Synergy software, Reading, PA, USA) for Macintosh Computer.

3. Results and discussion

3.1. Effect of acetaminophen on fluorescence spectrum of ALDH

Fluorescence spectra provide a sensitive and veritable means to characterize the biopolymer and their conformations [33]. ALDH intrinsic aromatic fluorophore was used to obtain information about conformational changes associated with interaction between ALDH and acetaminophen. ALDH has a strong fluorescence emission at 346 nm upon excitation at 280 nm. This is unconnected with to exposed tryptophan fluorescence due to solvent relaxation. So also, the fluorescence emission, peak, shape and intensity of ALDH are not unconnected with microenvironment position of the intrinsic fluorophores due to solvent relaxation [36]. The addition of acetaminophen, as a ligand, caused fluorescence quenching of ALDH fluorescence emission spectra (Fig. 2); and the quenching solely depend on the concentration of the ligand. The quenching was effective with average efficiency above 75%. This observation strongly indicates binding of acetaminophen with ALDH. This is not unusual. ALDH has identified as a major acetaminophen binding protein [28]. Acetaminophen was a non-fluorescent at the 280 nm excitation wavelength and has weak UV absorption at 280 and 346 nm. The inner filter effects caused by the absorption of acetaminophen were corrected. Fluorescence quenching is a decrease of the quantum yield of fluorescence from a fluorophore due to a variety of molecular interactions: excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching [36,37].

The fluorescence quenching were analyzed using the Stern-Volmer equation:

$$F/F_0 = 1 + K_{sv} [Q] = 1 + K_q [Q]$$  \hspace{1cm} (1)

$$K_q = K_{sv}/F_0$$  \hspace{1cm} (2)

where $F$ and $F_0$ are the intensity of fluorescence intensities with and without quencher, respectively. The $K_q$, $K_{sv}$, $\tau$ and [Q] are the quenching rate constant of the biomolecule, the quenching constant, the average life time of the biomolecule without quencher and the concentration of quencher, respectively. $K_{sv}$ is the slope of linear regressions and were analyzed at different temperatures (288, 293, 298, 303, 308 K). The Stern–Volmer plots (F/F against [Q]) were initially linear and later become exponential at above 35 μM (Fig. 3a). An initial linear slope of Stern Volmer plot is generally indicative of a single class of fluorophores, which are all equally accessible to the quencher [38]. The structural vicinity of the acetaminophen –OH group as a major acetaminophen binding site. The Stern Volmer constant is directly proportional to the temperature, indicating that it was a dynamic quenching mechanism (Fig. 3c). Dynamic quenching refers to a process that the fluorophore and the quencher come into contact during the transient existence of the excited state [36,37]. Dynamic quenching depends upon diffusion. However, at higher concentrations (above 35 μM of acetaminophen), the results depart from the initial linearity and demonstrated both static and dynamic quenching. The quenching rate constants, $K_q$, was calculated using the above equation. The values of $K_{sv}$ and $K_q$ are listed in Table 1. Generally, the maximum scatter collision quenching constant, $K_{sv}$, of various kinds of quenchers with biopolymer is $2 \times 10^{10}$ L mol$^{-1}$ s$^{-1}$. 
However, the rate constants for the quenching of ALDH caused by acetaminophen are less than the $K_q$ for the scatter mechanism. This demonstrates that the fluorescence quenching is not the result of static collision quenching, rather a consequence of dynamic quenching [41]. It showed that the binding constant between acetaminophen and ALDH increases with the increase of temperature, resulting in a reduction of the stability of the acetaminophen–ALDH complex. It concluded that acetaminophen was a good quencher of ALDH intrinsic fluorophores.

