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In Vitro Study of Acriflavine Interaction with Horseradish Peroxidase C

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ABSTRACT

Acriflavine (3,6-diaminoacridine) is an anticeptic drug developed in 1912. Previous research has focused on investigation of the intercalating features of acriflavine, but little is known about its interaction with proteins. Drug-receptor interaction is of major interest in clinical science. The aim of the present study was to evaluate the ability of acriflavine to induce alterations in conformation and function of peroxidase, a critical enzyme in cell survival. Horseradish peroxidase C (HRPC) activity was determined by measuring H_2O_2-dependent oxidation of o-dianisidine at 460 nm using an extinction coefficient of 11.3 Mm^{-1} cm^{-1}. Apparent Km and Vmax values were then calculated. The electronic absorption spectra were recorded for 300-700 nm. Both K_d and ΔG were calculated from changes in the absorbance of 403 nm. Intrinsic fluorescence was detected for the 297 nm excitation and the emission was recorded for 300-700 nm wavelengths. The Stern-Volmer constant and Hill coefficients were then obtained. All measurements were performed in 0.1 M citrate buffer, pH 4.0. Results indicated that acriflavine either stimulated or inhibited HRPC activity depending on concentration and pre-incubation time. The Drug-receptor complex formation occurred via binding of four molecules of acriflavine in two different binding sites on HRPC, and the heme environment became more polar. Finally, acriflavine quenched the only tryptophan residue of HRPC. The alterations in HRPC conformation identified in the present study, suggest that drugs that induce apoptosis could alter critical cell protein conformation and function.

Abbreviations: HRPC: Horseradish peroxidase C, IC50: The half maximal inhibitory concentration, ROS: Reactive oxygen species, UV-VIS: Ultra violet-visible

Keywords: Drug-protein interaction, Acriflavine, Horseradish peroxidase C, Enzyme activity, Spectroscopy

INTRODUCTION

Drug-receptor interactions are fundamental to almost all processes in the cell occurring at the molecular level in organisms. These interactions occur through molecular mechanisms involving conformational and functional changes in proteins [1]. Thus, elucidation of these drug effects using simplified *in vitro* systems and various spectroscopic methods is important [2,3].

Acriflavine, 10-methyl-3,6-diaminoacridinium chloride, has been used as an antibacterial, fungicidal, antiviral and trypanocidal drug from the time of its discovery by Ehrlrich and Beneda in 1912 [4-7]. The potential effects of acriflavine in antitumor activity [8,9] and *Candida utilis* apoptosis induction have also been reported [10].

Apoptosis, the terminal morphological and biochemical event of programmed cell death described for the first time by Kerr, Wyllie, and Currie in 1972 [11], has been of substantial interest in research for years. Increasing interest in apoptosis has resulted from the detection of several compounds and multiple proteins that can regulate the apoptotic procedures in the cell [12-14]. Reactive oxygen species (ROS) play a major role in inducing programmed cell death [15] on the other hand research supports that antioxidant defense enzymes are critical in cell protection against oxidative stress [16,17]. So any changes in conformation and function of antioxidant enzymes that reduce their ability of removing reactive oxygen species

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could be effective in inducing apoptosis. We have previously reported that, acriflavine, the drug which induces apoptosis and necrosis in yeast *candida utilis*, makes a complex with catalase, induce conformational changes and reduce the enzymatic activity [18]. In this investigation, an *in vitro* study was conducted evaluating the effect of acriflavine on horseradish peroxidase C, as another model of antioxidant defense enzyme.

Horseradish peroxidase (HRP), an important heme-containing enzyme (EC 1.11.1.7) which catalyzes the oxidation by hydrogen peroxide within an extensive range of organic and inorganic substrates, has been studied for more than a century [19,20]. Its most abundant isoenzyme, isoenzyme C (HRPC) is a well characterized enzyme. Plenty information on its three-dimensional structure and the mechanisms of catalysis has become accessible in recent years, that makes it an ideal candidate to study the relationship between protein conformation and enzymatic activity. In addition, the high substrate binding capacity and ability to accommodate substrates with diverse structural rearrangements is another reason that numerous studies have been carried out with HRPC [21,22]. Major advances in our understanding of the structure and function of HRPC have been completed recently, by the successful production of recombinant enzyme [21].

MATERIALS & METHODS

Materials

Horseradish peroxidase C type XII in the form of freeze-dried powder, o-dianisidine dihydrochloride and acriflavine were obtained from Sigma Chemical Co. Hydrogen peroxide (30% solution) and all the other chemicals used in this work were obtained from Merk Chemical Co. and were of reagent grade.

