Journal of Chromatography B, 873 (2008) 37–40

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Biochromatographic framework for analyzing magnesium chloride salt dependence on nor-NOHA binding to arginase enzyme

T. Bagnost, Y.C. Guillaume*, M. Thomassin, A. Berthelot, C. Demougeot, C. André

Equipe Sciences Séparatives Biologiques et Pharmaceutiques (2SBP/EA-4267), Faculté de Médecine et de Pharmacie, Université de Franche-Comté, Place St. Jacques, 25030 Besançon Cedex, France

ARTICLE INFO

Article history:
Received 5 March 2008
Accepted 7 July 2008
Available online 29 July 2008

Keywords:
Arginase
Nor-NOHA
Magnesium

ABSTRACT

Our group demonstrated recently that arginase I inhibition reduces endothelial dysfunction and blood pressure rising in spontaneously hypertensive rats [C. Demougeot, A. Prigent-Tessier, C. Marie, A. Berthelot, J. Hypertens. 23 (2005) 971; C. Demougeot, A. Prigent-Tessier, T. Bagnost, C. Andre, Y. Guillaume, M. Bouhaddi, C. Marie, A. Berthelot, Life Sci. 80 (2007) 1128]. This discovery opens interesting perspectives in the development of new drugs against hypertension. As well, in a previous paper [T. Bagnost, Y.C. Guillaume, M. Thomassin, J.F. Robert, A. Berthelot, A. Xicluna, C. Andre, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 856 (2007) 113], a novel biochromatographic column was developed in our laboratory for studying the binding of N^{ω}-hydroxy-nor-L-arginine (nor-NOHA), an arginase inhibitor, with this enzyme. In this manuscript, using this novel biochromatographic concept, the effect of magnesium chloride on the nor-NOHA/arginase binding was analyzed for the first time. This study demonstrated that the salt ions interacted with arginase and played a great role in the nor-NOHA/arginase association. For a salt concentration (x) in the medium less than 3 mM, the nor-NOHA/arginase binding decreased with x due to a decrease of the charge–charge interactions between nor-NOHA and its arginase binding site. Above 3 mM of salt in the medium, the affinity of nor-NOHA to arginase increased slightly with x because the net number of ions (n) (Mg^{2+} or Cl^{-}) released or bound upon complex formation is low. As well, it was clearly demonstrated, that above 3 mM the n value depend on the salt concentration in the bulk solvent and was approximately nil for $x = 12$ mM. This dependence was due to a gradual and conformational change of the arginase enzyme which around 12 mM adopted a less flexible structure; its binding site was thus less accessible to nor-NOHA and nor-NOHA–arginase association decreased slightly.

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 [4,5]. 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 [6–8]. 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. The arginase structure is the first atomic resolution structure of a functional metalloenzyme with a specific catalytic site and 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 [8,9].

In mammals, arginase exists in two isoforms. Arginase I, a protein that is elevated in inflammatory diseases, is located in the cytosol, and it is mainly expressed in liver and macrophages [10]. Arginase II is primarily expressed in extrahepatic tissues, with high abundance in the kidney [10]. Recently, our group demonstrated that arginase I inhibition reduces endothelial dysfunction and blood pressure rising in spontaneously hypertensive rats [1,2]. A novel chromatographic support was as well developed to determine and quantify the forces driving association between N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) which is a very good arginase inhibitor [3]. 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 [3]. Magnesium cation Mg^{2+} is an important factor in the physiology of cardiovascular apparatus and the pathogenesis of cardiovascular diseases. In some hypertensive patients, a magnesium deficit because of its numerous noxious

* Corresponding author. Tel.: +33 3 81 66 55 44.
E-mail address: yves.guillaume@univ-fcomte.fr (Y.C. Guillaume).

1570-0232/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2008.07.024

actions on the cardiovascular apparatus must be controlled and may behave as a cofactor of constitutional or acquired hypertensive factors [11–13]. This seems to be particularly the case with stress sensitive patients with labile hypertension because of the dose links between magnesium deficit and stress [14–16]. Interaction of arginase with divalent metal ions, like Zn^{2+} [17], Ni^{2+}, Co^{2+} [18], Cu^{2+}, Hg^{2+} [19], and Cd^{2+} [20], were studied. But no study examines the potential Mg^{2+} effect on arginase. In this study the effect of magnesium chloride salt on the nor-NOHA/arginase binding was analyzed for the first time using our novel biochromatographic approach.

