Biochemistry and Biophysics Reports 10 (2017) 198–207

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

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

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ARTICLE INFO

Keywords:
Aldehyde dehydrogenase
Acetaminophen
Binding site
Fluorescence spectrum
Viscosity

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 4.91×10^3 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 α-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 −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

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http://dx.doi.org/10.1016/j.bbrep.2017.03.010
Received 8 September 2016; Received in revised form 8 March 2017; Accepted 28 March 2017
Available online 06 April 2017

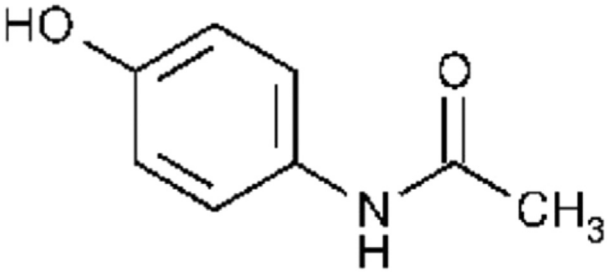

Fig. 1. Structure of Acetaminophen.

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. Materials and methods

2.1. Materials

Aldehyde dehydrogenase (ALDH; molecular weight 200,000 Da) was obtained from Sigma-Aldrich Fine Chemicals, USA and was used without further purification. Acetaminophen concentrate (≥99% purity,) was a generous gift from Deshalom Pharmaceuticals Nig. Ltd., Ilesha, Nigeria. All reagents were of analytical grade unless specialized. All solutions were prepared with double distilled water. Acetaminophen stock solution was prepared in analytical grade double distilled ethanol. All glass Ostwald viscometer (VWR, USA) was used to measure the intrinsic and extrinsic relative viscosity. ALDH protein concentration was measured using Bradford method. Protein sample, ligand solutions and buffers were filtered through a Millipore membrane filter (0.45 μm membrane filter) immediately before use. The pH was checked with a Sartorius PP-50 standardized pH meter (Germany).

2.2. Methods

2.2.1. Fluorescence spectra

All fluorescence spectra were measured with a Hitachi F-4500 Fluorescence Spectrophotometer (Hitachi Ltd., Tokyo, Japan) equipped with a refrigerated circulating water bath (Pharmacia Biotech) and interfaced with HP Window XP Computer. The equipment was furnished with a 150 W Xenon lamp and a 1 cm quartz cell. The spectra were recorded in the wavelength range of 300–500 nm upon excitation at 280 nm when ALDH samples were titrated with acetaminophen. Both excitation and emission bandwidths were set on 5 nm with a scan speed at 900 nm/min. The response time was set to 2 s with a high sensitivity signal. Titrations were performed manually by using trace syringes. A 2.0 mL solution containing an appropriate concentration of ALDH (0.120 μM) in 25 mM Tris–HCl pH 7.4 containing 0.1 M NaCl was titrated manually by successive additions of ethanol stock solution of Acetaminophen to a very saturating concentrations of 125 μM. The final ethanol concentrations never exceeded 1% (v/v), and all fluorescence readings were corrected for the dilution effect. The presence of this volume of ethanol in the assay mixtures had no effect on the fluorescence measurements. Also, respective blanks of the buffer were used for the correction of all fluorescence spectra.

Synchronous fluorescence spectroscopy (SFS) was used to study the environment of amino acid residues. It involves the measurement of any shift, to reflect the changes of polarity around the chromophore molecule, in the emission maximum on addition of ligand molecules. Synchronous fluorescence spectra of solutions prepared as above were measured on the same fluorescence spectrophotometer. The excitation wavelength (λex) was set at 280 nm. The excitation and emission slit widths were set at 5.0 nm. The D-value (Δλ) between the excitation and emission wavelengths was set at 15 or 60 nm. PMT voltage was 700 V.

2.2.2. UV–Visible absorption spectroscopy

All absorbance spectra and equilibrium ligand binding experiments were measured in 25 mM Tris-HCl buffer, pH 7.4, at 25 °C using Shimadzu double beam UV-Visible spectrophotometer (UV-1800) equipped with a Pharmacia refrigerating circulator for temperature control (25 ± 0.1 °C) unless otherwise stated. The scan speed and slit of absorbance (λ_{abs}) were set to medium and 1.0 nm respectively. The spectra were recorded between 200–500 nm. A 1.0 mL solution of 0.120 μM ALDH was titrated with successive addition of acetaminophen. Circular dichroism (CD) measurements were made on a J-810 Spectropolarimeter (Jasco, Tokyo, Japan) at room temperature under constant nitrogen flush. The Circular dichroism (CD) spectra were measured from 190 to 240 nm at a scan speed of 200 nm/min. Each result was the average of the three scans.