Enzyme Assay and Dialysis

HRPC activity was assayed at room temperature and under steady-state kinetics conditions by monitoring the H_2O_2-dependent oxidation of o-dianisidine at 460 nm and using an extinction coefficient of 11.3 mM^{-1} cm^{-1} [23]. HRPC solutions (1 mg ml^{-1}) were prepared by dissolving the enzyme in distilled water. Enzyme concentrations were determined spectrophotometrically using an extinction coefficient of 93 mM^{-1} cm^{-1} at 403 nm and molecular weight of 44,000 [24]. O-dianisidine solutions (10 mM) were prepared by dissolving o-dianisidine in distilled water. The stock solutions of H_2O_2 (0.3%) were prepared daily by dilution of 30% H_2O_2 in distilled water and acriflavine stock solutions were prepared by dissolving the acriflavine powder in distilled water. For each assay, a mixture of o-dianisidine (6-28 μM), H_2O_2 (1.6 mM), HRPC (45 nM), and acriflavine (0.03-5.46 mM) was pre-incubated at 25°C up to 60 min and in citrate buffer 0.1 M at pH 4.0 [20]. The concentration of H_2O_2 was kept high and constant with respect to the hydrogen donor (o-dianisidine) during the course of reaction to ensure providing pseudo-first-order condition. The enzyme's apparent K_m and V_{max} values were calculated from inverse plots of 1/rate *vs.* 1/o-dianisidine.

Recovery studies were conducted by pre-incubating HRPC (45 nM) with acriflavine (3.12 and 5.46 mM) at room temperature for 60 min then dialyzing the mixture at room temperature against 1 l of assay buffer for 0.5, 1, 2, 4 and 24 h with one change of buffer after each hour. Finally, the enzyme activity was measured as described above immediately after dialysis.

All results were the averages of at least three separate experiments, and Standard Deviations reported also.

Spectroscopic Studies

Electronic absorption spectra were recorded for 300-700 nm on a Cary 100 Bio UV-Vis spectrophotometer. For any given spectrum, HRPC (6.3 μM), citrate buffer (0.1 M, pH 4.0), and acriflavine (15-180 μM) were added to the sample cuvette then citrate buffer (0.1 M, pH 4.0) and acriflavine (at the same concentration as in the sample cuvette) was added to the reference cuvette. For each assay the enzyme was pre-incubated up to 60 min and all measurements were carried out at 25 °C. The profile of tertiary conformational changes was obtained from changes in the absorbance at 403 nm, related to the Soret band.

Intrinsic fluorescence was detected on a Cary Eclipse fluorescence spectrophotometer equipped with temperature controller. The excitation wavelength of 297 nm specific for tryptophan residues was chosen. The emission spectra were recorded from 300-600 nm after pre-incubation of 4.26 μM HRPC with various concentrations of acriflavine (15-180 μM) for increasing period of time (0-60 min). All

experiments were performed in citrate buffer 0.1 M at pH 4.0 and 25 °C.

In order to finding the affinity of acriflavine binding sites in the complex of HRPC-acriflavine the apparent dissociation constant, K_d of the complex, was calculated according to Eq. (1).

$$1/\Delta A = K_d/\Delta A_{max}\ 1/[\text{acriflavine}] + 1/\Delta A_{max} \quad (1)$$

Where ΔA is the absorbance change at a specific wavelength caused by certain acriflavine concentration, ΔA_{max} is the maximum change in absorbance and [acriflavine] is the concentration of free drug, which is assumed equal to the initial acriflavine concentration [25,26].

Values for Gibbs free energy of binding calculated to confirm the stability of HRPC-acriflavine complex, for each K_ds one $\Delta G_{binding}$ calculated according to Eq. (2).

$$\Delta G_{binding} = RT\ln(K_d) \quad (2)$$

To identify the type of quenching, which could be static or dynamic, fluorescence studies, were conducted at 25 °C, 35 °C and 45 °C upon excitation at 297 nm. The Stern-Volmer constant, K_{SV}, was calculated according to Eq. (3) and the plot of F_0/F *vs.* [Q].

$$F_0/F = 1 + K_{SV}\,[Q] \quad (3)$$

Where F_0 is the integrated area of the fluorescence spectrum of the sample before quenching, F is the integrated area of the sample after quenching, and [Q] is the concentration of the quencher. The Lehrer plot obtained from $F_0/\Delta F$ *vs.* 1/[Q] provides the accessible fluorephores at infinite quencher concentration. $F_0/\Delta F$ was also calculated according to Eq. (4), using the modified Stern-Volmer plot.

$$F_0/\Delta F = [1/K_{SV}f_a]\ [1/[Q]] + 1/f_a \quad (4)$$

Where ΔF is equal to F_0-F and f_a is the fraction of accessible fluorefores; F_0, F and [Q] are as defined above [27,28].