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 bovin native iver arginase I enzyme (NAIE) was obtained from Sigma Aldrich (Paris, France). Magnesium chloride, 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 preparation of the arginase column (50 mm × 4.6 mm) via the in situ technique was given in [3]. Throughout the study, the flow-rate was maintained constant and equal to 1 mL/min.

2.3. 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. When not used, the arginase column was stored at 10 °C in the loading buffer (phosphate buffer (pH 7.4; 5 mM))

2.4. Chromatographic operating conditions for the thermodynamic approach and magnesium effect study

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 pH 7.4 with a Mg^{2+} concentration varying from 0.5 mM to 15 mM (these values include cellular and cytosol Mg^{2+} concentrations). To avoid the presence of significant nonlinear effects, the solute amount added onto the column corresponded to the smallest sample size allowing the detection of nor-NOHA in all operating conditions. Nor-NOHA solutions were prepared in the mobile phase at a concentration of 7 μM, and 20 μL was injected at least three times.

2.5. Thermodynamic relationships

The solute retention on the arginase stationary phase can be evaluated using the retention factor k':

$$k' = \frac{t - t_0}{t_0} \quad (1)$$

where t is the retention time of the solute, and t_0 is the column void time. To obtain the thermodynamic retention time, i.e., the accurate measure of solute retention, t was determined by calculating the first moment of the peak as previously described [21].

The void time was determined using the mobile phase peak. The retention time and the column void time were corrected for the extra-column void time. It was assessed by injections of solute onto the chromatographic system when no column was present. At infinite dilution, i.e., under linear elution conditions, and assuming that non specific interactions between solute and chromatographic support was negligible (see below), the retention factor can be related to the association constant K between nor-NOHA and arginase as follows:

$$k' = \sigma K \quad (2)$$

where σ is equal to the ratio of the active binding site number in the column (m_L) over the void volume of the chromatographc column (V_M).

3. Results and discussion

In order to study the salt concentration effect on the nor-NOHA association to arginase, the plot $\ln k'$ versus $\ln x$ was studied. The salt concentration x varied from 0.5 mM to 15 mM (Fig. 1). To assess if this retention factor change with increasing x was due to a variation in the binding capacity of the arginase column, the concentration dependencies of the nor-NOHA retention were measured at different x values [22]. No change in the number of binding sites was observed when x varied. The knowledge of the salt effect operative in the nor-NOHA–arginase interacting system could provide valuable information on the role of the Coulomb interactions in the association process. Previous papers have reported that relations derived from the Wyman concept constitute a valuable tool to describe the salt dependence on the solute retention in affinity chromatography [23–27]. The salt effects on the equilibrium constant K between the nor-NOHA and arginase can be modeled at a thermodynamic level in terms of the direct stoichiometric participation of ions and water in the association reaction. The dependence of K on the mean ionic activity a can be formulated as follows, via the linkage Wyman relations [23–27]:

$$\frac{\partial \ln K}{\partial \ln a} = n - \frac{pm}{55.6}N \quad (3)$$

where n and N are respectively the net number of salt ions and water displaced or bound in forming the nor-NOHA arginase complex, m is the molal concentration of salt and p is the total number of ions released or bound upon complex formation. At low enough salt concentration, the consequences of water release are insignificant. So, the dependence of K on salt activity provides a measure of the

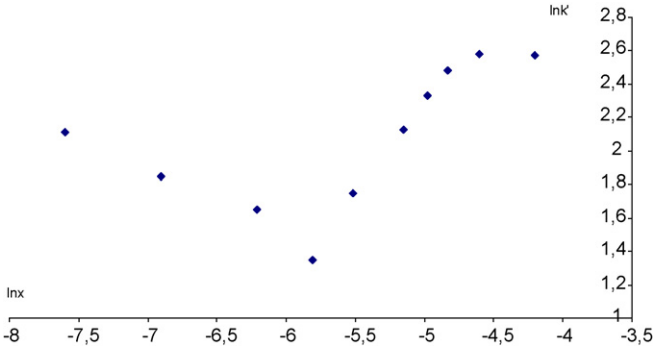

Fig. 1. logarithm of the salt concentration x (M) dependence of the $\ln k'$ values of nor-NOHA, at 298 K.