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 micro viscosogen 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 $[(K_a)^\circ/(K_a)] = (\eta^{rel}/\eta^\circ)^{exp}$. 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. A) 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 (SDF) 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.ccmb.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].

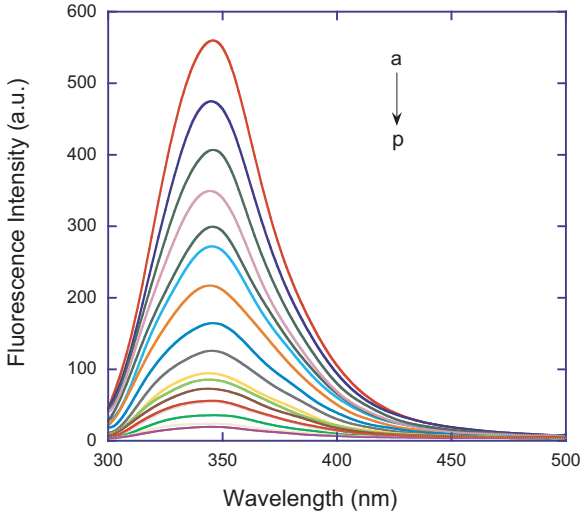

Fig. 2. Fluorescence emmision spectra of ALDH (1.20×10^{-7} mol L^{-1}) in 25 mM Tris–HCl pH 7.4 containing 0.1 M NaCl in the prescence of different concentrations of acetaminophen (0–1.25×10^{-5} mol L^{-1}). Excitation wavelength was set at 280 nm and the emmission spectra from 300 to 500 nm.

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

$$F_o/F = 1 + K_{sv}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

$$K_q = K_{sv}/\tau_0 \quad (2)$$

where F and F_0 are the intensity of fluorescence intensities with and without quencher, respectively. The k_q, K_{SV}, τ° 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_0/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 (Fig. 1) group might be responsible for the quenching [38]. In order to avoid the inner filter effects [39], the quenching mechanism were analyzed within the linear part of Stern–Volmer dependence (Fig. 3b).

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_q, of various kinds of quenchers with biopolymer is 2×10^{10} L mol^{-1} s^{-1}