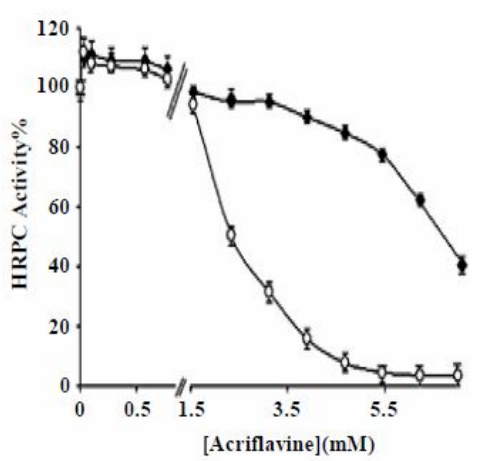

Fig. 1. Residual enzymatic activity; 45 nM of HRPC was pre-incubated for 0 min (●) and 60 min (○) with acriflavine (0.03-7.02 mM) in 0.1 M citrate buffer, pH 4.0 at 25 ºC.

RESULTS AND DISCUSSION

Enzyme Activity

HRPC activity was assayed by following the rate of H_2O_2-dependent oxidation of o-dianisidine. As shown in Fig. 1, the pre-incubation of HRPC (45 nM) with acriflavine (0.03-7.40 mM) led to either an increase or a decrease in enzyme activity depending on acriflavine concentration and the length of pre-incubation period. The addition of lower concentrations of acriflavine (0-0.58 mM) to HRPC causes stimulation in the enzyme activity, which occurred immediately and after 60 min pre-incubation. The addition of higher concentrations of acriflavine (1.50-7.40 mM) resulted in inhibition of HRPC activity, apart from the pre-incubation length. Determination of HRPC`s IC_{50} in 0 and 60 min pre-incubations showed 7.05 and 2.10 mM for acriflavine, respectively.

Inverse plots of the enzyme activity obtained immediately (Fig. 2a) and after 60 min pre-incubation (Fig. 2b) of HRPC (45 nM) with acriflavine at 0-0.58 mM concentrations were shown in Figs. 2a and b. The enzyme apparent V_{max} and K_m values obtained are presented in Table 1. As showed in Fig. 2a, b the enzyme V_{max} was increased compared to that of control for both 0 and 60 min pre-incubations, while its K_m remained unaffected for both

