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Immobilization of arginase and its application in an enzymatic chromatographic column: Thermodynamic studies of nor-NOHA/arginase binding and role of the reactive histidine residue

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Abstract

A biochromatographic approach is developed to measure for the first time changes in enthalpy, heat capacity change and protonation for the binding of nor-NOHA to arginase in a wide temperature range. For this, the arginase enzyme was immobilized on a chromatographic support. It was established that this novel arginase column was stable during an extended period of time. The affinity of nor-NOHA to arginase is high and changes slightly with the pH, because the number of protons linked to binding is low. The determination of the enthalpy change at different pH values suggested that the protonated group in the nor-NOHA–arginase complex exhibits a heat protonation of approximately −33 kJ/mol. This value agrees with the protonation of an imidazole group. Our result confirmed that active-site residue Hist 141 is protonated as imidazolium cation. Hist 141 can function as a general acid to protonate the leaving amino group of L-ornithine during catalysis. The thermodynamic data showed that nor-NOHA–arginase binding, for low temperature (<15 °C), is enthalpically unfavourable and being dominated by a positive entropy change. This result suggests that dehydration at the binding interface and charge–charge interactions contribute to the nor-NOHA–arginase complex formation. The temperature dependence of the free energy of binding is weak because of the enthalpy–entropy compensation caused by a large heat capacity change, $\Delta C_p = -2.43$ kJ/mol/K, of arginase. Above 15 °C, the thermodynamic data ΔH and ΔS became negative due to van der Waals interactions and hydrogen bonding which are engaged at the complex interface confirming strong enzyme–inhibitor hydrogen bond networks. As well, by the use of these thermodynamic data and known correlations it was clearly demonstrated that the binding of nor-NOHA to arginase produces slight conformational changes in the vicinity of the active site. Our work indicated that our biochromatographic approach could soon become very attractive for studying other enzyme–ligand binding.

Keywords: Enzyme; HPLC; Ligand

1. Introduction

Arginase, a 105 kDa homotrimer containing a binuclear manganese cluster in each protomer, catalyzes the hydrolysis of L-arginine to form L-ornithine and urea through a metal-activated hydroxide mechanism [1–2]. The binuclear manganese cluster is located at the base of a 15-Å deep active-site cleft in each monomer. The metal ion that is more deeply situated in the active site cleft (designed Mn_A^{2+}) is coordinated by four amino acids and a solvent molecule, with square pyramidal geometry [3–6]. The second metal ion Mn_B^{2+} is coordinated by four amino acids and the bridging solvent molecule in distorted octahedral fashion. The Mn_A^{2+}–Mn_B^{2+} separation is 3.3 Å. All metal ligands except for aspartate 128 (Asp 128) make hydrogen-bond interactions with other protein residues, and these interactions contribute to the stability of the metal binding site [5]. The arginase structure is the first atomic resolution structure of a functional metalloenzyme that has a specific catalytic site and