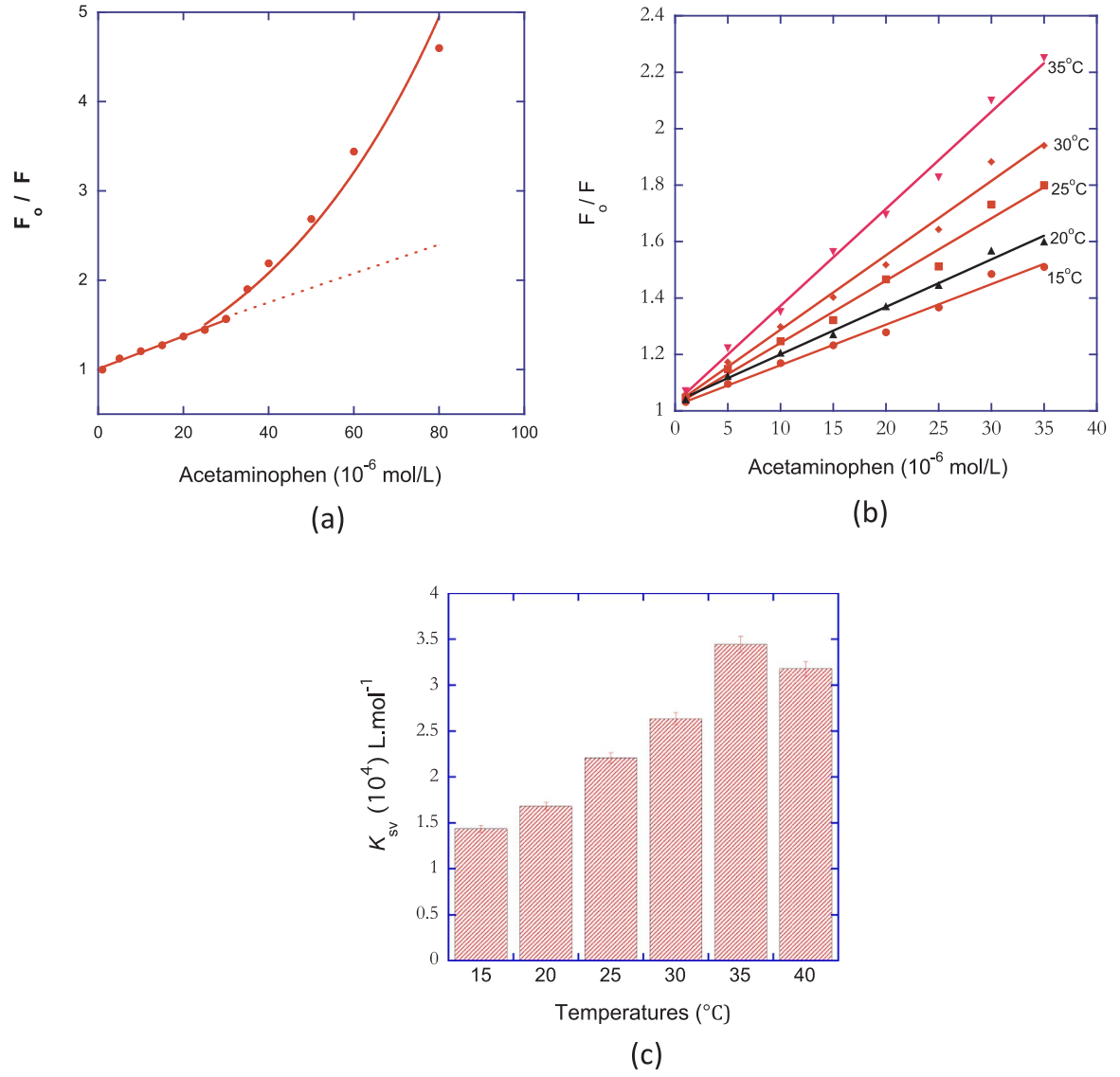

Fig. 3. (a)The Stern–Volmer plots for the binding of acetaminophen to Aldehyde dehyrogenase at 25 °C (298 K). The saturation concentration of acetaminophen was from 0 to 1.25×10^{-5} mol L^{-1} b: Stern-Volmer plots for the quenching of ALDH by acetaminophen (0- 35 μM) at different temperatures (15 °C, 20 °C, 25 °C, 30 °C and 35 °C). The linearity of portion Fig. 3a was used for analysis Stern-Volmer constant c: The plot of Stern-Volmer quenching constants (K_{sv}) and temperature.

Table 1
Stern-Volmer quenching constants for the acetaminophen-ALDH system at different temperature.

pH	T (K)	K_{sv} X 10^4 (L mol^{-1})	K_q X 10^{12} (L mol^{-1} S^{-1})	R	SD
7.4	288	1.44	1.44	0.999	0.063
	293	1.68	1.68	0.982	0.084
	298	2.21	2.21	0.945	0.101
	303	2.63	2.63	0.993	0.072
	308	3.44	3.44	0.984	0.17
	313	3.18	3.18	0.814	0.36

[40]. 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:

$$log\ (F_0 - F/F) = logK_a + nlog[Q] \quad (3)$$

K_a is the effective quenching constant for the accessible fluorophores [42], which are analogous to associative binding constants for the quencher–acceptor system. In the linear range of Stern–Volmer curve, the numbers of binding sites were obtained according to Eq. (3). The fluorescence quenching was mainly a dynamic quenching process. From the slope of the regression curve based on above equation and as shown in Fig. 4. The linearity of the Scatchard plot for acetaminophen–ALDH was obvious. The differences between the values calculated for both non-substrate ligands are within the experimental error. The value of n is approximately 1.18, indicating that there is one type of binding site for acetaminophen in ALDH, and the value of K_a is 4.91×10^4 L mol^{-1}, reflecting a strong interaction between acetaminophen and ALDH. Using Job's plot [43] gave syllogistic evidence that the stoichiometric ratio ALDH-acetaminophen at 25 C and pH 7.4 is 1:1