net number of ions released or bound upon complex formation. Assuming that replacing the ionic activity a by salt concentration x introduces little error over the experimental salt concentration range, an approximate form of Eq. (3) is obtained:

$$\frac{\partial \ln k'}{\partial \ln x} = n \tag{4}$$

Combining Eqs. (2) and (4) the following relation can be obtained:

$$\ln k' = \alpha + \beta \ln x + \delta(\ln x)^2 \tag{5}$$

The plot $\ln k'$ versus $\ln x$ can be divided into two salt concentration domains demonstrating a change on the nor-NOHA/arginase binding (Fig. 1). At the lowest salt concentrations (0.5 mM < x < 3 mM) (domain 1), the Cl^- anion of the $MgCl_2$ salt and the positively charged α-amino group of the substrate [28] formed an ion pair. Consequently, the ionic attraction between the negatively charge side chain of Asp 181 of arginase and the positively charged α-amino group of the substrate [28] decreased and the nor-NOHA–arginase affinity was decreased. This result was in accordance with some authors who indicated that electrostatic interactions are critical for catalysis [29,30]. Moreover, modeling studies have shown that divalent cations can be bound to negatively charge side chain of Asp 181 of arginase [30]. Thus the nor-NOHA complexation decrease with the enzyme was also attributed to a competition effect between the salt ions and the charged substrate nor-NOHA for binding with arginase. Integrating form of Eq. (5) is obtained as:

$$\ln k' \approx \ln k'_0 + n \ln x \tag{6}$$

where k'_0 is the retention factor in a hypothetical 1 M salt concentration reference state. From the linear $\ln k'$ versus $\ln x$ plot in domain 1, i.e., 0.5 mM < x < 3 mM, the n value was determined using Eq. (6) (r^2 = 0.969). The obtained value n = −0.40 was in accordance with the one obtained with other ligand/receptor binding such as ligand/HSA [31].

For 3 mM < x < 15 mM (domain 2), looking at the experimental data, it is evident that the trend is not linear (Fig. 1 in domain 2).

This is highlighted by the following quadratic function:

$$\ln k' = \alpha + \beta \ln x + \delta(\ln x)^2 \tag{7}$$

where α, β and δ were constants. These constants were determined using Eq. (7) (r^2 = 0.985). The α, β and δ values were respectively equal to −9.75, −5.62 and −0.64. 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. From Eq. (5) the slope of the curve $\ln k'$ versus $\ln x$ (i.e.; by derivation of Eq. (7)) gives the net number of salt ions, n displaced or bound in forming the nor-NOHA–arginase complex:

$$n = -1.28 \ln x - 5.62 \tag{8}$$

Fig. 2 and Eq. (8) showed how the magnitude of n decreased linearly with ln x and is practically zero for x ≅ 12 mM. In principle, solubility modifiers such as $MgCl_2$ can affect (i) the energy required to produce a solvent cavity (cavitation process) into which the solute (such as nor-NOHA) can go and (ii) the energy of solute medium solvation interaction [32]. Thus in domain 2, the Mg^{2+} cation increased the nor-NOHA–arginase association by electrostriction that squeezed out free space, made cavity creation harder, and increased the surface tension [32]. As well, ion-induced effects on surface tension, in addition to affecting hydrogen bonding [33–34], can also affect hydrophobic interactions within the protein [35], resulting in the burial of certain amino acid residues into the protein hydrophobic core and slowing down hydrogen

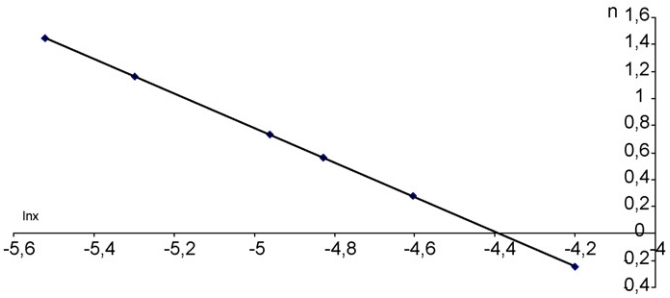

Fig. 2. logarithm of the salt concentration x (M) dependence of the linked salt ions (per mol arginase), n, at 298 K.