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physiological requirement for two Mn^{2+} ions. The catalytic metal requirement is rooted in the preferred geometry of manganese coordination, which properly orients the metal-bridging solvent molecule for catalysis [3]. As a metal-bridging solvent molecule must satisfy the coordination preferences of two manganese ions simultaneously, its position, and therefore its optimal catalytic activity, would be highly sensitive to the substitution of one or both Mn^{2+} ions. Coordination of a catalytic group to two metals rather than one may enhance the dependence of optimal catalytic activity on proper metal selectivity. Only two other polar residues are found in the immediate active site: glutamate 277 (Glu 277) and histidine 141 (Hist 141). Glu 277 is located deep in the active-site cleft 4.5 Å away from $Mn_A{}^{2+}$. A $\approx 20^{\circ}$ conformational change about side chain torsion angle χ^2 to orient Glu 277 would yield an ideal salt link with the substrate guanidinium group. Moreover, this interaction would position the electrophilic guanidinium carbon of the substrate directly over the metal-bridging solvent molecule, which is likely to be nucleophilic hydroxide ion in the active catalyst. It is unlikely that the deprotonated substrate guanidinium group binds directly to the metal(s) because of its high pK_a of 13.5 [7]. The side chain of His 141 is located about half-way out of the active-site cleft. Interestingly, arginase with asparagine (Asn or N with amino acid nomenclature) substituted for histidine at position 141 (Hist 141 → Asn arginase) retains roughly 10% residual activity compared with the wild-type enzyme [7]. Given its location 4.2 Å away from the metal-bridging solvent molecule, it is possible that Hist 141 is a proton shuttle in catalysis, mediating proton transfer to and from bulk solvent. Direct proton transfer with bulk solvent may be operative in the absence of Hist 141, which could account for the significant residual catalytic activity of Hist-141 → Asn arginase. A proton shuttle function for Hist 141 of arginase would be analogous for Hist 64 in the zinc metalloenzyme carbonic anhydrase II [8]. On opposite sides of the active-site lip, charged residues are found which may contribute to the exquisite specificity of substrate recognition [9]. Two isoenzymes have been identified in mammals: arginase I catalyses the final cytosolic step of the urea cycle in liver, and arginase II is a mitochondrial enzyme that functions in L-arginine homeostasis in non-hepatic tissues. For example, arginase I may regulate substrate L-arginine bioavailability to NO synthase in the immune response. Macrophage arginase I and NO synthase are reciprocally regulated at the level of transcription: NO synthase is induced by T-helper type 1 (TH1) cytokines, and arginase I is induced by T-helper type 2 (TH2) cytokines [10–13]. As a modulator of NO-dependent macrophage cytotoxicity, arginase I is implicated in the regulation of macrophage activity in wound healing [14] and the suppression of the tumoricidal activity of macrophages [15] and T cells [16]. Recently, our group demonstrated that arginase I inhibition reduces endothelial dysfunction and blood pressure rising in spontaneously hypertensive rats [17]. An interesting feature observed in the active site of this enzyme is the presence of a catalytically important, non-coordinating histidine residue. The crystal structure of rat arginase I reveals that the enzyme contains a $Mn_2{}^{2+}$ cluster bridged by a water molecule/hydroxide ion believed to be the catalytic nucleophile [18–20]. pH rate profiles for rat arginase I indicate that a Hist 141 must be deprotonated for maximal catalytic activity [21]. Arginase I contains a histidine residue, Hist 141, located partway out of the active site cleft and 4.2 Å from the metal-bridging solvent molecule. Residue Hist 141 is strictly conserved in all arginases, as well as in the arginase family-members agmatinase and proclavaminate amidino hydrolase [22]. A variety of data implicate Hist 141 in catalysis: (i) arginase I is inactivated by treatment with diethyl pyrocarbonate (DEPC), but Hist 141 → Asn arginase I (i.e., Hist 141 N arginase I) is unaffected [23], (ii) *N*-bromosuccinimide (NBS) inactivates arginase I at Hist 141 [24], (iii) the Hist 141 N arginase I variant displays 11% of wild-type activity [23], (iv) Hist 141 → leucine (L) human arginase I (i.e., Hist 141 L human arginase I) exhibits 2.6% activity as compared to a wild-type control [25], (v) human arginase I is inactivated by DEPC and photoinactivated by rose Bengal, while Hist 141 → phenylalanine (F) arginase (i.e., Hist 141 F arginase) is unaffected by these treatments [26], and (vi) human arginase I is inactivated by Woodward's reagent K at Hist 141 [27]. Interestingly, Hist 141 F human arginase I and Hist 141 N rat arginase I exhibit only modest ($\leq$10-fold) changes in K_M values, so a significant interaction of Hist 141 with substrate arginine is unlikely [7,23,26]. The crystal structure of rat arginase I complexed with a boronic acid inhibitor shows that Hist 141 hydrogen bonds to a water molecule which in turn donates a hydrogen bond to the α-carboxylate group of the inhibitor [28]. Consistent with these data, it is proposed that Hist 141 serves as a proton shuttle that helps regenerate the nucleophilic metal-bound hydroxide ion for catalysis [18], analogous to Hist 64 of carbonic anhydrase [29,30]. The technique usually employed to immobilize enzymes on solid supports are mainly based on chemical mechanisms. These chemical immobilization methods mainly include enzyme attachment by covalent bonds between enzyme and matrix. The most widely used method is based on the activation of amino supports, independently of their nature: porous [31–38], siliceous [39–43], polymeric [44], or monolithic [45,42]. The arginase enzyme has been immobilized by employing *N*,*N*′-disuccinimidylsuberate (DSS) as activating agent [39,40]. This novel chromatographic support was used to determine and quantify the forces driving association between N^{ϖ}-hydroxy-nor-L-arginine (nor-NOHA) which is a very good arginase inhibitor [46] and the bovine liver arginase I enzyme. The energetic of binding of the inhibitor to the enzyme as both a function of temperature and pH was studied using this novel biochromatographic approach. Those experiments allowed us also to calculate the number of protons linked to ligand binding and to probe the catalytic function of Hist 141.

2. Experimental and method

2.1. Reagents

Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. nor-NOHA was obtained from Bachem (Germany) and crystalline bovine liver arginase I was obtained from Sigma–Aldrich (Paris, France). *N*,*N*′-disuccinimidyl suberate

was purchased from Sigma–Aldrich (Paris, France). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate used for the preparation of the mobile phases were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Apparatus

The HPLC system for these measurements consisted of a Merck Hitachi Pump L-7100 (Nogent sur Marne, France), a Rheodyne injection valve with a 20 μL sample loop (Montluçon, France) and a Merck L-4500 diode array detector (Nogent sur Marne, France). The MODULO-CART HS UPTISPHERE 3 NH_2 (50 mm × 4.6 mm) was purchased from Interchim (Montluçon, France). The arginase column prepared via the in situ technique is given below. This arginase column temperature was controlled with a cryoimmerser for low temperature. Throughout the study the flow-rate was maintained constant and equal to 1 mL/min.

