Acrylamide-hemoglobin adduct: a spectroscopic study

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Abstract

Acrylamide is a neurotoxic and carcinogenic organic compound that is able to bind to several biomolecules and form adducts, through nucleophilic addition and the *in vivo* by the Maillard Reaction, interfering with the biological functions of these molecules. Hemoglobin is one of the most abundant intracellular blood proteins, and thus it is of high interest to understand whether the binding of acrylamide can alter its properties. The interaction of acrylamide with hemoglobin was assessed in a 20:1 ratio, and after a 72h-incubation period, a decrease of ca. 50% in the absorbance of the hemoglobin's Soret band was observed at 37ºC. This together with the analysis of circular dichroism spectra indicate that acrylamide binds in close proximity to the heme group. These perturbations were confirmed to not correspond to the loss of the heme group and were mostly reverted after passing the protein through a size-exclusion chromatographic matrix, suggesting a dominant non-covalent interaction for the observed effect. The thermodynamic parameters of unfolding in the absence and presence of acrylamide, suggest an interaction based on H-bonds and van der Waals forces that slightly stabilizes hemoglobin. The oxygen binding capacity of hemoglobin does not seem to be hindered, as no differences in the Q bands were observed in the adduct.

Keywords: hemoglobin, acrylamide adduct, visible spectroscopy, circular dichroism, thermal stability

1. Introduction

Acrylamide is a carcinogenic and neurotoxic organic compound of synthetic source [1, 2], with a wide application in industry, such as in the textile and paper sectors, as well as in biological sciences with protein electrophoresis [3]. Moreover, acrylamide can also be generated by the Maillard Reaction, a reaction between a sugar and an amino acid that occurs during food processing and cooking [4]. The presence of acrylamide in human food along with industrial production has led to an increasing exposure of the western general population to acrylamide, with an estimated daily uptake of 0.2 - 1.9 μg/kg [5, 6]. Therefore, concerns over its effects in the human body have increased.

Acrylamide's toxicity was first acknowledged in the early 1950's [7], which prompted numerous studies to monitor its adverse effects in the organism [8-11] and more recently prompted the development of therapies to protect against those effects [12-14]. The nature behind acrylamide's damaging effect in the organism is the formation of an adduct with biomolecules, such as DNA [15] and neuronal proteins [16, 17], that leads to the emergence of altered biomolecules. Being a soft electrophile, acrylamide will preferably react with soft nucleophiles, consisting mainly on cysteine residues, although it can react, at a lesser reaction rate, with lysine, histidine and the N-terminal amine of proteins [3, 17].

Hemoglobin is a metalloprotein present in red blood cells responsible for the transport of oxygen from the lungs to the tissues [18]. Structurally, hemoglobin is a tetramer with each subunit consisting of a polypeptide binding non-covalently a heme group. The subunits, designated as α and β, differ in their primary sequence, with a sequence identify of 43%, as well as in the number of residues, with 142 and 147 residues, respectively. The quaternary structure of hemoglobin is composed of two α-subunits and two β-subunits with a total of four heme groups [18, 19]. Due to its abundance in the human body and its lengthy lifespan of 120 days [20], hemoglobin is a good candidate to react with acrylamide. The first report of this interaction dated from 1970 in a study by Hashimono *et al.* that proved that acrylamide binds covalently to hemoglobin through its cysteine residues, forming the compound S-carboxyethyl-cysteine [21]. More recent studies by Springer et al. [22] and Basile et al. [23] using liquid chromatography - electrospray ionization mass spectrometry, showed that acrylamide binds residues Cys93 of the β-subunit and Cys95 of the α-subunit of human hemoglobin. In fact, the acrylamide-hemoglobin adduct has been used as a biomarker for acrylamide exposure. Many studies on this adduct have been reported in the past decades to monitor and clarify acrylamide's

exposure routes [24-26] and metabolic pathways in the organism [27, 28] mostly to explain its induced health damages.

Besides acrylamide, it has been shown that hemoglobin forms adducts with other Maillard reaction's products (MRP) through the N-terminal valine, lysine and arginine residues [29-32]. These hemoglobin adducts have mostly been studied with mass spectrometry [29, 30], Fourier-transform infrared [31, 33], UV-visible [29, 33] and fluorescence [34] spectroscopy. The studies of Hb-MRP adducts by UV-visible spectroscopy revealed a decrease in intensity and shift of the Soret band and a decrease in intensity at 280 nm [30, 33]. Moreover, the stability of hemoglobin has also been proven to be altered, specifically increased, in a study with methylglyoxal [30]. All these data suggested that hemoglobin undergoes structural changes when forming these adducts, which might influence its stability and function. Nonetheless, there are no studies regarding the influence of these adducts in the O_2 binding function of hemoglobin or in its stability after interaction with acrylamide.