exchange rates. Such conformational changes would result in a more compact and less flexible structure [36–39]. Therefore in domain 2, the decrease of the net number of ions released or bound upon complex formation when the salt concentration increased (Fig. 2) demonstrated changes in the enzyme conformation. Around $x \cong$ 12 mM, the arginase enzyme adopted a less flexible structure, its binding site was thus less accessible to nor-NOHA and the nor-NOHA–arginase association decreased slightly. This confirmed the importance of the arginase conformation for the interaction between the arginase and the nor-NOHA and the conformation change on arginase immobilized on silica. The arginase immobilization on the chromatographic support did not alter its conformation change capacity. As well, our result confirmed 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 hydrogen bonding [3]. In addition, the results presented here provide evidence for an Mg^{2+}-mediated variation of nor-NOHA/arginase association, suggesting a contribution to the regulation of the arginase activity.

4. Conclusion

For the first time, the effect of magnesium chloride on the nor-NOHA/arginase binding was analysed using a novel biochromatographic column developed in our laboratory. The affinity of nor-NOHA to arginase was high and changed slightly with the salt concentration. For a salt concentration in the range 3–10 mM, the nor-NOHA/arginase binding increased and could thus improve the enzyme activity inhibition by nor-NOHA. As well, the results obtained in the presence of salt could be presented in a coherent plot in which the concentration of salt was expressed by the number of salt ions (n) displaced or bound in forming the nor-NOHA–arginase complex. A conformational change of the arginase enzyme due to a variation in the number of ions displaced or bound was vizualized. Further experiments are now in progress in our laboratory in order to demonstrate the role of other divalent salt cation (Zn^{2+}, Ca^{2+}) on both nor-NOHA-arginase binding and the enzyme activity.

Acknowledgments

This work was supported by Grants 2007 from Direction Générale de la Recherche from Programme National de Biotechnologie, Ministère de la Recherche, France.

References

[1] C. Demougeot, A. Prigent-Tessier, C. Marie, A. Berthelot, J. Hypertens. 23 (2005) 971.