2.3. Covalent immobilization technique of arginase on DSS-activated aminopropyl silica

The in situ immobilization technique was considered in this study. The immobilization of arginase via the amino groups of the enzyme on aminopropyl silica pre-packed column activated with DSS was carried out as follows [39,40]. Briefly, the column was first washed (1 h for each eluent at flow rate 0.5 mL/min) with acetonitrile and with $NaHCO_3$ (0.1 M)–CH_3CN (67/33, v/v). Then the stationary phase was activated by recycling 0.450 g DSS in 30 mL acetonitrile for 12 h followed by washing with 15 mL $NaHCO_3$ (0.1 M) at 0.5 mL/min, 20 mL of water–acetonitrile (33/67, v/v) and finally with 100 mL of $NaHCO_3$ (0.1 M). A solution of 90 mg arginase in 40 mL of a NaHCO3 solution (0.1 M) was recirculated through the column at a flow-rate of 0.5 mL/min for 16 h, flushing and back flushing every 15 min during the first hour, every 30 min during the following 3 h. After the immobilization procedure, the column was rinsed for 1 h with phosphate buffer (pH 7.0; 5 mM) at flow rate of 0.5 mL/min. After that, the column was flushed with 50 mL of a glycine solution (1 M) in phosphate buffer (pH 7.0; 5 mM) and then rinsed with the same phosphate buffer. The amount of immobilized enzyme on the activated DSS aminopropyl silica column, as determined by elemental analysis, was found to be 81.56 mg/g solid support. For this analysis, four fractions of the stationary phase were removed from the head to the end of the column. The maximum relative difference of the amount of immobilized enzyme between these different measurements was always 0.5%, making a homogeneous enzyme distribution in the column from the ends to the core.

2.4. Chromatographic operating conditions

The mobile phase consisted of 5 mM phosphate buffer. The phosphate buffer was prepared by mixing equimolar solutions of mono- and dibasic sodium phosphate to produce the desired eluent pH. The mobile phase pH range was 5.0–6.5, the column temperature ranged from −2 to 20 °C. To avoid the presence of significant non-linear effects, the solute amount added onto the column corresponded to the smallest sample size allowing the detection of nor-NOHA in all operating conditions. The sample was injected at least three times.

2.5. Column stability

The column stability was evaluated by comparing the nor-NOHA retention factor before and after more than 4 months in the same conditions. No significant change in retention was observed. This column is thus stable during a long period of time.

2.6. Column binding properties

In order to confirm the binding properties of the immobilized enzyme on the chromatographic support, the study of the NOHA (N^{ϖ}-hydroxy-L-arginine, an other well-known arginase inhibitor which is known to bind on the same active site than nor-NOHA) displacement of its arginase binding site by nor-NOHA was investigated using the Langmuir approach [47–51]. For this, single and multi-component isotherms were determined using the perturbation technique [47]. This method was described previously for the analysis of the progesterone displacement of its human binding site by β-estradiol [52]. Briefly, single component isotherms of NOHA and nor-NOHA (each in the concentration range 0.01–0.1 mol L^{-1}) and two-component isotherms of a mixture of NOHA and nor-NOHA (at a constant concentration ratio 0.01:0.01 to 0.1:0.1 mol L^{-1}) were measured in the phosphate buffer (5 mM; pH = 6.5) at 20 °C. Each isotherm data point was measured in 11 subsequent steps after equilibration of the arginase column with a solution containing a single compound (NOHA or nor-NOHA (0, 0.0025, 0.005, 0.0075, 0.01, 0.0125, 0.015, 0.0175, 0.02, 0.0225, 0.025 mol L^{-1})) or a mixture of NOHA and nor-NOHA ($C_{NOHA} + C_{nor\text{-}NOHA} = 0$, 0.0025, 0.005, 0.0075, 0.01, 0.0125, 0.015, 0.0175, 0.02, 0.0225, 0.025 mol L^{-1}) until a stable detector response was obtained. Small volume (5 μL) of the most concentrated sample (single or the mixture) was injected onto the column and the apparent retention times were measured.