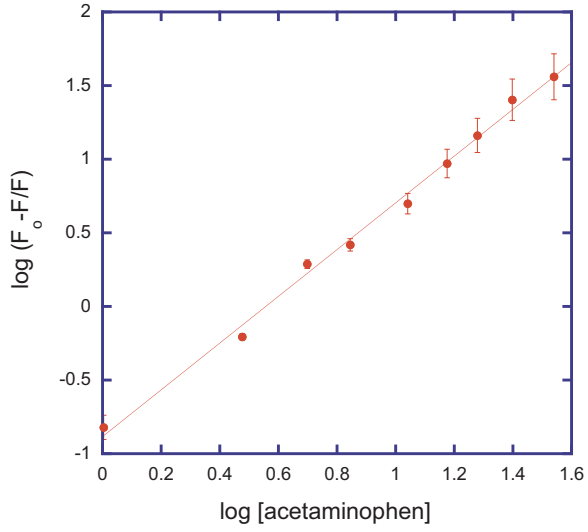

Fig. 4. Scatchard plots for the interaction between acetaminophen with ALDH. [ALDH] = 0.120 μM, λ = 280 nm at pH 7.4 in phosphate buffer 25 mM.

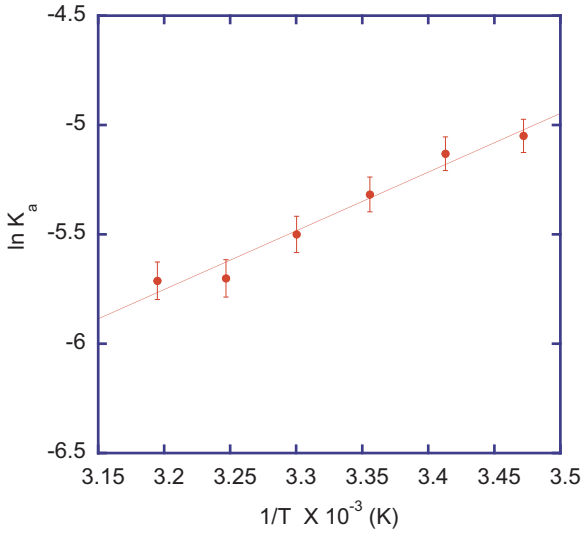

Fig. 5. Van't Hoff plots for the interaction between acetaminophen bound to ALDH. The temperatures used were between temperatures of 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C.

(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 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 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-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 versus 1/T (T, absolute temperature) allows the determination of ΔH and ΔS using Eq. (4):

$$lnK_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

The free energy change (ΔG) was estimated from the following relationship:

$$\Delta G = \Delta H - T\Delta S = -RTlnK_a \quad (5)$$

The enthalpy change (ΔH) 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 (ΔG, ΔH and ΔS) 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 ΔS value. From Table 3 it can be seen that ΔH and ΔS have negative value (−22.32 kJ/mol) and a negative value (−119.24 J/mol K), respectively. The positive sign for ΔG means that the binding process was non-spontaneous. The ALDH-acetaminophen, at pH7.4, is enthalpically favourable and entropically unfavorable (negative TΔS). 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]. The signs and magnitude of the thermodynamic parameters for proteins reaction can account for the main forces contributing to protein stability. The sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes have been characterized [46–48]. Here, the negative entropy and enthalpy is frequently taken as evidence for Van der Waals and hydrogen bonding.

3.4. pH dependence of acetaminophen-ALDH binding

ALDH must acquire a unique conformation in order to be function-

Table 2
Binding parameters obtained from the acetaminophen-ALDH interaction.

T (K)	$K_a \times 10^{-3}$ L mol^{-1})	SD	n	R^2
288	6.41	0.53	0.99	0.9921
293	5.91	0.23	1.2	0.9918
298	4.91	0.27	1.18	0.9929
303	4.09	0.33	1.08	0.9956
308	3.34	0.42	1.05	0.9913
313	3.30	0.12	1.22	0.8562

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

T (K)	ΔH (kJ mol^{-1})	ΔS (J mol^{-1})	ΔG (kJ mol^{-1})
288			12.02
293			12.62
298	−22.32	−119.24	13.21
303			13.81
308			14.41
313			15.00