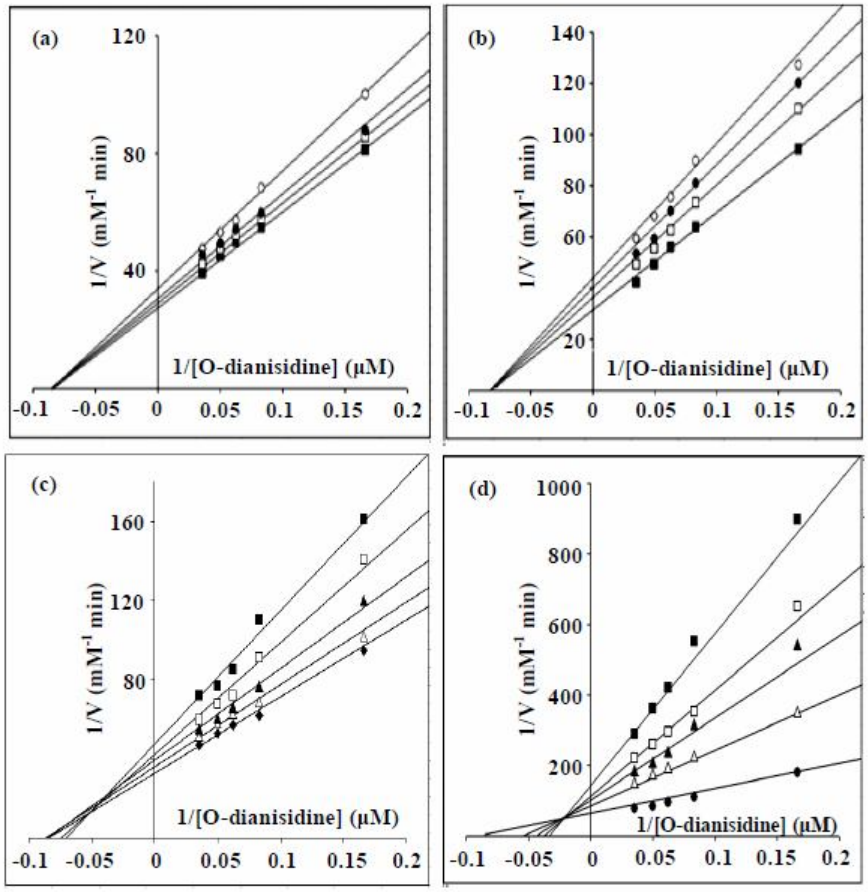

Fig. 2. Inverse plots of 1/rate *vs.* 1/[o-dianisidine] obtained after 0 min (A) and 60 min (B) pre-incubation of HRPC with 0 (○), 0.03 (■), 0.28 (□), and 0.58 (●) mM of acriflavine at 25 ºC. Inverse plots of 1/rate *vs.* 1/[o-dianisidine] obtained after 0 min (C) and 60 min (D) pre-incubation of HRPC with 0 (●), 3.12 (Δ), 3.90 (▲), 4.68 (□), and 5.46 (■) mM of acriflavine. Aliquots of the HRPC and acriflavine were placed in a 1-ml reaction mixture of 0.1 M citrate buffer, pH 4.0, 6-28 µM o-dianisidine, 1.6 mM hydrogen peroxide and 45 nM HRPC. The rate of oxidized o-dianisidine formation was calculated using $\varepsilon_{460} = 11.3\ mM^{-1}\ cm^{-1}$.

(Figs. 2a, b). This indicates HRPC stimulation at low concentrations of acriflavine which could be affected by pre-incubation time (Table 1). On the other hand the inhibition was observed when acriflavine concentration increased to 3.12 mM and more (Figs. 2c, d). Without pre-incubation (Fig. 2c), the type of inhibition was noncompetitive for acriflavine concentrations up to 4.68 mM and V_{max} decreased while K_m remained unaffected. When acriflavine concentration increased above 4.68 mM, the type of inhibition changed from noncompetitive to mix. Both the K_m and V_{max} were modified (Table 1). When the enzyme was pre-incubated for 60 min with 3.12 mM and higher acriflavine concentrations, not only was the inhibition more severe, but also showed mixed inhibition (Fig. 2d). Increasing the concentrations of acriflavine, inhibited the enzyme activity and the pre-incubation time affected on changing the type of inhibition.

Acriflavine induced variations in enzyme catalytic

Table 1. Values for HRPC K_m, V_{max}, and K_{cat}/K_m with O-dianisidine as the Varying Substrate, after 0 and 60 min Pre-Incubation with Various Concentrations of Acriflavine (0-5.46 mM)

	0 min pre-incubation			60 min pre-incubation		
[Acriflavine] (mM)	K_m (μM)	V_{max} (mM min^{-1})	K_{cat}/K_m (10^6 mM^{-1} min^{-1})	K_m (μM)	V_{max} (mM min^{-1})	K_{cat}/K_m (10^6 mM^{-1} min^{-1})
0	12.3 ± 0.4	0.030 ± 0.004	5.4 ± 0.2	12.3 ± 0.4	0.030 ± 0.004	5.4 ± 0.2
0.03	12.3 ± 0.4	0.036 ± 0.002	6.5 ± 0.1	12.3 ± 0.4	0.031 ± 0.001	5.3 ± 0.1
0.28	12.3 ± 0.4	0.036 ± 0.003	6.5 ± 0.1	12.3 ± 0.4	0.031 ± 0.001	5.6 ± 0.1
0.58	12.3 ± 0.4	0.032 ± 0.003	5.7 ± 0.1	12.3 ± 0.4	0.030 ± 0.006	5.4 ± 0.1
3.12	12.3 ± 0.4	0.026 ± 0.001	4.6 ± 0.1	19.5 ± 0.5	0.011 ± 0.005	1.2 ± 0.1
3.90	12.3 ± 0.4	0.025 ± 0.005	4.5 ± 0.1	25.0 ± 0.5	0.009 ± 0.005	0.8 ± 0.06
4.48	13.6 ± 0.5	0.023 ± 0.004	3.7 ± 0.1	35.5 ± 1	0.008 ± 0.002	0.5 ± 0.05
5.46	14.5 ± 0.5	0.020 ± 0.005	3.0 ± 0.1	55.0 ± 2	0.006 ± 0.001	0.2 ± 0.01
Type of inhibition	Non competitive & mixed			Mixed		

efficiency was shown in Table 1. For up to 0.58 mM acriflavine, the value of K_{cat}/K_m was larger than that of control that indicates on HRPC stimulation at low concentrations of acriflavine. In 3.12 mM and higher concentrations of acriflavine, the catalytic efficiency decreased that confirmed the inhibition of HRPC in these concentrations.