3. Results and discussion

3.1. Column binding properties

The Langmuir approach was found to describe adequately the experimental data (non-linear coefficients of the models were always higher than 0.991). It was found that the column saturation factor for the two compounds was identical ($\alpha = 95.20$) (the difference for the two inhibitors was always lower than 0.02%) justifying the use of the competitive Langmuir isotherm equation for this study [52]. For the evaluation of the coefficients of the two-components competitive bi-Langmuir isotherms, the iterative Marquadt approach was used to fit the best isotherm coefficients values as shown previously described [48,49,52]. There is a good agreement between the theoretical and experimental data, also confirmed by the low standard devi-

ation ($\varepsilon = 1.15$) for all total isotherm derivative. These results confirmed the importance of the competitive effect between nor-NOHA and NOHA to bind on the same active binding site. The corresponding equilibrium affinity constants K for nor-NOHA and NOHA were, respectively, $19.10^5 \pm 16.10^4\ M^{-1}$ and $9.10^4 \pm 8.10^3\ M^{-1}$. The reverse of these K-values ($1/K$) was in the μM range, (0.5 μM and 11 μM, respectively), in the same order magnitude than those obtained by other authors for these two arginase inhibitors [46]. This confirmed that bonded arginase on silicea do not modified the structure of the active site and the binding properties.

3.2. Bulk solvent pH effects

Valuable information about the processes driving the arginase–nor-NOHA association mechanism can be further gained by examining the pH dependence on nor-NOHA retention. The nor-NOHA retention on the arginase stationary phase can be evaluated using the retention factor k':

$$k' = \frac{t - t_o}{t_o} \tag{1}$$

where t is the retention time of nor-NOHA and t_o is the column void time. To obtain the thermodynamic retention time, i.e., the accurate measure of nor-NOHA retention, t was determined by calculating the first moment of the peak as previously described [53]. The void time was determined using the mobile phase peak. The retention times and column void time were corrected for the extracolumn void time. It was assessed by injections of solute onto the chromatographic system when no column was present. As well, the nor-NOHA retention factor can be related to the association constant between arginase and nor-NOHA as follows:

$$k' = \sigma K \tag{2}$$

where σ is equal to the ratio of the active binding site number in the column over the void volume of the chromatographic column. When the pH of the bulk solvent changed, a full description is essential, which explicitly maintains conservation of mass of each species and take into account binding of H^+ to arginase (Arg_{ase}), nor-NOHA and the complex Arg_{ase}·nor-NOHA:

$$\mathrm{Arg_{ase}}(\mathrm{H^+})_A + \mathrm{nor-NOHA}(\mathrm{H^+})_B + n_{\mathrm{H^+}}\mathrm{H^+} \leftrightarrow \mathrm{Arg_{ase}} \cdot \mathrm{nor-NOHA}(\mathrm{H^+})_C \tag{3}$$

where $n_{H^+} = C - (A + B)$ is the number of protons linked to this nor-NOHA binding reaction of arginase.

The association constant of this equilibrium was given by:

$$K = \frac{\left[\mathrm{Arg_{ase}} \cdot \mathrm{nor-NOHA}\right]}{\left[\mathrm{Arg_{ase}}\right]\left[\mathrm{nor-NOHA}\right]\left[\mathrm{H^+}\right]^{n_{\mathrm{H^+}}}} \tag{4}$$

Eq. (4) can be rewritten as:

$$K = \frac{K^0}{\left[\mathrm{H^+}\right]^{n_{\mathrm{H^+}}}} \tag{5}$$

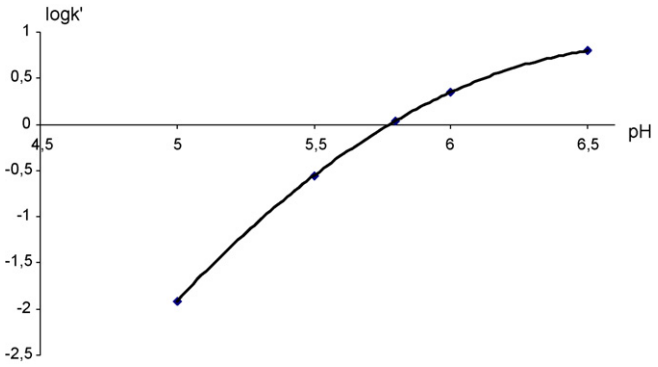

Fig. 1. pH dependence of the log k' values of nor-NOHA at 271 K.

where K^0 is the K value for $[H^+] = 1$ M. Taking the logarithm of Eq. (5) gives:

$$\log K = \log K_0 - n_{\mathrm{H^+}} \log[\mathrm{H^+}] \tag{6}$$

As, $-\log[H^+]$=pH, Eq. (6) can be rewritten as:

$$\log K = \log K_0 + n_{\mathrm{H^+}}\mathrm{pH} \tag{7}$$

Derivation of Eq. (7) gives:

$$\frac{\partial \log K}{\partial \mathrm{pH}} = n_{\mathrm{H^+}} \tag{8}$$

Combining Eqs. (2) and (8) the following is obtained:

