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Specific anion effects on urease activity: A Hofmeister study

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ABSTRACT

The effects of a range of electrolytes on the hydrolysis of urea by the enzyme urease is explored. The autocatalytic behavior of urease in unbuffered solutions and its pH clock reactions are studied. The concentration dependence of the experimental variables is analyzed in terms of specific ion-enzyme interactions and hydration. The results offer insights into the molecular mechanisms of the enzyme, and on the nature of its interactions with the electrolytes. We found that urease can tolerate mild electrolytes in its environment, while it is strongly inhibited by both strong kosmotropic and strong chaotropic anions. This study may cast light on an alternative therapy for *Helicobacter pylori* infections and contribute to the design of innovative materials and provide new approaches for the modulation of the enzymatic activity.

1. Introduction

Enzymatic mechanisms remain a subject of enduring basic interest due to their high selectivity, fast kinetics, and biocompatibility [1]. And too for pragmatic reasons like drug delivery, environmental remediation and material science [1]. Urease belongs to the superfamily of amidohydrolases and phosphotriesterases [2]. It catalyzes the hydrolysis of urea to ammonium and hydrogen carbonate ions with pH increase in unbuffered aqueous solutions (Scheme 1) [3].

Most studies on urease focus on its biomedical applications as for the search for inhibitors [2], on environmental applications, *e.g.* in the recovery of phosphate and ammonium from wastewaters [2,4], and in the degradation of polyurethane [5]. Moreover, due to its pH feedback-driven activity, urease can be exploited in stimulus-responsive materials, and can give rise to non-linear dynamic processes [6]. The nonlinear behavior of the reaction dynamics of urease is also seen as a viable tool in the design of pH-oscillators in unbuffered media [3,7].

The hydrolysis follows Michaelis-Menten kinetics with a bell-shaped pH-rate. It shows an activity maximum around pH 7. So a pH change provides a feedback to the enzyme activity [8]. Starting from acidic conditions, the pH increases with the acceleration of the reaction up to pH ~ 7. But a further pH increase inhibits the enzyme activity, with complete damping around pH 9.2 [8–10]. This pH dependence can be used to induce a clock-type reaction - a sort of controllable autocatalysis - by adjusting the initial pH of the solution [3].

Specific ion effects on proteins and enzymes induce changes in the protein folding, stability, solubility, aggregation, fibrillation and kinetics [11–13]. Some examples of Hofmeister phenomena with enzymes include the effects on glucose oxidase [14], alcohol dehydrogenase [15], lipase A [16], and chymotrypsin [17] just to mention a few.

Here we investigate the effect of some potassium electrolytes on the enzymatic activity of urease, their specific interactions with the enzyme, and the possibility of tuning the activity by adding different ions to the reaction mixture. This is the first systematic investigation of specific ion effects on the catalytic activity of urease. While the activity inhibiting effect of F^- and $H_2PO_4^-$ are known [2,18,19], previous works are limited to the investigation of few electrolytes and do not offer an extensive study within the framework of the Hofmeister phenomena [20–23]. Moreover, most of these studies have focused only on the effect of different buffers in the presence, and absence of additional electrolytes [20–23].

Specific ion effects are widespread [24,25], from bulk solutions, interfacial systems, water and non-aqueous polar organic liquids [26,27], to soft matter materials (including enzymes). Since the early intuitions of John Dalton in 1840 and of Franz Hofmeister in 1888 [28] specific ion effects are still an evergreen research topic [24–29]. By

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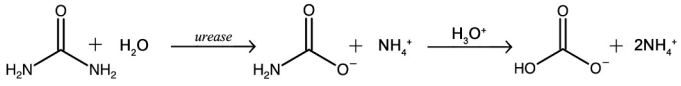

Scheme 1. Urease-catalyzed hydrolysis of urea into ammonium and hydrogen carbonate ions.

specific ion effects we mean effects that deviate from the classical theory of electrolytes for any physico-chemical observable [30,31]. A complete theory for Hofmeister effects is still open. The classical theory is valid only for low salt concentrations. So specific ion effects occupy center stage in chemistry and biology, and any complex entity like supramolecular self-assemblies, nanoparticles, chemical oscillators and living organisms. The overall effects result from a combination of different simultaneous mechanisms [24,32–34].

Chemistry, life and environmental sciences are strongly ion-specific [35]. However, the effect of specific ions usually emerges only when their concentration becomes moderately high. The threshold depends on the system investigated. In general it is about 0.15 M, where electrostatic Coulombic interactions vanish, and ion-specific non-electrostatic (including van der Waals and hydration) forces come into play [24,36,37].

In this work we explore effects of specific ions on the autocatalytic behavior of urease in unbuffered solutions. We focus on the dependence of the clock time (Fig. 1), *i.e.* the time lag comprised between the start of the reaction, and the point of maximum rate during the autocatalysis (inflection point in the sigmoid-like curve, see Fig. 1), as a function of the nature and concentration of various potassium salts. We also evaluate the effect of different anions on the value of the maximum reaction rate (see the inset in Fig. 1).

Our results indicate a significant and reproducible effect of a number of anions on the kinetic features of urease in unbuffered water at 25 °C. These effects can be related to the specific nature of the anions, and, in particular, to their interfacial and hydration properties. This suggests that ions adsorb specifically at the enzyme/water interface. We believe that this study will help highlight the interactions between background ions and urease, contribute to the design of innovative pH-responsive autonomous materials [38,39] and provide new approaches for the modulation of the enzymatic activity in medical and biochemical applications.