Overall, the current study on HRPC activity revealed that acriflavine activated HRPC at low concentrations (0.03-0.58 mM). Then, at higher concentrations of acriflavine (0.58-5.46 mM), inhibition occurred. On the other hand, at lower concentrations of acriflavine, the drug would enhance rather than inhibit enzymatic activity. Similar results were described in a study of the effect of Ni^{2+} on horseradish peroxidase C activity [20]. As previously suggested, this transient stimulation of activity could be due to stabilization of the enzyme in its best possible conformation. About one-third of all proteins are completely or partially unfolded, and drugs bind with higher affinity to the folded state than that the unfolded state or partially folded state of a protein [27,28]. It is possible that when acriflavine was present in low concentrations, binding occurred first with the completely folded enzyme, make it more stable and enhance the enzyme activity. With expanded pre-incubation time or higher drug concentration, binding to the other states of enzyme took place and eventually multiple binding sites were filled leading to increasing inhibition of the activity. As data reported in spectroscopic parts this could also be due to differential affinity of acriflavine (concentration dependent) for various states of the enzyme. When acriflavine concentrations increased, the type of inhibition changed from noncompetitive to mixed, which indicates that two steps of alterations occurred in HRPC conformation.

The inhibition constant (K_i) values were obtained as the cutoff of the abscissa from replots of the slopes ($K_{m.app}/V_{max.app}$) *vs*. [acriflavine] (mM). K_i decreased from 4.2 ± 0.1 mM to 1.1 ± 0.1 mM after 60 min pre-incubation respectively, an indication of the stabilization of the drug-enzyme complex increase during the pre-incubation time. Moreover the remaining percentages of activities after 0.5, 1, 2, 4 and 24 h dialysis are listed in Table 2. Following the earlier results, HRPC activity decreased during pre-incubation time and with acriflavine concentration.

Spectroscopic Studies

The electronic absorption spectrum of the HRPC used in the present work was similar to the spectrum that of previous work was reported [29]. The native spectrum contains a Soret band at 403 nm, a β-band at 500 nm and a charge transfer band at 642 nm (CT_1 band). In current study, the effect of acriflavine on Soret band was investigated.

Table 2. The Enzymatic Activity after 0.5, 1, 2, 4 and 24 h of Dialysis of the HRPC Pre-incubated for 60 min with 3.12 µM and 5.46 µM of Acriflavine

Dialysis duration (h)	0 min pre-incubation [Acriflavine] (µM)			60 min pre-incubation [Acriflavine] (µM)		
	0	3.12	5.46	0	3.12	5.46
½	95 ± 4.2	90 ± 3.5	61 ± 4.2	95 ± 4.5	40 ± 3.1	11 ± 0.5
1	95 ± 4.2	92 ± 2.7	62 ± 3.7	95 ± 4.2	43 ± 4.1	14 ± 1.0
2	96 ± 2.1	94 ± 3.2	64 ± 2.3	96 ± 4.0	45 ± 3.5	17 ± 0.7
4	96 ± 3.1	95 ± 2.5	67 ± 2.2	96 ± 5.1	50 ± 3.2	19 ± 0.8
24	96 ± 3.0	95 ± 5.1	70 ± 2.2	96 ± 5.0	59 ± 3.4	28 ± 1.2