$$\frac{\partial \log k'}{\partial \mathrm{pH}} = n_{\mathrm{H^+}} \tag{9}$$

Fig. 1 reports, at −2 °C, all the data acquired on the evolution of the retention factors of nor-NOHA when the bulk solvent pH increased from 5.0 to 6.5. Looking at the experimental data, it is evident that the trend is not linear. This is highlighted by the following quadratic function:

$$\log k' = \log k'_o + \lambda_1 \mathrm{pH} + \lambda_2 \mathrm{pH}^2 \tag{10}$$

where k'_o is the retention factor extrapolated at pH = 0 and $\lambda_{1,2}$ were constants related to the structure of nor-NOHA. From the non-linear log k' versus pH plot, these constants were determined using Eq. (10) ($r^2 = 0.997$). The log k'_o, λ_1 and λ_2 values were, respectively, equal to −40.66, 12.32 and −0.91. From a full regression model, a Student's t-test was used to provide the basis whether or not the model's coefficients were significant. Results of this test show that no variable can be excluded from the model. The line showed in Fig. 1 was obtained by fitting the experimental data to the quadratic function of the pH. The agreement between the experimental data and this second-order relationship between log k' and pH is excellent and suitable for accurate description of the nor-NOHA–arginase association in the whole range analyzed. From Eq. (9) the slope of the curve log k' versus pH gives the number of proton n_{H^+} linked to the nor-NOHA-binding reaction of arginase. Fig. 2 showed how n_{H^+} decreased linearly with pH and are practically zero at pH = 6.5. The protonation changes for nor-NOHA-arginase binding can be viewed as arising from a shift in the pK_a of one or more groups on complex formation. Alterations in the protonation state of certain

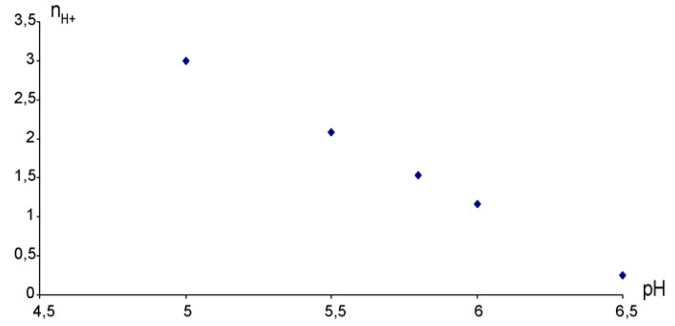

Fig. 2. pH dependence of the linked protons (per mol arginase), n_{H^+}, at 271 K.

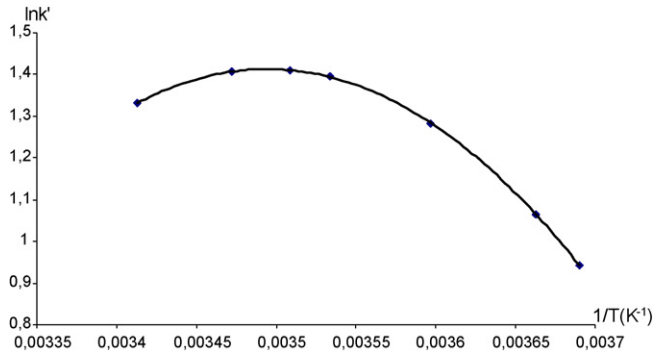

Fig. 3. Temperature dependence of the $\ln k'$ values of nor-NOHA at pH = 6.5.

residues in the vicinity of the arginase binding site may explain the variation of the number of protons at the pH examined. At this point, it is interesting to point out the role of histidine 141 (Hist 141) residue. Considering the possible pK_a value of this His-141 residue in the enzyme and its ionization enthalpy change, a shift in its pK_a value can explain the uptake of protons obtained at the pH range studied. There are several reasons that can explain these changes in pK_a values. For instance, a variation in the micropolarity of the environment surrounding the side chains of certain active site residues as a result of nor-NOHA binding is a possibility. Alternatively, a protonated form could be stabilized by forming hydrogen bound with a neighbouring group. Crystallographic analysis of all arginase structures determined to date yields Hist 141–Glu 277 interactions ranging 2.8–5 Å, largely due to the conformational flexibility of Hist 141; such flexibility would be consistent with the general acid function contemplated for Hist 141 [54]. Thus, we conclude that His141 which is not coordinated to Mn^{2+} may become stabilized with a positively charged imidazolium group by its hydrogen bound with Glu 277. This result led to a revised mechanistic proposal for arginase. In the first step of the arginase reaction, the metal bridging hydroxyde ion attacks the guanidinium carbon of L-arginine to form a tetrahedral intermediate which subsequently collapses to form products ornithine and urea. Our results suggest that imidazolium group of Hist 141 protonates the amino leaving group of L-ornithine in the collapse of the tetrahedral intermediate to form L-ornithine and urea. Carboxylate–histidine imidazolium pairs serve as general acids to protonate amino leaving group in other metalloenzymes. For example, in the zinc deacetylase enzyme, the side chain of Hist 265 is protonated at physiological pH and protonates the amino leaving group in the collapse of the tetrahedral intermediate to form UDP-3-*O*-((R)-3-hydroxymyristoyl)-*N*-acetylglucosamine and acetate [55,56].