It is possible to obtain important results related with protein-ligand interactions by UV-visible and circular dichroism spectroscopies [35, 36].

In the present work, UV-visible spectroscopy was used to monitor the formation of the adduct between acrylamide and hemoglobin, while circular dichroism was chosen to investigate its effects in the tertiary structure and heme pocket. The effect of acrylamide in hemoglobin's thermal stability and oxygen binding ability was also explored using visible spectroscopy.

2. Materials and Methods

2.1 Hemoglobin, acrylamide-hemoglobin adduct and acrylamide solutions

Stock solutions of 100 μM bovine hemoglobin (Sigma Aldrich) and 1 mM acrylamide (Sigma Aldrich 99 %) were prepared in 100 mM sodium phosphate buffer, pH 7.4. The acrylamide-hemoglobin solutions used in all the experiments, with exception of the acrylamide-hemoglobin interaction assay, were prepared by the addition of acrylamide to a hemoglobin solution in a 20:1 proportion, followed by incubation at 37 °C for a time period between 24 and 48 hours. The hemoglobin and acrylamide-hemoglobin samples analyzed throughout the work were prepared from the same stock solution of hemoglobin.

2.2 Hemoglobin and acrylamide interaction

UV-visible spectra of hemoglobin and acrylamide-hemoglobin solutions, prepared at different ratios (4:1, 20:1 and 80:1), were acquired in a UV-1800 Shimadzu spectrophotometer, at room temperature. The formation of the adduct was monitored at 0, 5, 30, 60 and 90 minutes after the addition of acrylamide, maintaining the samples at room temperature in between measurements. The acrylamide-hemoglobin solution at a 20:1 ratio was incubated at 37 °C and its UV-visible spectrum acquired at 0, 5, 10, 20, 45, 75 minutes and 72 hours. Prior to any measurement the solutions were centrifuged during 5 minutes at 17900 g.

2.3 Circular dichroism spectroscopy

Circular dichroism spectra of 1.45 µM hemoglobin and 1.22 µM acrylamide-hemoglobin samples prepared in 100 mM sodium phosphate buffer, pH 7.4, were acquired in an Applied Photophysics Chirascan^{TM} qCD spectrometer. The experiments were performed at 21°C with three repetitions, an integration time of 2 sec/nm and spectral window of 260-500 nm.

2.4 Heme and protein quantification

The metal and protein content were quantified using Inductively Coupled Plasma - Atomic Emission Spectrometry and Bradford assay (Bio-Rad), respectively. The samples were analyzed after elution from a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 7.6, 150 mM NaCl. Elution was monitored by following the absorbance at 210 nm and 410 nm. The UV-visible spectra of these hemoglobin and acrylamide-hemoglobin samples were also acquired before and after elution.

2.5 Thermal stability of the acrylamide-hemoglobin adduct

The stability of hemoglobin and acrylamide-hemoglobin adduct was examined by UV-visible spectroscopy, between 25 °C to 80 °C (above 65 ºC precipitation of the protein was observed). The spectra were acquired in an Agilent Diode Array associated with a Lauda Ecoline cryostat.

The data was analyzed considering a two-state equilibrium between the native (N), folded, state, and the unfolded (U) state (Equation 1).

$$N \rightleftharpoons U \qquad (1)$$

The absorbance at 405 nm was used to determine the fraction of unfolded protein, f_u, at each temperature through Equation 2,

$$f_u = \frac{A - A_n}{A_u - A_n} \qquad (2)$$

in which, A is the absorbance value at a specific temperature value; A_n is the absorbance value of the native folded form (100 % folded) at 25 ºC and A_u is the absorbance value of the unfolded form (0 % folded) at 65 ºC. The fraction of folded protein, f_n, was calculated using Equation 3, at each temperature.

$$f_n = 1 - f_u \qquad (3)$$

The equilibrium constant (K_u), describing the unfolding equilibrium at each temperature was given by Equation 4,

$$K_u = \frac{f_u}{f_n} \qquad (4)$$

The changes in enthalpy and entropy associated with the unfolding transition were estimated from the linear least-square fit of $\ln K_u$ versus 1/T, the van't Hoff plot [37], which is a representation of Equation 5.

$$lnK_u = \frac{-\Delta H^\circ}{R}\frac{1}{T} + \frac{\Delta S^\circ}{R} \qquad (5)$$

In Equation 5, T is the temperature in Kelvin (K); ΔH° is the variation of enthalpy in Joule (J); ΔS° is the variation of entropy in J/K; R is the gas constant that has a value of 8.3145 J/mol.K. The T_m (temperature of unfolding) is estimated when the fraction of unfolded is equal to the fraction of folded protein (meaning each are 0.5), and thus $\ln K_u$ equals zero.