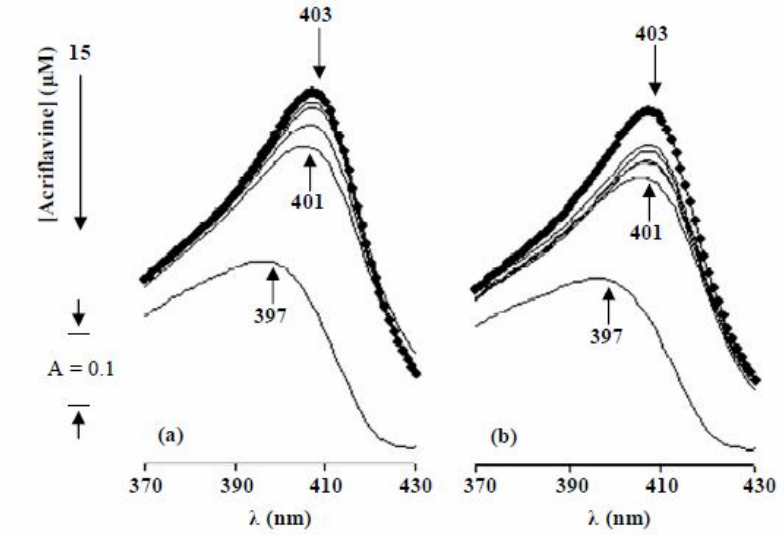

Fig. 3. Electronic absorption spectra of HRPC incubated with acriflavine. For any given spectrum, HRPC (6.3 µM), acriflavine (15-180 µM), and citrate buffer (0.1 M, pH 4.0) were added to the sample cuvette, whereas buffer (0.1 M, pH 4.0) and acriflavine (at the same concentration as in the sample cuvette) were added to the reference cuvette. Spectra were recorded after 0 (A) and 60 (B) min pre-incubation of HRPC with acriflavine.

Absorption spectra were obtained immediately after adding 15-180 µM of acriflavine to HRPC (Fig. 3a) and after 60 min pre-incubation (Fig. 3b). As shown in this figure, changes in the enzyme absorption occurred at 403 nm indicating the alterations around heme pocket in HRPC. After the addition of acriflavine, a progressive decrease observed in the intensity of the Soret band in both 0 and 60 min pre-incubation times. The level of these spectral alterations was time and drug dependent. The blue-shift was observed immediately and after 60 min pre-incubation by adding 120 µM and 180 µM of acriflavine (Figs. 3a, b) whereas the Soret band maximum was shifted from 403 nm to 401 nm after adding 120 µM of the drug and from 403 nm to 397 nm after adding 180 µM acriflavine.

Coincidence of hypochromisity and maximum band shift indicate that the heme pocket environment became more polar due to conformational changes [30]. In order to finding the affinity of acriflavine binding sites in the complex of HRPC-acriflavine the apparent dissociation constant, K_d of the complex, was calculated.

As an illustration, plots of $1/\Delta A_{403}$ *vs.* 1/[acriflavine] related to 0 and 60 min pre-incubation of the enzyme with acriflavine are shown in Figs. 4a, b. All the plots displayed 2 slopes, one equivalent to acriflavine lower concentrations (filled line, 15-60 µM) and the other equivalent to higher acriflavine concentrations (dotted line, 60-180 µM). Thus, two K_d values, K_{d1} and K_{d2} were calculated which both decreased with increasing pre-incubation time whereas, K_{d1} decreased from 990 ± 21 µM to 58 ± 2 µM after 60 min and K_{d2} decreased from 22 ± 1 µM to 14.5 ± 0.2 µM after 60 min pre-incubation (Table 3). K_d values also decreased time and drug concentration dependent.

To characterize the manner of acriflavine binding with HRPC, the plot of $\Delta A_{403}/\Delta A_{max}$ *vs.* [acriflavine] for Soret band after 0 and 60 min pre-incubation times was obtained (Fig. 5). ΔA_{max}, was calculated from the cut off the plot of $1/\Delta A_{403}$ *vs.* 1/[acriflavine] by extrapolation of low drug concentration. According to these results, acriflavine binds with HRPC in two different manners, at first the plot is linear which indicates the independent binding of acriflavine with HRPC, and then turned to a sigmoid trend which indicates a cooperative manner of binding to the enzyme.

The ΔG values for 0 and 60 min pre-incubation times were shown in Table 3. As pre-incubation time increased, a decrease in ΔG occurred, which indicates a progressive stabilization of acriflavine-HRPC complex. Also, the lower values of $\Delta G_{binding2}$ indicated that the binding sites, which filled at higher acriflavine concentrations have a higher affinity for the drug.

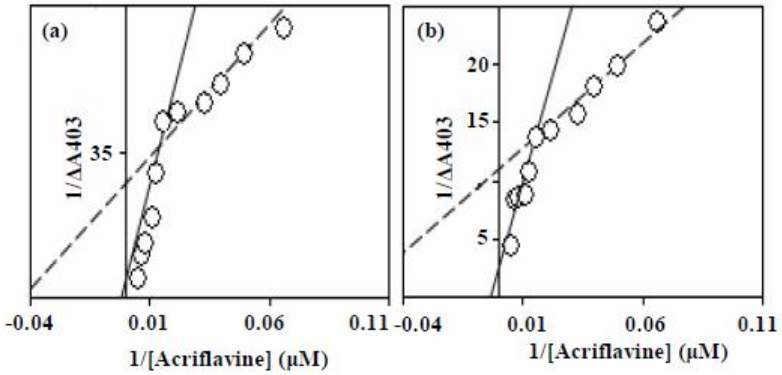

Fig. 4. Inverse plots of $1/\Delta A_{403}$ *vs.* 1/[acriflavine], giving apparent dissociation constants K_{d1} (filled line) and K_{d2} (dotted line). K_{d1} and K_{d2} calculated for 15-60 µM and 60-180 µM of acriflavine, respectively. The slopes decreased as the pre-incubation time increased. The values for K_d are presented in Table 3.