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:

$$Y = \frac{\Delta F_i}{\Delta F_{max}} = \frac{K_a\,[L]}{1 + K_a\,[L]} \quad (6)$$

where ΔF_i indicates the fluorescence-quenching change observed at non-saturating ligand concentrations of acetaminophen, and ΔF_{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:

$$[L] = [L]_t - nY[ALDH]_t \quad (7)$$

where $[L]_t$ 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 Δλ (Δλ = λem − λex) 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 Δλ = 60 nm and red shift (from 295 to 305 nm) when Δλ = 15 nm. The red-shifts effect implied that the interaction of acetaminophen affect the microenvironment 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 (λ_{max}) 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-ligandin 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 (ΔG). 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].

$$\Delta F = \frac{\Delta F_{max}[L]}{K_d + [L]} \quad (8)$$

The equation was linearized using

$$[L]/\Delta F = (K_d/\Delta F_{max}) + ([L]/\Delta F_{max}) \quad (9)$$

where ΔF_{max} is the maximum decrease in fluorescence observed when the enzyme is saturated by acetaminophen. The plot of ΔF against the ligand (Acetaminophen) concentration obey a Michaelis-Menten 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]/ΔF 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 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.

	pH 5.0					pH 9.0			
T (K)	K_a [L.mol^{-1}] X10^3	n	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1})	ΔG° (kJ mol^{-1})	n	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1})	ΔG° (kJ mol^{-1})
288	9.74	2.1			11.21	2			14.93
293	5.15	1.9			12.47	1.8			13.95
298	4.59	1.9	−61.14	−251.22	13.72	1.7	71.26	195.59	12.97
303	2.39	1.9			14.98	1.8			12
308	1.81	2.1			16.24	2.1			11.02

[a] K_A = association constant
[b] n = number of binding sites
[c] ΔH = Enthalpy change
[d] ΔS = Entropy change
[e] ΔG = Gibb's free energy change

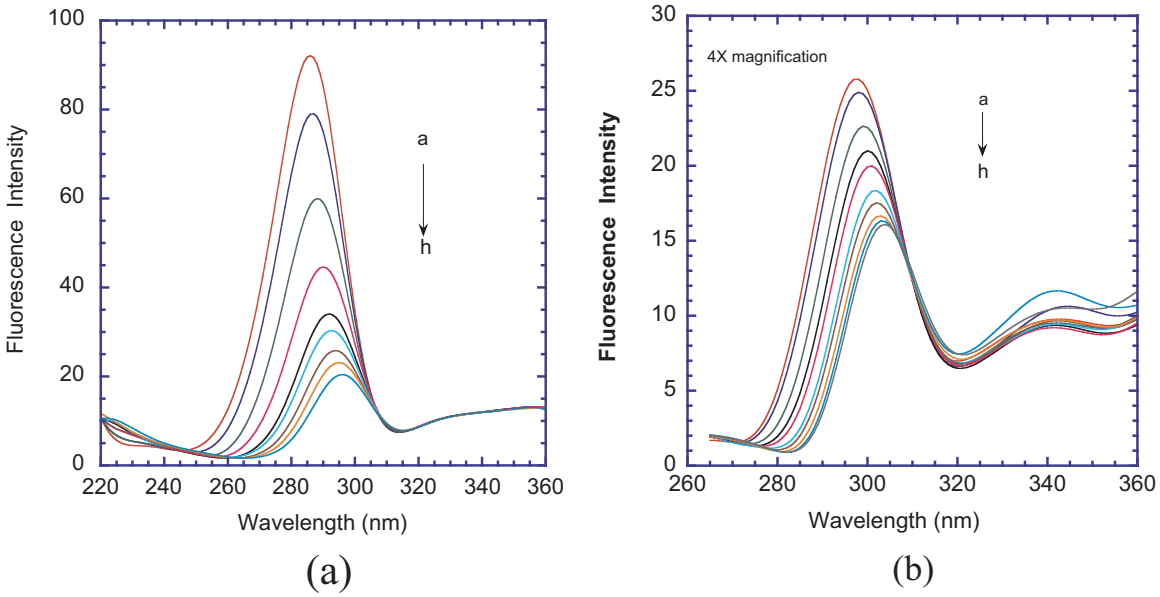

Fig. 6. Synchronous fluorescence spectra of ALDH (1.20×10^{-7} mol L^{-1}) in the presence of different concentrations of acetaminophen (0- 1.25×10^{-5} mol L^{-1}). (a) $\Delta\lambda=60$ nm (b) $\Delta\lambda=15$ nm (b. The spectra was magnified 4X). Both at pH 7.4; T = 25 °C.