2.6 Oxygen binding capacity of acrylamide-hemoglobin adduct

Solutions of concentrated hemoglobin and acrylamide-hemoglobin adduct were diluted in Argon-equilibrated 100 mM sodium phosphate buffer pH 7.4. The samples were reduced with sodium dithionite, corresponding to the deoxyhemoglobin species, and UV-visible spectra were acquired. Subsequently, sodium dithionite was removed by applying the samples onto a PD-10 column equilibrated in the same buffer, as to obtain the oxyhemoglobin species, and UV-visible spectra of these samples were acquired. The UV-visible spectra, acquired at 25 ºC on Agilent Diode Array associated with a Lauda Cryostat, were normalized for hemoglobin concentration.

3. Results

3.1 Analysis of the formation of acrylamide-hemoglobin adduct using spectroscopic techniques

3.1.1 UV-visible spectroscopy

The formation of an adduct between acrylamide and hemoglobin was monitored by UV-visible spectroscopy at two different temperatures (25 ºC and 37 ºC).

At 25 ºC, the formation of the acrylamide adduct was monitored in solutions with increasing ratio of acrylamide to hemoglobin (4:1, 20:1 and 80:1) during 90 minutes of incubation. During this incubation period it was observed a decrease in intensity of the Soret band (Figure 1), with the 280 nm absorption band being relatively unaltered (Figure S1, in Supplementary Material).

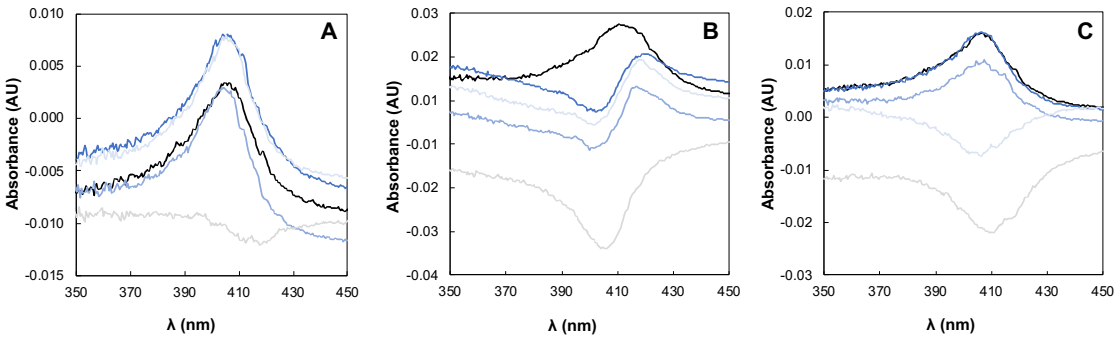

Figure 1 - Formation of acrylamide-hemoglobin adduct at 25 °C. The difference spectra between acrylamide-hemoglobin solution and hemoglobin solution, at each incubation time point, is presented for (A) 4:1 (B) 20:1 and (C) 80:1 acrylamide-hemoglobin ratio. The spectra were acquired after 0 (black), 5 (dark blue), 30 (blue), 60 (light blue), and 90 (light grey) minutes of incubation.

At 37ºC, the solution with an acrylamide-hemoglobin ratio of 20:1 was monitored for 72 hours (Figure 2), and a similar effect to the one reported at 25 ºC was observed up to 75 minutes, with a decrease in intensity of the Soret band (Figure 2, inset) and the 280 nm region relatively unaltered (Figure S1, in Supplementary Material). After 72 hours of incubation, it was observed a decrease in the absorbance at 280 nm (Figure 2), which can be attributed to hemoglobin unfolding [38]. Therefore, a time frame of 48 hours was chosen for the following studies with the adduct being formed in a ratio of 20:1 acrylamide to hemoglobin.