Table 3. Values for HRPC-acriflavine Complex Dissociation Constants (K_d) and Free Energy of Binding (ΔG) Obtained for the Soret Band (403 nm)

Incubation time (min)	K_{d1} (µM)	ΔG_1 (cal mol^{-1})	K_{d2} (µM)	ΔG_2 (cal mol^{-1})
0	990 ± 21	-25 ± 1	22 ± 1.0	-947 ± 41
60	58 ± 2	-7068 ± 33	14.5 ± 0.2	-10509 ± 67

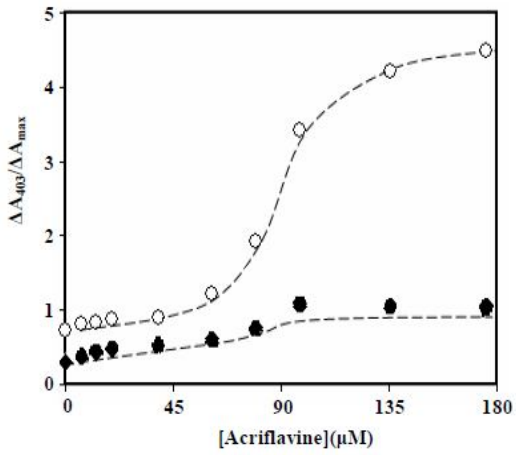

Fig. 5. Plots of the ratio of $\Delta A_{403}/\Delta A_{max}$ *vs.* [acriflavine] ΔA_{403} is the absorbance change caused by a given acriflavine concentration at the specified wavelength and ΔA_{max} is the absorbance change for complete formation of the HRPC-acriflavine complex as seen at the wavelength. Data were obtained after 0 (●) and 60 (○) min pre-incubation of HRPC with acriflavine. The arrow indicates the transition between the two acriflavine concentration ranges, below 15-60 μM and above 60-180 μM.

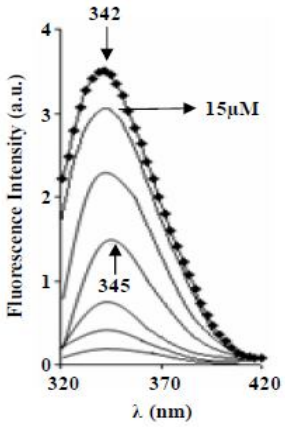

Fig. 6. Fluorescence emission spectra of HRPC (4.2 μM) (♦) and HRPC with various concentrations of acriflavine 15-180 μM, obtained upon excitation at 297 nm. The peak at 342 nm attributable to the tryptophan residue was decreased as acriflavine concentration increased and 3 nm blue-shifts performed with acriflavine concentration of 60 μM and above.

The Hill coefficient (h) calculated from the plot of $\log[\Delta A_{403}/(\Delta A_{max}-\Delta A_{403}]$ *vs.* log[acriflavine], to reveal the number of acriflavine which interacts with HRPC. For both pre-incubation times, it was equal to one for acriflavine concentrations from (15-60 μM) and three for acriflavine concentrations up to 180 μM [30,31].

Overall the results of electronic absorption spectrum indicating the existence of 2 types of sites for acriflavine binding in HRPC K_{d1} and K_{d2}. The sites with lower affinity could be filled at low concentrations of acriflavine (15-60 μM) described by K_{d1}, and the sites with higher affinity filled at acriflavine concentrations of 60 μM or greater are described by K_{d2}. For both sites, the complex of acriflavine-HRPC became more stabilized by time, which is confirmed by $\Delta G_{binding}$ values. In addition, the plots of $\Delta A_{403}/\Delta A_{max}$ *vs.* acriflavine showed 2 steps for acriflavine binding in HRPC. In the first step, the linear plot indicated the independent binding of drug to the enzyme and in the second step acriflavine binds to HRPC in a cooperative manner. The Hill plot also revealed the number of drug binding in each step, which was only one molecule of drug at first, and three molecules of drug at the second step.