### 3.2. Equilibrium binding stoichiometry and parameters

Using the intrinsic fluorescence decrease, the association constants $K_a$ of acetaminophen-ALDH complex at different temperatures and number of binding site can be obtained from the regression of:

$$\log (F_0 - F/F) = \log K_a + n\log(Q)$$

Using Job’s plot [43] gave syllogistic evidence that the stoichiometric ratio ALDH-acetaminophen at 25 °C and pH 7.4 is 1:1.
The interaction of acetaminophen with Human Serum albumin has previously been studied [25]. The results indicated that the interaction of acetaminophen with HSA is stronger than ALDH- acetaminophen complex. The reason might be connected to the structure of the protein. The distinct $K_a$ values of HSA-acetaminophen and ALDH-acetaminophen showed that acetaminophen is loaded more strongly by HSA, which is crucial for transportation than detoxification. The number of binding site in concentration of acetaminophen-ALDH and acetaminophen-HSA were similar [25]. A consistent 2:1 stoichiometry. The binding parameters are helpful in the design of dosage forms and pharmacokinetics between therapeutics and toxicity. The stoichiometry of binding apparently varies according to the size of the ligand. Large ligands have less stoichiometry of ligand per bio-macromolecules [44]. At pH 7.4, there is possibility that the conformation of ALDH and ligand-steric effects might explain the 1:1 binding stoichiometric of acetaminophen to ALDH. However, the issue still remains if the acetaminophen binds distantly from the active site and/or possibly at a site peripheral to the recognized substrate cavity. There is possibility of coordination of phenyl group of acetaminophen with hydrophobic residues of ALDH.

### 3.3. Thermodynamic parameters of Binding modes

Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for this interaction. Therefore, The thermodynamic parameters dependent on temperatures were analyzed in order to characterize the acting forces between ALDH and acetaminophen during the quenching process. The thermodynamic parameters were calculated from the Van’t Hoff plots. The temperatures were ranged between 288 to 313 K. The plot of log $K_a$ versus $1/T$ (T, absolute temperature) allows the determination of $\Delta H$ and $\Delta S$ using Eq. (4):

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{4}$$

The free energy change ($\Delta G$) was estimated from the following relationship:

$$\Delta G = \Delta H - T\Delta S = -RT\ln K_a$$ \tag{5}

(Figure not shown). However, the stoichiometric ratio increases to 2:1 when the concentration of acetaminophen was above 35 μM with a slight increase of $K_a$. This glaringly showed the existence of a second binding site of ALDH for acetaminophen. The multiple binding site underscored the exceptional capability of the enzyme as regulator of intracellular and intercellular fluxes.

The enthalpy change ($\Delta H$) was calculated from the slope of the van’t Hoff relationship (Fig. 5) Here, there was a good linear relationship between ln $K_a$ and reciprocal absolute temperature, 1/T. $R$ is the universal gas constant (8.314 J mol⁻¹ K⁻¹). The thermodynamic values ($\Delta G$, $\Delta H$ and $\Delta S$) were obtained from the slopes and the ordinates at the origin of the fitted lines are presented in Table 2. At pH 7.4, the formation of the complex was an exothermic reaction accompanied by negative $\Delta S$ value. From Table 3 it can be seen that $\Delta H$ and $\Delta S$ have negative value (−22.32 kJ/mol) and negative value (−119.24 J/mol K), respectively. The positive sign for $\Delta G$ means that the binding process was non-spontaneous. The ALDH-acetaminophen, at pH7.4, is enthalpically favourable and entropically unfavorable (negative $\Delta T\Delta S$). The interaction of drug with protein has been reported to be an entropically unfavorable process in aqueous conditions [27]. The net balance in the solvation free energies of acetaminophen and ALDH from the Acetaminophen-ALDH provides the binding free energy of the acetaminophen with ALDH. There are essentially four types of non-covalent interaction existing between quencher and biological macromolecules; and are hydrogen, Van der Waals and electrostatic and hydrophobic forces [45].