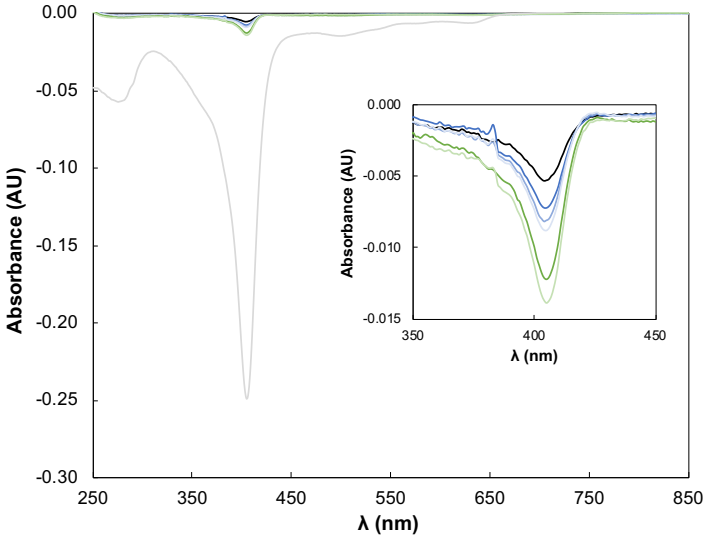

Figure 2 - Formation of acrylamide-hemoglobin adduct at 37 ºC. The adduct was formed with a ratio of 20:1 acrylamide to hemoglobin. The difference spectra between acrylamide-hemoglobin solution and hemoglobin solution, at each incubation time point, is presented. The spectra were acquired after 0 (black), 5 (dark blue), 10 (blue), 20 (light blue), 45 (green), 75 (light green) minutes and 72 hours (light grey) of incubation. Inset: Soret band region.

The interaction between acrylamide and hemoglobin resulted in a significant decrease in intensity of the Soret band (Figure 1 and 2). Thus, to establish whether this effect was not due to hemoglobin losing its heme group, samples of hemoglobin and acrylamide-hemoglobin were compared before and after size-exclusion chromatography.

The elution profile of hemoglobin has a single peak at 12 mL, which corresponds to hemoglobin (64 kDa), while that of acrylamide-hemoglobin has a peak at the same elution volume and additional one at 22 mL, when it is monitored at 220 nm and that corresponds to acrylamide (1.3 kDa) (see Figure S2 in Supplementary Material), which was in excess in this sample. The main peak at 12 mL has a Fe:protein ratio of 1 in both cases.

The UV-visible spectra of acrylamide-hemoglobin before and after the chromatography were compared (see Figure S3 in Supplementary Material), showing an increase in the A_{405nm}/A_{274nm} ratio from 3.1 to 3.5 (see Table S1 in Supplementary Material). The value of the later compares well with the one of free hemoglobin after chromatographic analysis (3.7).

3.1.2 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was used to analyze whether significant structural changes were induced in hemoglobin upon formation of the acrylamide adduct.

CD spectra were acquired in the near-UV and visible spectral regions, which are sensitive to changes in the tertiary structure (as it reflects the environment of the aromatic residues) and heme pocket, respectively [39]. The heme group CD band in the visible region, specifically in the Soret region, arises from the interaction between the heme vinyl and propionate groups and the side chain of aromatic residues and it can also be affected by the heme's level of planarity [40, 41]. The near-UV spectra of hemoglobin and acrylamide-hemoglobin do not show any alteration (Figure 3), whereas the positive CD band, in visible region, shows a noticeable decrease in the molar ellipticity when acrylamide is present (Figure 3, grey line).

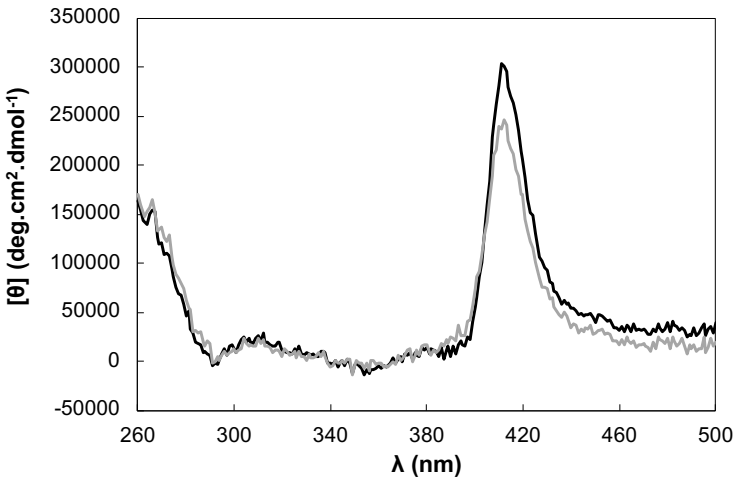

Figure 3 - Circular dichroism spectra of hemoglobin (black) and acrylamide-hemoglobin adduct (grey).