3.3. Possible thermodynamic origins of the nor-NOHA binding to arginase

The temperature dependence of the nor-NOHA retention factor is given by the well known thermodynamic relation [57,58]:

$$\frac{\partial \ln k'}{\partial T} = -\frac{\Delta H}{RT^2} \tag{11}$$

where ΔH is the binding enthalpy and R is the gas constant. The analysis of the thermodynamics was carried out by measuring the nor-NOHA retention factor in the temperature range −2 to 20 °C at pH = 6.5. The van't Hoff plot for the nor-NOHA exhibits a significant non-linear behaviour as shown in Fig. 3. From this non-linear plot and using Eq. (11) the ΔH values were determined (Table 1). ΔH depends linearly on the temperature in the range −2 to 20 °C. At low temperature, the binding enthalpy contributes non-favourably to the free energy of binding. At about 13 °C, the enthalpy change of association was nil and above this value became negative indicating that the complex formation is enthalpically governed. This means that van der Waals interactions and hydrogen bonding (both characterized by negative enthalpy changes at these temperatures) are engaged at the complex interface confirming strong enzyme–inhibitor hydrogen bond networks. In the temperature range −2 °C to 13 °C, as temperature increases, the binding enthalpy becomes less endothermic (more favourable). As can be seen in Fig. 5 the enthalpy change decreases quickly with temperature due to a large negative heat capacity change $\Delta C_p = -2.43$ kJ/mol/K obtained from the slope ΔH versus temperature in Fig. 4. A rather high negative ΔC_p value is normal in binding studies and is a distinctive feature of site specific binding. As well, a corresponding change in the ΔH changes with pH given by the following relation will take place [59,60]:

$$\left(\frac{\partial \Delta H}{\partial \mathrm{pH}}\right)_T = -2.3RT^2\left(\frac{\partial n_{H^+}}{\partial T}\right)_{\mathrm{pH}} \tag{12}$$

For example, at pH 6.0, the plot n_{H^+} versus temperature was given in Fig. 5. As the temperature increased the number of protons decreased and $(\partial n_{H^+}/\partial T)_{\mathrm{pH}}$ had a negative value. Thus, from Eq. (12), $(\partial \Delta H/\partial \mathrm{pH})$ has a positive value, and as the pH

Table 1
Apparent thermodynamic parameters for the binding of nor-NOHA to arginase at pH = 6.5

T (°C)	ΔH (kJ/mol)	ΔS (J/mol/K)
−2	37.4 (1.9)[a]	161.2 (4.8)
0	32.5 (1.3)	140.9 (4.0)
5	20.4 (1.1)	92.1 (5.2)
10	8.2 (1.2)	45.7 (6.0)
12	3.4 (1.4)	27.8 (2.5)
15	−3.8 (1.2)	1.7 (0.9)
20	−16.0 (1.9)	−39.9 (4.0)

[a] Standard deviations in parentheses.

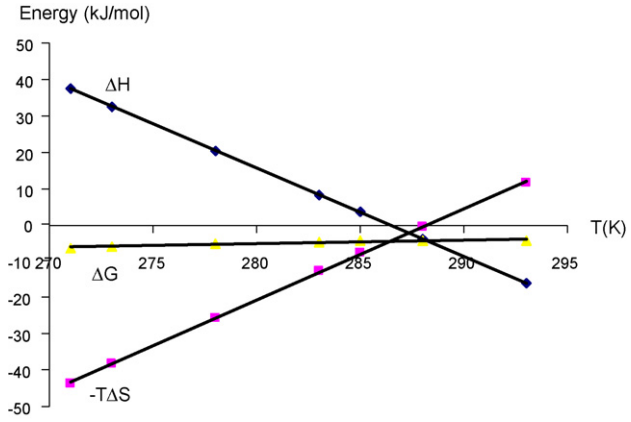

Fig. 4. Temperature dependence of the thermodynamic parameters for the binding of nor-NOHA–arginase at pH = 6.5.