### 3.4. pH dependence of acetaminophen-ALDH binding

ALDH must acquire a unique conformation in order to be function-
ally effective catalytically, pH change tends to alters the conformation of enzyme and hence could affect the association constant of ligand binding [49]. This will assumed we consequently affect the energetics of binding. The influence of acidic pH (5.0) and alkaline pH (9.0) on the interaction between acetaminophen and ALDH was explored. The result is shown in Table 4. The stoichiometric of binding was altered. The bonding was non-spontaneous at pH 9.0 and is essentially hydrophobic bonding. The bonding did not change as at pH of 5.0 compared to physiological pH of 7.4. The significant change is that it is more enthalpically driven at pH 5.0 compared to the entropically motivation at pH of 9.0. The reason for this is not immediately clear but pH 5.0 is outside the ALDH enthalpy of ionization and its optimum pH. The lowering of the pH which increases the rate of agonist-induced conformational change is consistent with the hypothesis of acidification, and thus presumably protonation of one or more amino acids. This might lessen the responsiveness of ALDH for acetaminophen and thus perhaps reflecting the lower stability of the ALDH. The binding stoichiometric between ALDH-acetaminophen was not affected by the change in pH either to 5 or 9.

The co-operativity of the binding or otherwise, when n > 1, was assessed on the assumption that ALDH with equal and independent n site, could have a characteristic association constant, $K_a$, for the acetaminophen, L. Then, the saturation fraction, $Y$, was expressed as:

$$ Y = \frac{\Delta F}{\Delta F_{\text{max}}} = \frac{K_a [L]}{1 + K_a [L]} $$

(6)

where $\Delta F$ indicates the fluorescence-quenching change observed at non-saturating ligand concentrations of acetaminophen, and $\Delta F_{\text{max}}$ is the maximum fluorescence-quenching variation detected at saturating ligand concentration. Where $L$ is the free concentration of Acetaminophen which can be derived from:

$$ [L] = [L]_0 - nY[\text{ALDH}] $$

(7)

where $[L]_0$ and [ALDH] are the total non-enzyme bound ligand and protein concentrations, respectively. The plot at pH 7.4 (not shown) gives the best fit to the data for a non-cooperative model.

3.5. Analysis of synchronous fluorescence

Synchronous fluorescence spectroscopy was used to investigate the acetaminophen-ALDH complex. The synchronous fluorescence spectra of ALDH provide the characteristic information for the Try residues and Trp residues when the wavelength interval $\Delta \lambda$ ($\Delta \lambda = \lambda_{em} - \lambda_{ex}$) is fixed at 15 and 60 nm, respectively [50]. The synchronous spectra are shown in Fig. 6. The ALDH synchronous fluorescence intensity is affected by acetaminophen concentration. This further demonstrated the occurrence of fluorescence quenching in the binding. It is apparent that the maximum emission wavelength red-shifts (from 284 to 294 nm) at the investigated concentrations range when $\Delta \lambda = 60$ nm and red shift (from 295 to 305 nm) when $\Delta \lambda = 15$ nm. The red-shifts effect implied that the interaction of acetaminophen affect the micro-environment around the Tyr and Trp residues of ALDH conformation. The polarity around the tryptophan residues was increased and the hydrophobicity was decreased. This exaggerated the results deduced in Fig. 2. ALDH tryptophan residues hydrophobicity was obvious based on maximum emission wavelength (60 nm) and was more sensitive to change while the microenvironment around the tyrosine residues has less discernable change during the binding process. It was apparent that the fluorescence of tyrosine residues was weak. We reckoned that fine linearity of the Stern Volmer quenching and a singular binding site derived from Scatchard plot in the same condition and conclude that acetaminophen would bind a hydrophobic cavity within the vicinity of ALDH tryptophan residue and consequently affect the conformation of ALDH. Fig. 7.