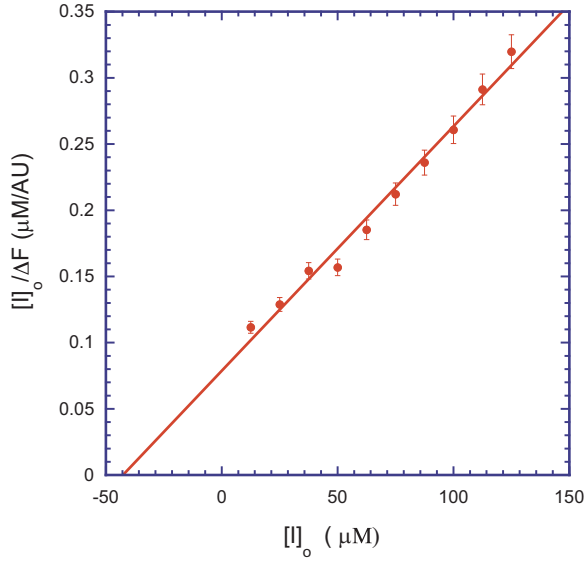

Fig. 7. Hanes-Woolf plots of Acetaminophen quenching the fluorescence of ALDH at 30 °C, pH 7.4. The experiment was done in triplicates at λ_{exc} = 280 nm. The $-K_d$ values of acetaminophen were obtained from the intercept at x-axis of the linear equation. Here, acetaminophen was assumed to be an inhibitor [I].

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 Diochroism

UV–Vis absorption spectroscopy and Circular Diochroism 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

Table 5
The pH-dependence on the relative thermodynamic parameters of the Aldehyde dehydrogenase – acetaminophen system from 15°C-40°C at pH 5.0, 7.4, and 9.0.

	pH 5.0			pH 7.4			pH 9.0		
T(*K*)	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1})	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1})	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1})	ΔG° (kJ mol^{-1})
288			43.76			22.72			26.98
293			44.57			23.46			27.23
298	−23.36	−161.7	45.38	−20.02	−148.4	24.2	12.82	−49.18	27.48
303			46.19			24.94			27.72
308			46.99			25.68			27.97

[a] K_d = dissociation constant
[b] ΔH = Enthalpy change
[d] ΔS = Entropy change
[e] ΔG = Gibb's free energy change

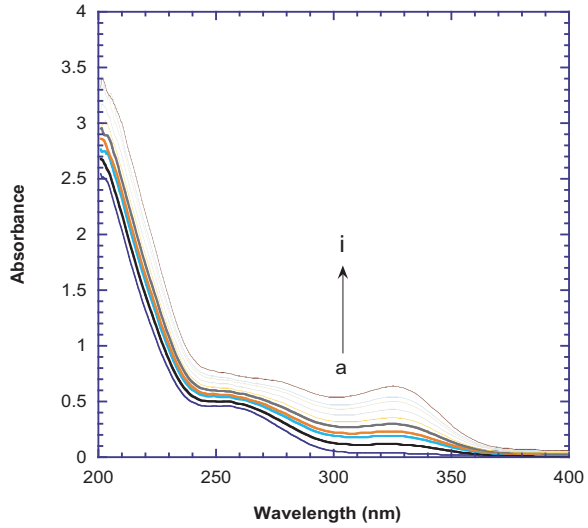

Fig. 8. The UV–Visible spectra of acetaminophen-ALDH binding.