3.2 Influence of acrylamide in the thermal unfolding of hemoglobin

The influence of acrylamide adduct in the thermal stability of hemoglobin was evaluated by visible spectroscopy. For both hemoglobin and acrylamide adduct was observed a decrease in absorbance of Soret band, between 25 ºC and 65 ºC, and an increase in the absorbance at 274 nm (between 25 ºC and 65 ºC) (Figure 4A and Figure S4 in Supplementary Material). This indicates that hemoglobin is denaturing and losing its heme, as described in the literature [38, 40, 41].

The temperature of unfolding, T_m, as well as the thermodynamic parameters of the thermal unfolding, $\Delta H°_{unfolding}$ and $\Delta S°_{unfolding}$, were estimated for hemoglobin and acrylamide-hemoglobin adduct using the van't Hoff's plot (Figure 4B). The presence of the acrylamide slightly increases hemoglobin's unfolding temperature (Table 1), with the thermodynamic parameters being also affected. $\Delta H°_{unfolding}$ and $\Delta S°_{unfolding}$ for hemoglobin were estimated to be 135±7 kJ/mol and 0.42±0.01 kJ/mol/K, respectively, while for acrylamide-hemoglobin adduct were estimated to be 109±10 kJ/mol and 0.33±0.03 kJ/mol/K, respectively.

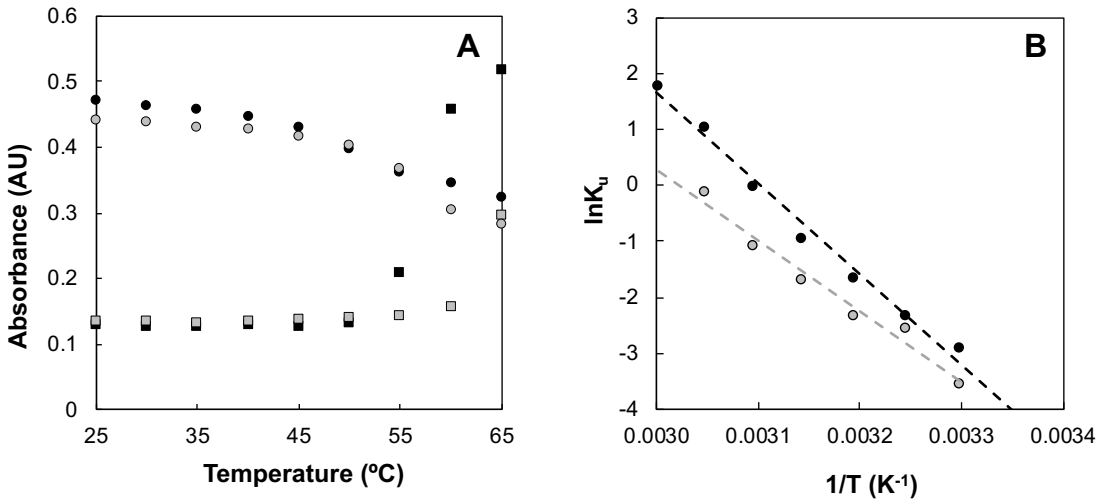

Figure 4 – Effect of temperature in the UV-visible spectra of hemoglobin and acrylamide-hemoglobin. **(A)** Absorbance at 405 nm (circles) and 274 nm (squares) as a function of temperature for hemoglobin (black) and acrylamide-hemoglobin (grey). **(B)** The van't Hoff plot of hemoglobin (black circles) and acrylamide-hemoglobin adduct (grey circles) used to estimate the thermodynamic parameters of thermal unfolding.

3.3 Ability of hemoglobin to bind oxygen

The oxygen binding capacity was studied by acquiring the UV-visible spectra between 490 nm and 600 nm (Q bands region) of the oxygenated and deoxygenated species of hemoglobin and acrylamide-hemoglobin (Figure 5). The spectra of deoxygenated hemoglobin and acrylamide-hemoglobin overlap (Figure 5A), whereas the oxygenated spectra are similar, with a slight decrease in intensity of the β-band and broader bands being observed in the presence of the acrylamide adduct (Figure 5B).