3.6. Dissociation constant $K_d$

Ligand-ligand binding interaction is a kinetically rapid reversible interaction [27]. The reversibility is a function of association constant ($K_a$), dissociation constant $K_d$ and binding free energy ($\Delta G$). The net balance between these dictates the possible ligands/drugs transportation or immobilization, or metabolism or toxicity [27]. Dissociation constant $K_d$ was calculated as described elsewhere [49].

$$ \Delta F = \frac{\Delta F_{\text{max}} [L]}{K_d + [L]} $$

(8)

The equation was linearized using

$$ [L]/\Delta F = \frac{(K_d/\Delta F_{\text{max}}) + ([L]/\Delta F_{\text{max}})}{[L]/\Delta F_{\text{max}}} $$

(9)

where $\Delta F_{\text{max}}$ is the maximum decrease in fluorescence observed when the enzyme is saturated by acetaminophen. The plot of $\Delta F$ against the ligand (Acetaminophen) concentration obey a Michaelis-Menton equation at all temperature and pH examined using Eq. (6) and was linear using the Hanes-Woolf plot (Eq. (9)) from the plot of $[L]/\Delta F$ against $[L]$. The concentration of the ligand was ranged to 125 μM. Acetaminophen exhibited a distinct $K_d$ value within the concentration range. This thus

### Table 3
Thermodynamic Parameters of Acetaminophen-ALDH interaction at pH 7.4.

| T (K) | $\Delta H$ (kJ mol$^{-1}$) | $\Delta S$ (J mol$^{-1}$ K$^{-1}$) | $\Delta G$ (kJ mol$^{-1}$) |
|------|------------------|-----------------|------------------|
| 288  | 12.62            |                 |                  |
| 293  | 12.62            |                 |                  |
| 298  | -22.32           | -119.24         |                  |
| 303  | 13.81            |                 |                  |
| 308  | 14.41            |                 |                  |
| 313  | 15.00            |                 |                  |

### Table 4
Association constants $K_a$ number of binding sites (n) and relative thermodynamic parameters of the Aldehyde dehydrogenase – acetaminophen system from 15 to 35 °C at pH 5.0 and 9.0.

| T (K) | $K_a$ (μM$^{-1}$) | n | $\Delta H^*$ (kJ mol$^{-1}$) | $\Delta S^*$ (J mol$^{-1}$ K$^{-1}$) | $\Delta G^*$ (kJ mol$^{-1}$) |
|------|------------------|---|-----------------|-----------------|------------------|
| 288  | 9.74             | 2.1| 11.21           |                 |                  |
| 293  | 5.15             | 1.9| 12.47           |                 |                  |
| 298  | 4.59             | 1.9| -61.14          | -251.22         |                 |
| 303  | 2.39             | 1.9| 14.98           |                 |                  |
| 308  | 1.81             | 2.1| 16.24           |                 |                  |
| 313  |                  |   |                  |                 |                  |

* $K_a$ = association constant
* n = number of binding sites
* $\Delta H^*$ = Enthalpy change
* $\Delta S^*$ = Entropy change
* $\Delta G^*$ = Gibbs' free energy change
further confirms that acetaminophen has a dissociation mode with ALDH. The $K_d$ value at other temperatures and pH were also estimated. The $K_d$ values were affected by pH and temperature changes. The thermodynamic of dissociation was calculated from Van Hoft equation using Eqs. (4) and (5). The thermodynamics of dissociation were ultimately affected by the change in pH (Table 5). From this, association of ALDH-acetaminophen complex is more favourable than its dissociation.

### 3.7. UV–Visible absorption spectroscopy and Circular Dichroism

UV–Vis absorption spectroscopy and Circular Dichroism were used to further explore protein structural changes. The UV–Vis absorption spectra of ALDH in the absence and presence of acetaminophen is shown in Fig. 8. Complex formed between acetaminophen and ALDH was evident from the data of UV–Vis absorption spectra. ALDH has two absorption peaks, the absorption peak at 208 nm showed the conformation of the peptide bonds, while the peak of 272 nm be evidence of the aromatic amino acids [51]. The maximum peak position of the acetaminophen -ALDH was clearly visible. The red shift indicated acetaminophen changed the peptide strands of the ALDH, the skeleton of acetaminophen became loosen and the hydrophobicity decreased [52]. The absorption peak at about 278 nm can provide us with information about the three buried aromatic amino acids: tryptophan, tyrosine, and phenylalanine. With the concentration of acetaminophen increasing, the absorption peak at 278 nm increases.