increased, the binding enthalpy contributes non-favourably to the free energy of binding. As well, using the above relations, the magnitude of the heat protonation of the protonated group in the nor-NOHA–arginase complex was approximately determined 33 kJ/mol. This value agrees with the heat protonation of an imidazole group (−30 kJ/mol) and confirmed that Hist 141 may become protonated in the complex. As well, the entropy change ΔS was determined from the ΔH obtained and using the value of ΔG calculated from the relation:

$$\Delta G = -RT(\ln k' - \ln \sigma) \tag{13}$$

The ΔS value was then calculated using the equation:

$$\Delta S(T) = -\frac{\Delta G(T)}{T} + \frac{\Delta H(T)}{T} \tag{14}$$

For the determination of ΔS, the number of moles of immobilized enzyme was used, assuming that the arginase immobilized on the column was available for an interaction with nor-NOHA. However, it is not always verified, and the number of active sites in an affinity enzyme based column can be lower than the number of moles of ligand effectively immobilized. Therefore, the ΔS values were also determined using a number of moles of immobilized enzyme representing 50% of the number of moles of enzyme effectively immobilized. The maximum relative difference observed of the ΔS values between these different measurements was always 0.7%. Therefore, neglecting these effects has no serious consequences on the interpretation of the thermodynamics. The ΔS is also displayed in Table 1. At about 15 °C, the entropy change was nil. Below this temperature value, the binding process is therefore accompanied by a positive entropy change, which depends also strongly on temperature, while ΔG changes little with temperature because of the enthalpy–entropy compensation (Fig. 4). This behaviour has been found in many ligand–protein interactions [61–66]. At low temperature, below ≈15 °C, the positive enthalpy change and positive entropy change of binding upon complex formation can be justified by charge–charge interactions and hydrophobic forces [67,68]. The nor-NOHA hydroxyguanidino group is bound to the Mn^{2+} ions [69]. Structure activity relationships for arginase analogues indicate that electrostatic interactions with α substituents of the substrate are critical for catalysis [69]: deletion of the α-carboxylate group or the α-amino group results in 10^2–10^5-fold reductions in k_{cat}/K_m [9]. Inspection of the arginase active site indicates that the positively charged side chain Arg 21 may interact with the negatively charged α-carboxylate group of the substrate, and the negatively charge side chain of Asp 181 may interact with the positively charged α-amino group of the substrate [9]. In the association of a protein to a ligand, several contacts between non-polar groups of nor-NOHA and arginase are engaged. Thus, substantial fraction of polar and non-polar surface is buried in the complex formation which is thus accompanied by negative heat-capacity changes of the system. Murphy and Freire [70] and Spolar and Record [71] have suggested that ΔC_p may be described as a phenomenon in hydration terms, pointing out that changes in vibrational modes apparently contribute little to ΔC_P. Similarly, Connely and coworkers have shown that the heat capacity of ligand binding can be approximated by contributions arising from dehydration of solvents exposed groups [72–74]. The interaction between apolar groups of nor-NOHA and arginase requires the dehydration of both protein and the drug and there is an entropic gain from the transfer of interfacial water into the bulk solvent. Assuming that ΔC_p value is due principally to the hydrophobic effect [75] and that the decrease in heat capacity per mol of water lost is, on average 24 kJ/mol/K [76], one can calculate that about 101 water molecules are released. As well, the enthalpy and heat capacity values provide an estimation of solvent accessibility changes during the binding. Murphy and Freire have suggested the following equations for ΔC_p and ΔH_{60} (enthalpy change at 60 °C) [70]:

$$\Delta C_p = 1.88\Delta ASA_{ap} - 1.09\Delta ASA_p \tag{15}$$

$$\Delta H_{60} = -35.3\Delta ASA_{ap} + 131\Delta ASA_p \tag{16}$$

where ΔC_p, ΔH_{60} and ΔASA are in J/K/mol, J/mol and Å^2 units, respectively [70,77]. ΔASA_{ap} and ΔASA_p represent the changes in non-polar and polar areas exposed to solvent (accessible surface area) that take place upon enzyme–inhibitor binding. The temperature of 60 °C in the expression is the mean value of the denaturation temperature of the model proteins used in the analysis. For example, using $\Delta H_{60} = -11.63$ kJ/mol, assuming a $\Delta C_p = -2.43$ kJ/mol/K, the changes in accessible surface

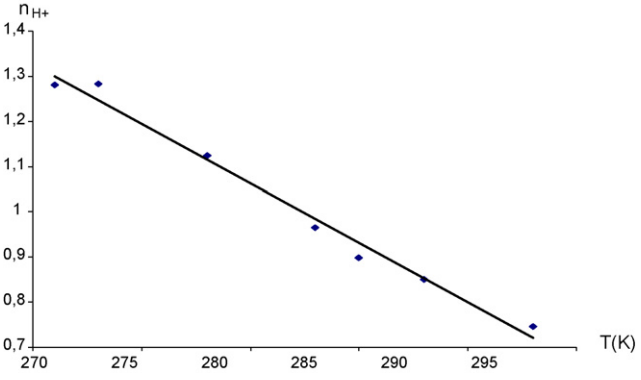

Fig. 5. Temperature dependence of the linked protons (per mol arginase) n_{H^+}.

areas are $\Delta ASA_{ap} = -2123.02$ Å^2 and $\Delta ASA_P = -1435.81$ Å^2. Therefore, the results of Murphy's approach indicated that the surface area buried on complex formation comprises 67% non-polar surface and 33% polar surface. The amount of non-polar surface involved appeared too large to be accounted for in "rigid body" association. That could justify the accessible surface area value calculated. At low temperature, below ≈15 °C, ΔH and ΔS values remained positive since the contributions of the desorption of the solvent molecules overweight that of the nor-NOHA adsorption on the enzyme surface. At 15 °C, $T\Delta S \approx 0$, it appears so that there should be some source of negative entropy compensating the positive entropy of dehydration.