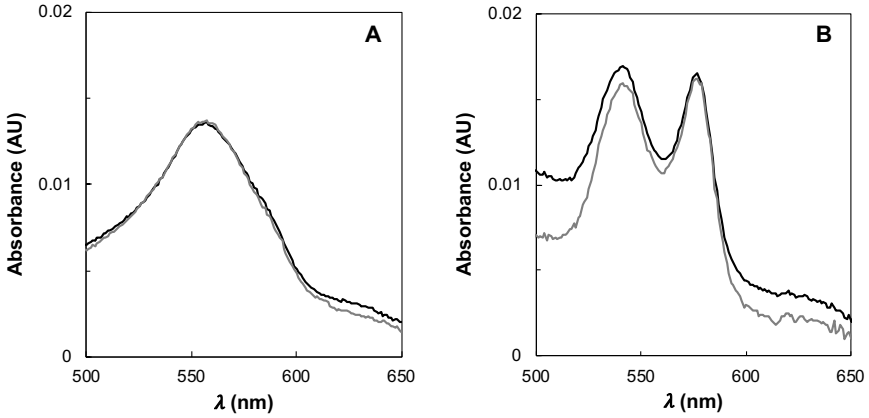

Figure 5 - UV-visible spectra of the Q bands region of deoxyhemoglobin (A) and oxyhemoglobin (B) of hemoglobin (black line) and acrylamide-hemoglobin adduct (grey line). The spectra were normalized for hemoglobin concentration.

4. Discussion

4.1 Properties of the acrylamide-hemoglobin adduct

The interaction between acrylamide and hemoglobin resulted in a significant decrease in intensity of the Soret band, which indicates that the binding region of acrylamide is close to the heme pocket. This decrease in absorbance could be due to acrylamide induced release of the heme or an alteration of the heme pocket hydrophobicity. As the iron/protein ration of hemoglobin with and without acrylamide incubation was the same, the heme was not lost, and therefore acrylamide changed de heme pocket. In fact, it was observed that after removal of acrylamide, using a size-exclusion chromatography, the A_{405nm}/A_{280nm} increases to a value close to the one of hemoglobin alone. This recovery of the Soret band implies the existence of a reversible non-covalent interaction between acrylamide and hemoglobin.

The structural changes in the heme pocket of hemoglobin upon formation of the adduct with acrylamide were also corroborated by the CD spectra in the visible region, with no change in the tertiary structure, as no differences were observed in the near-UV region between the CD spectra of hemoglobin and that of the adduct. The absence of change in the near-UV region indicated that the aromatic residues are not being altered. The decrease in the molar ellipticity of the Soret CD band in the presence of acrylamide is

attributed to a small distortion of the heme's planarity, to which this band is sensitive [42].

The covalent binding of acrylamide to hemoglobin occurs preferentially through cysteine residues [22, 23]. Moreover, a recent fluorescence spectroscopy study of human Hb-MRPs adducts by Ioannou and Varotsis demonstrated that acrylamide binds near β-Trp37 residue, located close to the heme pocket [34]. However, the hemoglobin in our study is of bovine source and has only two cysteine residues, Cys92, one in each β-subunit located in proximity to the heme, adjacent to His91, the proximal histidine (Figure S5 in Supplementary Material), with their side chains pointing in the opposite direction of this prosthetic group (data not shown). Additionally, the side chain of Trp36 (equivalent to Trp37 in human hemoglobin) points in the direction of the heme group. Thus, the data in the literature together with the results presented here indicate that acrylamide can be covalently bound to the cysteine residues, Cys92, but acrylamide interacts mainly non-covalently with the heme group. The cysteine-bound acrylamide units can induce structural changes on the side chain of the heme proximal histidine, which together with the non-covalent acrylamide molecules binding in this region results in distortion of heme's planarity leading to the changes observed in the visible region of the CD spectra.

4.2 Effects of acrylamide in the function and thermal stability of hemoglobin

After establishing the formation of the acrylamide adduct, functional and stability studies of the adduct were performed. Regarding the thermal stability, the difference between hemoglobin (T_m of 49.3±0.2°C) and acrylamide-hemoglobin (T_m of 55.8±0.2°C) of 6 ºC suggests a slight stabilization of the protein when forming the adduct, indicating that acrylamide is functioning as a kosmotropic agent, contributing to the maintenance and stability of the water-water interactions, and influencing the intramolecular interactions in hemoglobin polypeptide chain. In fact, in the CD spectra indicated that acrylamide did not change the tertiary structure of hemoglobin, with an overlap of the near-UV spectral region of hemoglobin and acrylamide-hemoglobin CD spectra (Figure 3).