### Table 5

| $T$ (K) | $\Delta H^\circ$ (kJ mol$^{-1}$) | $\Delta S^\circ$ (J mol$^{-1}$ K$^{-1}$) | $\Delta G^\circ$ (kJ mol$^{-1}$) |
|---------|---------------------------------|--------------------------------------|-----------------------------|
| 288     | 43.76                           | -23.36                               | 22.72                       |
| 293     | 44.57                           | -171.7                               | 23.46                       |
| 303     | 46.19                           | -20.02                               | 24.2                        |
| 308     | 46.99                           | -161.7                               | 25.68                       |
| 308     | 26.98                           | -148.4                               | 24.94                       |
| 308     | 12.82                           | -49.18                               | 27.72                       |

$\Delta H =$ Enthalpy change
$\Delta S =$ Entropy change
$\Delta G =$ Gibbs free energy change

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increased, the ALDH molecules gradually become less compact without necessarily denaturing ALDH. This might affect the activity of the enzyme.

Changes in the ALDH secondary structure conformation from acetaminophen complexation was explored using CD spectroscopy. The CD measurements were expressed in terms of mean residue ellipticity (MRE) in deg cm$^2$ dmol$^{-1}$, which can be estimated with Eq. (10). The conformational information of ALDH in the absence and presence of acetaminophen is shown in Fig. 9. The two negative bands at 208 and 222 nm as well as a strong positive band at 200 nm indicate a significant amount of both α-helix and β-sheet structures [53]. Obviously, acetaminophen had a marked effect on the ellipticity of ALDH structure. This might not be unconnected to ALDH activity reduction. However, the slight alteration in the CD spectra together with a significant decrease in the fluorescence intensity was observed. Acetaminophen caused a notable increase in the intensity of the bands at 208 and 222 nm. The α-helical content was calculated from Eq. (11) [54,55]; (Fig. 10)

\[
MRE = \frac{\text{Observed CD} (\text{m. deg})}{C_p n l \times 10}
\]

where $C_p$ is the molar concentration of the protein, $n$ is the number of amino acid residues and $l$ is the path length:

\[
\alpha - \text{helix}(\%) = \frac{\text{MRE}_{208} - 4,000}{33,000 - 4000} \times 100
\]

where MRE$_{208}$ is the observed MRE value at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm, and 33,000 is the MRE value of a pure α-helix at 208 nm. From the above equations, the α-helix content of ALDH in absence and presence of acetaminophen was calculated. The content of α-helix decreased from 41.4% to 36.6% when acetaminophen was added up to 150 μM. The decrease of α-helix content indicates that acetaminophen combines with the amino acid residues of the main polypeptide chain of the protein and alters secondary structure bond [56] and loses native secondary structures as α-helix and β-sheet elements are converted to random coil and/or turn. The protein skeleton of ALDH became looser, the amino acid residues were exposed, and the hydrophobicity decreased. The possibility of ALDH-acetaminophen modifying the kinetic and thermodynamic stability ALDH still remains a mystery.

3.8. Energy transfer from ALDH to acetaminophen

Fluorescence resonance energy transfer (FRET) is a convenient ‘spectroscopic ruler’ for measuring molecular distances in biological and macromolecular systems by exploring the fluorescence emission from a donor to be absorbed by an acceptor [57,58]. Energy transfer is likely to happen consequent upon (1) the donor can produce fluorescence light; (2) fluorescence emission spectra of the donor and UV–Vis absorption spectra of the acceptor have more overlap; (3) the distance between the donor and the acceptor is 2–8 nm [59]. The energy transfer effect is related not only to the distance between the acceptor and the donor, but also to the critical energy transfer distance. The spectral studies have revealed that the ALDH could form a complex with acetaminophen. The distance between the donor (ALDH) and the acceptor (acetaminophen) can be calculated according to the Förster’s non-radiative energy transfer theory using this equation [60,61]:

\[
E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6}
\]

where $R_0$ is the Förster critical distance when the transfer efficiency is 50%, and $r$ is the distance between the donor and the receptor. $F$ and $F_0$ are the fluorescence intensities of ALDH in the presence and absence of
acetaminophen, respectively:

\[ R_0^2 = 8.79 \times 10^{-25} K^2 \frac{\phi}{N} \]  