The overall entropy change at 15 °C can be split up in the following way [71]:

$$\Delta S = \Delta S_{hydr} + \Delta S_{trans} + \Delta S_{specific} \quad (17)$$

where ΔS_{hydr} is the contribution by the hydrophobic effect. ΔS_{trans} accounts for the reduction in the overall rotational and translational degrees of freedom, as well as the immobilization of amino acid side chain at the complex interface. $\Delta S_{specific}$ describes system-specific contributions such as reduction of main chain mobility and entropic contributions from polar interactions. ΔS_{hydr} can be estimated from

$$\Delta S_{hydr} = 1.35 \Delta C_p \ln\left(\frac{T}{386}\right) \quad (18)$$

where ΔC_p (in J/mol/K) is the measured heat capacity change, T the absolute temperature and 386 the reference temperature at which the entropy of transfer of non-polar liquids to water vanishes [71]. For nor-NOHA–arginase complex at 15 °C we obtained $\Delta S_{hydr} = +0.96$ kJ/mol/K. From $T\Delta S \approx 0$ J/mol/K, we calculated that $\Delta S_{trans} + \Delta S_{specific} = -0.96$ kJ/mol/K. For a great number of bimolecular association reactions, ΔS_{trans} has been thought to contribute −0.21 kJ/mol/K of rotational and translational entropy [71]. Hence, the remaining entropic loss of −0.75 kJ/mol/K must be contributed by the loss in the conformational restrictions of nor-NOHA and arginase. This unfavourable conformational change entropy could proceed from fixation of side chains at the interface and structural changes in the interacting molecules upon complex. Our results showed that adaptive conformational transitions are associated with the nor-NOHA–arginase complex formation where both components are able to adjust their recognition surfaces in order to maximize complementarities through tightly packed contacts involving Coulomb interactions and hydrogen bonding [78,79]. This result can be objectivized by the following considerations. It was previously shown that both the length of the chain linking the amino-acid and *N*-hydroxyguanidine functions and the bulky nature of the hydroxyguanidino group of nor-NOHA are very important for recognition by arginase [46,69].

Nor-NOHA was found to be much more potent than NOHA (addition of a CH_2 moiety) to inhibit the arginase-dependent hydrolysis of L-arginine [46]. The addition of a CH_2 moiety may restrict the α-substituents of the inhibitor molecule from achieving an optimal constellation of hydrogen bond interactions in the arginase active site, suggesting well, that slight conformational changes in the vicinity of the active site of the enzyme were also coupled to binding for an optimal association between the ligand and the enzyme.

4. Conclusion

For the first time, the binding nor-NOHA–arginase was analysed in the large temperature range −2 °C to 20 °C using a biochromatographic approach. This novel arginase column was stable during a long period of time and allowed us the determination of the thermodynamic data of this association. The results of this study presented here can be summarized as: (i) the affinity of nor-NOHA to arginase was high and changed slightly with the pH. The binding is accompanied by a proton uptake which can be attributed to an increase in the pK_a of one or more groups of the drug and/or enzyme in the complex. The present results confirmed that (i) Hist 141 in the active site which is not coordinate to Mn^{2+} may become protonated in the complex and that it could function as a general acid to protonate the leaving amino group of L-ornithine during catalysis. (ii) The binding in a low temperature domain (<15 °C) is entropically driven, indicating a contribution from hydrophobic effect due to the release of water molecules when nor-NOHA and arginase associate. (iii) The large negative ΔC_p suggest that, in this low temperature range, the driving force for the binding of nor-NOHA to arginase is provided by electrostatic interactions and several contacts between non-polar groups of nor-NOHA and arginase. (iv) Above 15 °C, the thermodynamic data ΔH and ΔS became negative due to van der Waals interactions and hydrogen bonding which are engaged at the complex interface confirming strong enzyme–inhibitor hydrogen bond networks. By the use of known correlations between the heat capacity change and the burial of non-polar surface area, the surface area that is burried in the nor-NOHA–arginase complex was estimated. These results demonstrated that the binding of nor-NOHA to arginase produces also slight conformational changes in the vicinity of the active site. This arginase column could find applications such as enzymatic activity study. Further experiments are now in progress in our laboratory in order to couple our arginase column through a switching valve to an analytical column to study the influence of various parameters on enzymatic activity.

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