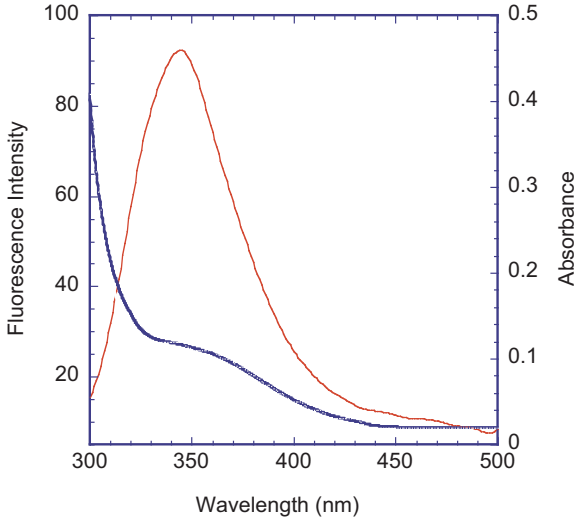

Fig. 10. The overlap of the fluorescence emission spectrum of ALDH with the absorption spectrum of acetaminophen T = 298 K; pH = 7.4; λex = 280 nm; λem = 300–500 nm.

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)

$$MRE = \frac{Observed\ CD \quad (m.\ deg)}{C_p nl \times 10} \tag{10}$$

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

$$\alpha - helix(\%) = \frac{-MRE_{208} - 4,000}{33,000 - 4000} \times 100 \tag{11}$$

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 filigree [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.

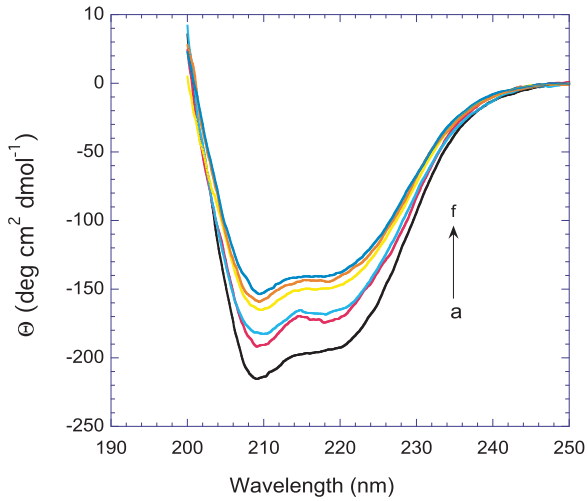

Fig. 9. CD spectra of the acetaminophen-ALDH system (pH 7.4; T = 298 K). The concentration of ALDH was set to 1.20×10^{-8} M). The concentration of acetaminophen was varied between 0 and 1.25×10^{-5} mol L^{-1}.

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 Forster's non-radiative energy transfer theory using this equation [60,61]:

$$E = 1 - \frac{F_o}{F} = \frac{R_O^6}{R_O^6 + r^6} \tag{12}$$

where R_0 is the Forster 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

Table 6
FRET data obtained from the Spectra overlap of ALDH emission and Acetaminophen absorption at 25 C and pH 7.4.

J (cm^3 L mol^{-1})	E	R_o (nm)	r (nm)
1.42×10^{-15}	0.0704	3.97	4.43

acetaminophen, respectively:

$$R_O^6 = 8.79 \times 10^{25} K^2 \varnothing n^{-4} J \quad (13)$$

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:

$$J = \Sigma F(\lambda)\varepsilon(\lambda)\lambda^{4\Delta}\lambda / F(\lambda)\Delta\lambda \quad (14)$$

where F(λ) is the donor fluorescence intensity at the wavelength of λ, and ε is the molar absorption coefficient of the receptor at the wavelength of λ. In the above equations, $k^2 = 2/3$, N = 1.336, and ϕ = 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 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 $[(K_a)^\circ/(K_a)]$ as a function of relative viscosity $(\eta^{rel}/\eta^\circ)^{exp}$ 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_a 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 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.