(13)

\( K^2 \) is the spatial orientation factor of the dipole related to the random distribution of the donor and the receptor. \( N \) is the refractive index of the medium. \( F \) is the fluorescence quantum yield of the donor and \( J \) is the overlap integral between the fluorescence emission spectra of the donor and the absorption spectra of the receptor, which can be calculated by the equation:

\[ J = \sum \frac{F(\lambda)\varepsilon(\lambda)\Delta\lambda}{\Delta\lambda} \]  

(14)

where \( F(\lambda) \) is the donor fluorescence intensity at the wavelength of \( \lambda \), and \( \varepsilon(\lambda) \) is the molar absorption coefficient of the receptor at the wavelength of \( \lambda \). In the above equations, \( k^2 = 2/3, N = 1.336, \) and \( \phi = 0.15 \) [62]. From Eqs. (12)–(14), \( J, R_0, E \) and \( r \) were calculated and are shown in Table 6. The binding distance was \(< 7 \, \text{nm} \) and \( 0.5 \, R_0 < r < 2.0 \, R_0 \). The results showed that the non-radiative energy transfer occurred between acetaminophen and ALDH.

3.9. Viscosity effect

Studies of the binding of Acetaminophen to ALDH as a function of solution viscosity were carried out to determine the possible contributions from the viscous solution. The effects of Ficoll 400 and Glycerol (viscosity-induced macromolecules) on the binding of Acetaminophen to ALDH was monitored and shown in Fig. 11a and b, respectively and the relative \( K_a \) values plotted as a function of the relative viscosity. The sensitivity of this binding constant \( K_a \) to viscosity was calculated from the exponential of such plot. It showed that \( K_a \) value decreases with increasing viscosity. Plot of the reciprocal of the relative catalytic efficiency \([K_o/u(K_o)]\) as a function of relative viscosity \( (\eta/\eta^o)^{1000} \) with glycerol as micro-viscosogen have an exponential value of 0.14. While the use of Ficoll 400, as macro-viscosogen, the exponential value is 0.21. These clearly showed that the second order rate constant, \( K_o \), is affected by the increased in solution viscosity by 14% and 21% by glycerol and Ficoll 400, respectively [62]. A viscosity dependence for \( K_a \) observed suggested that acetaminophen binding to ALDH could be partially rate-determining. The viscosity effect of 0 mean the rate of the reaction is completely independent of solvent viscosity whereas the effect of 1 indicate a completely diffusion–limited event while viscosity effect > 1 indicate a conformational change accompanying binding of the substrate. This also demonstrates that the acetaminophen-ALDH bonding is dynamic quenching [36,37].

3.10. Molecular docking study of ALDH-Acetaminophen interaction

Molecular docking was employed to simulate the binding mode of the acetaminophen to ALDH. The possible binding mode and pattern is presented in Fig. 12. This revealed that acetaminophen, as a ligand, is a good molecule which docks well with ALDH. The binding region of the acetaminophen on the ALDH is located in the interior hydrophobic cavity of the enzyme. The ALDH-acetaminophen complex is stabilized by the hydrogen and Van der Waals bonding between the drug and the Ile-365, Arg-136, Leu-217, Phe-219, Glu-220 and Gln-321 amino acids within the active site. As calculated by Auto Dock Vina, acetaminophen showed good binding affinity with a minimum binding energy of \(-5.3 \, \text{kcal/mol}\).