The thermal unfolding of proteins in the presence of ligands directly influences the $\Delta H°_{unfolding}$ and $\Delta S°_{unfolding}$ as a result of the ligand binding during this process [43, 44]. The $\Delta H°_{assoc}$ and $\Delta S°_{assoc}$ associated with acrylamide was estimated through the difference of the $\Delta H°_{unfolding}$ and $\Delta S°_{unfolding}$ of hemoglobin in the presence and absence of acrylamide, being of -26±12 kJ/mol and -90±0.04 J/mol/K, respectively. These values

imply that acrylamide's interaction with hemoglobin is governed by H-bonds and van der Waals forces. The negative $\Delta H°_{assoc}$ indicates a enthalpy-driven binding process [44-47]. The changes observed in the Q-band region of the spectra between hemoglobin and acrylamide-hemoglobin samples in the presence of oxygen suggest that the altered conformation of the pocket does not hinder the binding of oxygen to the heme group. However, during the procedure to remove the reducing agent, the non-covalently bound units of acrylamide were also removed, remaining in the heme pocket solely the covalently bound acrylamide units. Thus, the minor changes observed between the oxyhemoglobin spectra of hemoglobin and the adduct arises from the cysteine-bound acrylamide, which account for only a small portion of the interaction. Therefore, it can be hypothesized that in the presence of the non-covalently bound acrylamide units, there will be significant differences between the oxy form of hemoglobin and adduct (as observed in the initial studies for the methemoglobin form, Figure 1 and 2). Nevertheless, further experiments are required to determine whether these units would hamper oxygen binding.

5. Conclusions

The spectroscopic data presented here for the formation of the acrylamide-hemoglobin adduct support that acrylamide binds close to the heme group, mainly non-covalently, interacting with residues in its vicinity and altering its planarity.

Moreover, it was shown that this binding slightly stabilizes hemoglobin without apparent effect in its oxygen binding capacity. However, these functional studies are not completely conclusive, as it is necessary to determine the contribution of the non-covalently bound acrylamide to hemoglobin oxygen binding capacity.

Nonetheless, the adduct formed between acrylamide and hemoglobin is a concern since it alters the heme pocket, with still unknown consequences to its physiological function. Therefore, caution with the injection of processed food and when working directly with the acrylamide is advisable to prevent serious damages to the organism.

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Declaration of competing interests

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported here.

Author Contributions

AGF, DSB, JNM and PO have designed and performed the experiments, data analysis and wrote the manuscript. SRP as contributed to the experimental design, data analysis, wrote and reviewed the manuscript with contributions from all the authors.

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Figure Legends

Figure 1 - Formation of acrylamide-hemoglobin adduct at 25 °C. The difference spectra between acrylamide-hemoglobin solution and hemoglobin solution, at each incubation time point, is presented for (A) 4:1 (B) 20:1 and (C) 80:1 acrylamide-hemoglobin ratio. The spectra were acquired after 0 (black), 5 (dark blue), 30 (blue), 60 (light blue), and 90 (light grey) minutes of incubation.

Figure 2 - Formation of acrylamide-hemoglobin adduct at 37 ºC. The adduct was formed with a ratio of 20:1 acrylamide to hemoglobin. The difference spectra between acrylamide-hemoglobin solution and hemoglobin solution, at each incubation time point, is presented. The spectra were acquired after 0 (black), 5 (dark blue), 10 (blue), 20 (light blue), 45 (green), 75 (light green) minutes and 72 hours (light grey) of incubation. Inset: Soret band region.

Figure 3 - Circular dichroism spectra of hemoglobin (black) and acrylamide-hemoglobin adduct (grey).

Figure 4 – Effect of temperature in the UV-visible spectra of hemoglobin and acrylamide-hemoglobin. **(A)** Absorbance at 405 nm (circles) and 274 nm (squares) as a function of temperature for hemoglobin (black) and acrylamide-hemoglobin (grey). **(B)** The van't Hoff plot of hemoglobin (black circles) and acrylamide-hemoglobin adduct (grey circles) used to estimate the thermodynamic parameters of thermal unfolding.

Figure 5 - UV-visible spectra of the Q bands region of deoxyhemoglobin (A) and oxyhemoglobin (B) of hemoglobin (black line) and acrylamide-hemoglobin adduct (grey line). The spectra were normalized for hemoglobin concentration.

Tables

Table 1 – Thermodynamic parameters of thermal unfolding of hemoglobin in the absence and presence of acrylamide.