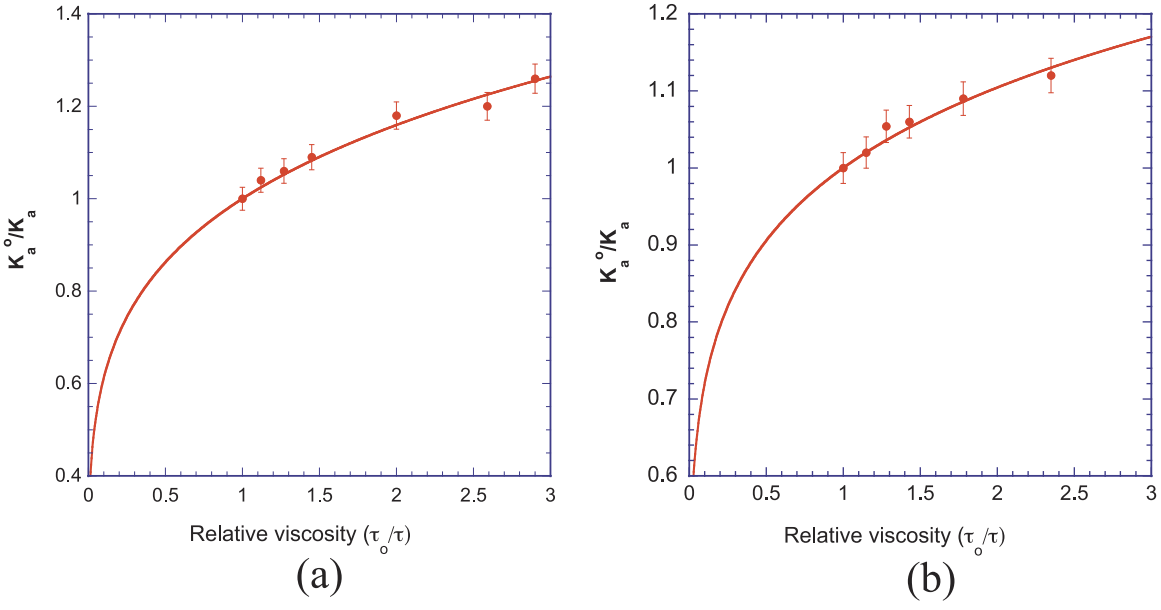

Fig. 11. Effect of solution viscosity upon the binding of Acetaminophen to ALDH using (a) Ficoll 400 as macro-viscosogen with *exp* = 0.21 and (b) glycerol as micro-viscosogen with *exp* = 0.14.

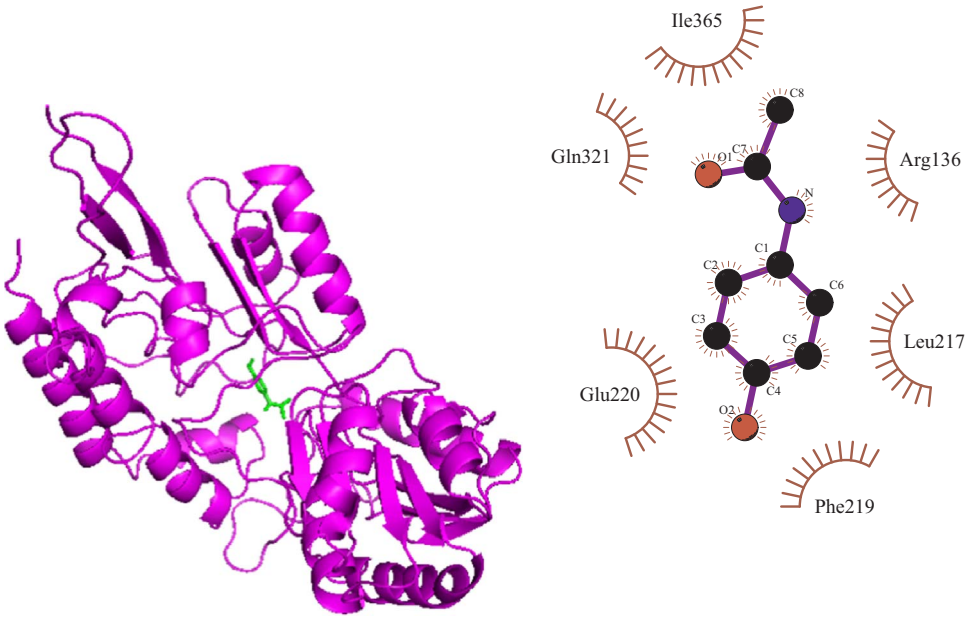

Fig. 12. Binding pose of acetaminophen (green colour) at the active site of Baker's yeast aldehyde dehydrogenase and the residues involved in the hydrogen and van der Waals bonding.

Acknowledgments

This work is supported by the TETFund Research Grant of the Senate of The Federal University of Technology, Akure. The author thanked IFS for the funds (F/4449 1F and F/4449-2F) used to purchase the equipments used and CCMB, Hyderabad, India for the use of their CD during his stay in the institute. The effort of Dr. Olusola Elekofehinti is highly regarded in the docking experiment.

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