[2] C. Demougeot, A. Prigent-Tessier, T. Bagnost, C. Andre, Y. Guillaume, M. Bouhaddi, C. Marie, A. Berthelot, Life Sci. 80 (2007) 1128.
[3] T. Bagnost, Y.C. Guillaume, M. Thomassin, J.F. Robert, A. Berthelot, A. Xicluna, C. Andre, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 856 (2007) 113.
[4] H. Hirsch-Kolb, D.M. Greenberg, J. Biol. Chem. 243 (1968) 6123.
[5] M.C. Yip, W.E. Knox, Biochem. J. 127 (1972) 893.
[6] Z.F. Kanyo, C.Y. Chen, F. Daghigh, D.E. Ash, D.W. Christianson, J. Mol. Biol. 224 (1992) 1175.
[7] D.W. Christianson, R.S. Alexander, J. Am. Chem. Soc. 111 (1989) 6412.
[8] R.S. Reczkowski, D.E. Ash, J. Am. Chem. Soc. 114 (1992) 10992.
[9] J. Custot, C. Moali, M. Brollo, J.L. Boucher, M. Delaforge, D. Mansuy, J.P. Tenu, J.L. Zimmermann, J. Am. Chem. Soc. 119 (1997) 4086.
[10] S.M. Morris Jr., Annu. Rev. Nutr. 22 (2002) 87.
[11] P.C. Zemel, M.B. Zemel, R.F. Urberg, F.L. Douglas, R. Geiser, J.R. Sowers, Am. J. Clin. Nutr. 51 (1990) 665.
[12] H. Ruddel, C. Werner, H. Ising, Magnesium Bull 11 (1989) 93.
[13] J. Durlach, M. Bara, A. Guiet-Bara, in: Y. Itokawa, J. Durlach (Eds.), Magnesium in Health and Disease, John Libbey, 1989, p. 173.
[14] A.M. Fehily, J.W. Yarnell, C.A. Bolton, B.K. Butland, Eur. J. Clin. Nutr. 42 (1988) 405.
[15] J. Durlach, Magnesium in Clinical Practice, John Libbey, London, 1988.
[16] V. Kuti, Magnesium Res. 2 (1989) 229.
[17] S.M. Green, A. Ginsburg, M.S. Lewis, P. Hensley, J. Biol. Chem. 266 (1991) 21474.
[18] N. Carvajal, C. Torres, E. Uribe, M. Salas, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 112 (1995) 153.
[19] C.D. Tormanen, J. Enzyme Inhib. 16 (2001) 443.
[20] C.D. Tormanen, J. Enzyme Inhib. Med. Chem. 21 (2006) 119.
[21] M. Goedert, G. Guiochon, Chromatographia 6 (1973) 39.
[22] Under moderate nonlinear conditions, Snyder and co-workers (J. Chromatogr. 1987, 384, 45–56) have established that the apparent solute retention factor k_a, can be described by the following single function: $k_a = k' \int [[k'/(k'+1)]N^{1/2}Q_s/m_L]$ where k' and N are respectively the retention factor and the number of theoretical plates (at infinite dilution), m_L is the number of active binding sites and Q_s, is the amount of solute injected. In two different operating conditions (name 1 and 2) where $\left\lfloor k'_1/(k'_1+1)\right\rfloor N_1^{1/2} \approx \left\lfloor k'_2/(k'_2+1)\right\rfloor N_1^{1/2}$, the k_{a1}/k_{a2} ratio is expected to be invariant with Q_s if m_L is constant. Overloading experiments were carried out at the different pH or temperature conditions for which $N_1 \approx N_1$ and $[k'_1/(k'_1+1)] \approx [k'_2/(k'_2+1)]$ (for high k' values with k'_1 not much different from k'_2). The k_a ratios were found to be invariant with the amount of solute injected, indicating that no changes in the m_L value occurred with varying magnesium concentration.
[23] I. Mazsaroff, L. Varady, G.A. Mouchawar, F.E. Regnier, J. Chromatogr. 499 (1990) 63.
[24] S.L. Wu, K. Benedek, B.L. Karger, J. Chromatogr. 359 (1986) 3.
[25] L.N. Lin, A.B. Mason, R.C. Woodworth, J.F. Brandts, Biochemistry 30 (1991) 11660.
[26] M.L. Doyle, G. Louie, P.R.D. Monte, T.D. Soboloski, Methods Ezymol. 259 (1995) 183.
[27] E. Ortiz-Salmeron, C. Baron, L. Garcia-Fuentes, FEBS Lett. 435 (1998) 219.
[28] R.S. Reczkowski, D.E. Ash, Arch. Biochem. Biophys. 312 (1994) 31.
[29] D.E. Ash, J.D. Cox, D.W. Christianson, Met. Ions. Biol. Syst. 37 (2000) 407.
[30] R.S. Reczkowski, Characterization of the kinetic and catalytic mechanism of rat liver arginase, Ph.D. Thesis, Temple University, 1991.
[31] C. André, Y. Jacquot, T.T. Truong, M. Thomassin, J.F. Robert, Y.C. Guillaume, J. Chromatogr. B 796 (2003) 267.
[32] M.R.J. Dack, Chem. Soc. Rev. 4 (1975) 211.
[33] T. Arakawa, S.N. Timasheff, Biochemistry 23 (1984) 5912.
[34] E. Goormaghtigh, V. Raussens, J.M. Ruysschaert, Biochem. Biophys. Acta 1422 (1999) 105.
[35] N.K.D. Kella, J.E. Kinsella, Int. J. Pept. Protein. Res. 32 (1988) 396.
[36] C. Nishimura, V.N. Uversky, A.L. Fink, Biochemistry 40 (2001) 2113.
[37] S.E. Harding, Prog. Biophys. Mol. Biol. 68 (1997) 207.
[38] D. Lairez, E. Pauthe, J. Pelta, Biophys. J. 84 (2003) 3904.
[39] C. Pace, R. Alston, K. Shaw, Prot. Sci. 9 (2000) 1396.