	T_m (ºC)	$\Delta H_{unfolding}$ (kJ/mol)	$\Delta S_{unfolding}$ (kJ/mol/K)
Hemoglobin	49.3 ± 0.2	135 ± 7	0.42 ± 0.02
Acrylamide-hemoglobin adduct	55.8 ± 0.2	109 ± 10	0.33 ± 0.03

Figures

Figure 1

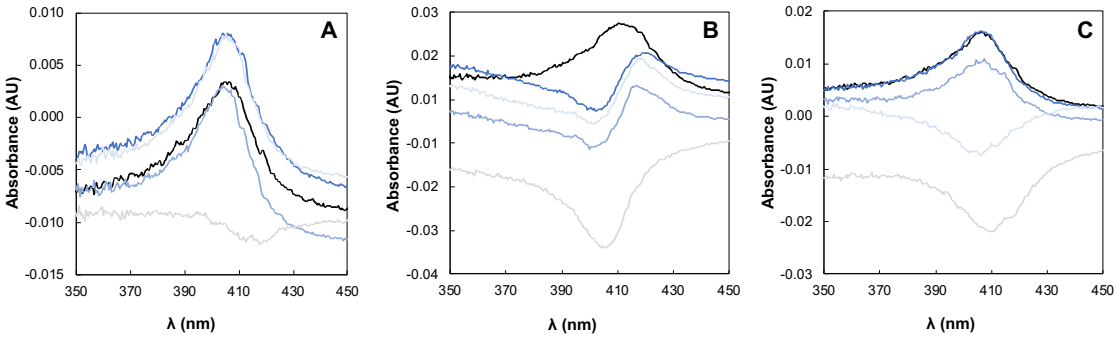

Figure 2

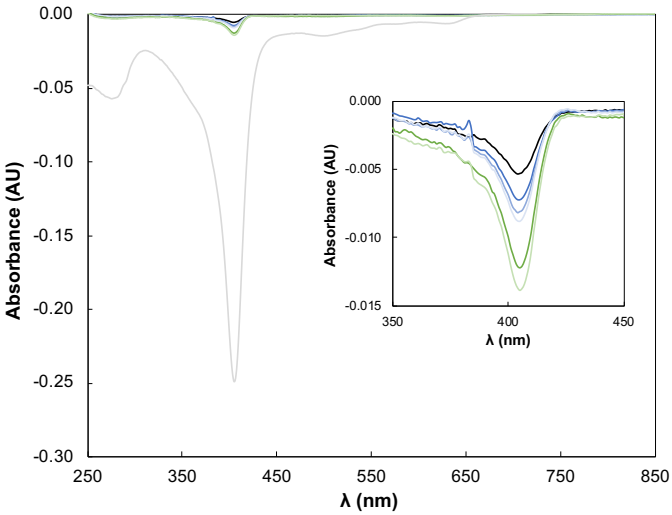

Figure 3

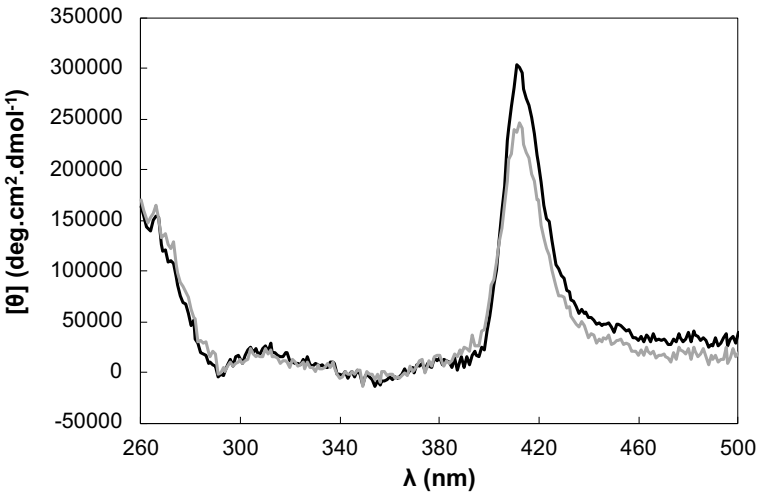

Figure 4

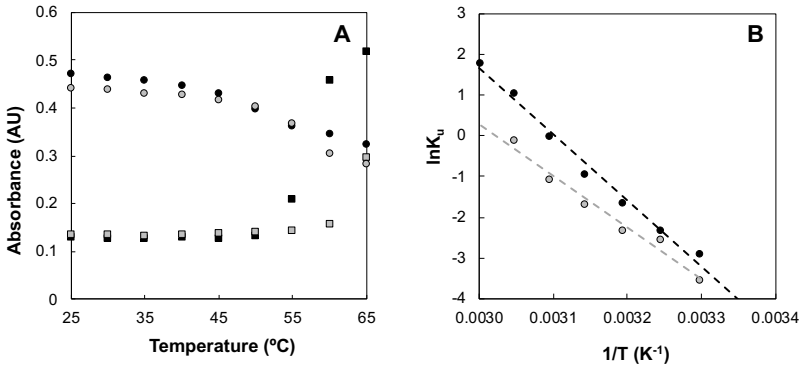

Figure 5