4. Conclusions

Studies on ALDH fluorescence quenching by acetaminophen have been presented. The results show that acetaminophen is a strong quencher and binds to ALDH with high affinity. This study shows that Acetaminophen quenches the intrinsic fluorescence of ALDH through a dynamic quenching mode and the binding of Acetaminophen to ALDH was sensitive to pH and concentration change. The bonding is predominantly Vander Waal force and was not spontaneous. Synchronous fluorescence spectra indicate that the microenvironments of tryptophan remarkably change. Results from UV-visible and CD spectrum suggested that ALDH underwent substantial conformational changes at both secondary and tertiary structure levels. These changes could indicate that the biological activity of ALDH would be weakened in the presence of the drug.
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References

[1] G. Muzio, M. Mazzagri, E. Pauzuzi, M. Oraldi, R.A. Canuto, Free Radic. Biol. Med. 52 (2012) 735-746.
[2] V. Vasiliiou, A. Pappas, T. Estey, Drug Metab. 36 (2004) 279-299.
[3] B. Persson, Y. Kalberg, J.E. Bray, E. Bruford, S.L. Dellaripa, A.D. Favia, R.G. Duarte, H. Jo¨rnvall, K.L. Kavanagh, N. Kedishvili, M. Kisieia, E. Maser, J. Chromatogr. B. 677 (1996) 1541.
[4] J.C. Jimenez-Lopez, E.W. Gachomo, M.J. Seu, A. Pappa, T. Estey, Drug Metab. 36 (2004) 279-299.
[5] B. Persson, Y. Kalberg, J.E. Bray, E. Bruford, S.L. Dellaporta, A.D. Favia, S.A. Marchitti, C. Brocker, D. Stagos, V. Vasiliou, Drug Metabol. Toxicol. 4 (2008) 94–106.
[6] S.A. Moore, H.M. Baker, T.J. Blythe, K.E. Kitson, T.M. Kitson, E.N. Baker, Structure 23 (2013) 283-303.
[7] M.F. Wang, C.L. Han, S.J. Yin, Chem. Biol. Interact. 178 (2009) 36-44.
[8] B. Jackson, C. Brocker, D.C. Thompson, W. Black, K. Vasiliiou, D.W. Nebert, J. Biochem. Mol. Toxicol. 17 (2003) 7-15.
[9] N.E. Sladek, J. Biochem. Mol. Toxicol. 17 (2003) 7–23.
[10] V. Vasiliou, Hum. Genom. 5 (2011) 283–299.
[11] Y.J. Hu, Y. Liu, J.B. Wang, X.H. Xiao, S.S. Qu, J. Pharm. Biomed. Anal. 36 (2004) 697–720.
[12] S.A. Marchitti, C. Brocker, D. Stagos, V. Vasiliiou, Drug Metabol. Toxicol. 4 (2008) 665–673.
[13] J.S. Landin, S.D. Cohen, E.A. Khairallah, Toxicol. Appl. Pharmacol. 141 (1996) 299–307.
[14] D. Tatlidil, M. Ucuncu, Y. Akdogan, Phys. Chem. Chem. Phys. 17 (2015) 1541–1551.
[15] J.R. Lakowicz, G. Freter, G. Weber, Biophys. J. 32 (1980) 591–601.
[16] Y.J. Hu, Y. Wang, Y. Ou-Yang, J. Zhou, Y. Liu, J. Lumin. 130 (2010) 1394–1399.
[17] Y. Ni, S. Su, S. Kokot, Analytica Chim. Acta 628 (2008) 49–56.
[18] P.L. James, P.M. Meade, J.A. Hinson, Drug Metab. Dispos. 31 (2003) 1499–1506.
[19] S.A. Moore, H.M. Baker, T.J. Blythe, K.E. Kitson, T.M. Kitson, E.N. Baker, Structure 6 (1998) 1541–1551.
[20] A.K.H. MacGibbon, L.F. Blackwell, P.D. Buckley, J. Biochem. 77 (1977) 93–100.
[21] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[22] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[23] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[24] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[25] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[26] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[27] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[28] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[29] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[30] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[31] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[32] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[33] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[34] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[35] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[36] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[37] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[38] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.
[39] A.R. Cottrell, R. Pietruszko, Biochem. Biophys. Acta 1674 (2004) 205–211.