Fluorescence studies were conducted in order to corroborate the observed conformational alterations induced by acriflavine to HRPC. The amino acid composition of HRPC includes one tryptophan residue that is quenched by heme [19,20], thus any alterations in protein conformation would change the quenching. Upon excitation at 297 nm a single fluorescence emission spectrum at 342 nm, due to the tryptophan residues, was displayed (Fig. 6). Immediately after the addition of 15 μM acriflavine to the enzyme, the emission peak quenched to 73.6% of the original value. An additional decrease in maximum band was also observed when the drug concentration increased. Moreover, the emission of maximum band showed 3 nm of red-shift in the presence of higher concentrations of acriflavine.

As shown in Table 4, the values of K_{SV} decreased as temperature increased, which indicates that the fluorescence quenching of HRPC occurs through a static mechanism.

The value found from Lehrer plot was 100%, which indicates that the only tryptophan of HRPC was quenched by acriflavine (Fig. 7).

The results obtained here indicated that acriflavine induced changes in HRPC conformation and led to

Table 4. Values for Stern-Volmer Constants (K_{SV}) at 25, 35, and 45 °C upon Excitation at 297 nm, Obtained from the Plot of F_0/F *vs.* [Q]

Temperature (C°)	K_{SV} at 297 nm of excitation
25	$2.0 \times 10^3 \pm 0.2 \times 10^3$ M^{-1}
35	$1.7 \times 10^3 \pm 0.4 \times 10^3$ M^{-1}
45	$1.5 \times 10^3 \pm 0.3 \times 10^3$ M^{-1}

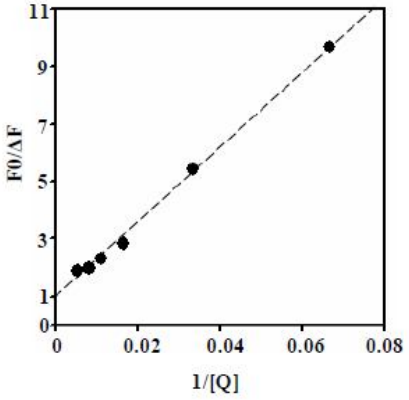

Fig. 7. Lehrer plot of HRPC quenching by acriflavine. The solution conditions were 0.1 M citrate buffer pH 4.0, containing HRPC (4.2 µM), and acriflavine 15-180 µM, at 25 °C. The Fluorescence emission spectra were recorded at the excitation wavelength of 297 nm.

quenching of the tryptophan in the HRPC structure. In addition, the red-shift, observed in fluorescence spectra, showed that the environment of tryptophan residue became more polar upon acriflavine binding [32,33]. The values of K_{sv}, obtained from fluorescence measurements, showed a decrease with raising temperature, which indicates that acriflavine quenched HRPC intrinsic fluorescence *via* a static mechanism, reflected in the interaction between acriflavine and the enzyme.

CONCLUSIONS

Acriflavine (10-methyl 3, 6-di amino acridinium chloride) is a drug that caused petite mutation in the yeast *Saccharomyces cerevisiae*, kinetoplast loss in Trypanosomatidae, and that interacted with DNA. As we previously reported elsewhere, acriflavine induces apoptosis and necrosis in yeast *Candida utilis* [10]. Apoptosis has been under scrutiny for decades and this is because of multiplicity of factors that induce programmed cell death. Reactive oxygen species have been generally identified as one of the factors associated with apoptosis and it has been shown that enzymes providing protection against oxidative stress could prevent apoptosis due to interaction with peroxides.

In the present study, *in vitro* changes in the HRPC activity and protein conformation induced by acriflavine were investigated. First, kinetic results showed stimulation and inhibition depending on acriflavine concentration and pre-incubation time. Then the spectroscopic results indicated that four molecules of acriflavine bound to HRPC in 2 types of sites and different manners. One molecule in low concentrations of acriflavine bound independently to HRPC, then three other molecules bound in higher concentrations, in cooperative manner and the resulting complex showed more stabilization by time. The environment of heme and tryptophan residue became more polar and the tryptophan residue is quenched. So in agreement with theses alterations mediated by acriflavine to HRPC, it is suggested that acriflavine have a direct effect on HRPC. We also know peroxidases, as a vital enzymes involved in protection against oxidative stress, mentioned as critical factors causing programmed cell death. There is probably a need to do more studies in yeast peroxidase enzymes to find some direct effects of these compounds in biochemical and physiological process of programmed cell death.

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