2. Materials and methods

2.1. Chemicals

Milli-Q water from Millipore with a resistivity of 18.2 MΩ•cm and a conductivity of 0.055 μS•cm^{-1} was used. The following salts were used in experiments: KH_2PO_4 (≥99%), KCl (≥99%), KBr (≥99%), KI (≥99%), $KClO_3$ (≥99%), $KBrO_3$ (≥99.8%), KIO_3 (≥99.5%), KNO_3 (≥99%), $KClO_4$ (≥99%), KSCN (≥99%). The salts, HEPES (≥99.5%) and urea (≥99.5%) were purchased from Merck (Milan, Italy). Urease (from Jack Bean, min. 300 unit/mg) was purchased from TCI-Europe N.V. (Zwijndrecht, Belgium). HCl (37%) was purchased from Merck (Milan, Italy) and used to adjust the initial pH of the solutions. All stock solutions of urease, urea, and salts were freshly prepared.

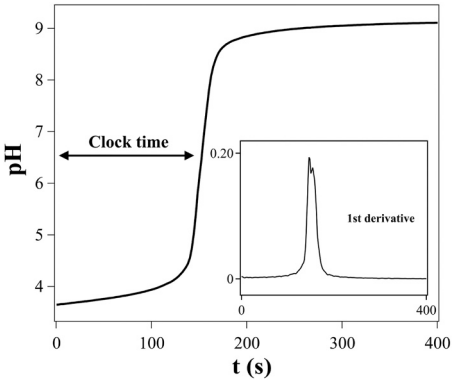

Fig. 1. Change of pH *vs.* time. The clock time (t_c) of the blank solution, *i.e.* the time lapsed between the start of the reaction and the point of inflection. Inset: the first derivative ∂pH/∂t *vs.* t from the same data.

2.2. Determination of enzyme units

The definition of a urease unit was taken as 1.0 μmol of NH_3 liberated from urea per minute at pH 7.0 and 25 °C (the equivalent of 1.0 I.U. or 0.054 Sumner unit). The enzyme activity was determined using an ammonium ion-selective electrode ISE NH_4^+ 9663 C purchased from Hach (Lainate, Italy). The electrode was calibrated with NH_4Cl standards using the same conditions of the reaction and in the concentration range of interest (from 1 to 10 mM). We used a HEPES buffer because it does not interfere with the enzymatic activity of urease [18]. The calibration was controlled daily and repeated whenever needed. The reaction was conducted in 0.1 M HEPES pH 7 buffer in the presence of 0.2 M urea at 25 °C (see Scheme S1a in the Supplementary Material). The solution was equilibrated for 10 min in a water bath before the measurements. Upon the addition of a known amount of urease, the ammonium ion concentration produced in a minute was obtained and the value was converted to units/mL of the enzyme using the calibration curve.

2.3. pH measurements

The enzyme reaction was investigated by pH measurements as a function of time in the presence of salts at different concentrations in non-buffered conditions. The salt-free reference system comprises urea 10 mM and urease 5 U/mL. To obtain the sigmoidal behavior, an initial acidic pH is required. The initial pH was set equal for all systems investigated by adding the amount of HCl required for a starting pH of 3.6. In these conditions, the reference clock time was approximately 180 s. The final pH was around 9.2. Specific ion effects cause changes in the measured pH [30]. In the concentration range relevant to our experiments the pH change with the addition of salts for the anion series was low enough to take the measured pH value as the real pH of the system.

Reactions were performed under magnetic stirring and thermostatted at 25 °C (see Scheme S1b in the Supplementary Material). pH measurements were made by using an XS pHsensor 201 T DHS (Carpi, Italy) and Amel pHmeter 2335 (Milan, Italy) by recording values every second. To estimate the speed of the process (∂pH/∂t), the derivatives of the pH value as a function of time were also calculated for each curve. An example of the typical curves obtained during the experiments is shown in Fig. S6 (Supplementary Material).

3. Results and discussion

3.1. Specific ion effects

The experimental clock times (t_c) and the maximum of the first derivative in the pH *vs.* time curve (see Fig. 1) were normalized to the blank, and gave the two benchmark parameters, τ and δ respectively, defined according to Eqs. 1 and 2:

$$\tau = \frac{t_{c,solution} - t_{c,blank}}{t_{c,blank}} \quad (1)$$

$$\delta = \frac{\max\left(\frac{\partial pH}{\partial t}\right)_{solution} - \max\left(\frac{\partial pH}{\partial t}\right)_{blank}}{\max\left(\frac{\partial pH}{\partial t}\right)_{blank}} \quad (2)$$

$t_{c,blank}$ and $t_{c,solution}$ are the clock time for the blank and for the solution, respectively. The maxima of the first derivative $\partial pH/\partial t$ were used.

The results were analyzed in terms of some descriptors, *i.e.* ionic physico-chemical parameters that reflect their nature and behavior in solution [30,31,35].

We tested a series of potassium salts assuming additivity of the effects induced by the cation and the anion. We also assumed that the contribution of the cation (K^+) does not depend on the anion with which it is paired in the salt. In particular, we tested the effect induced by KH_2PO_4, KCl, KBr, KI, $KClO_3$, $KBrO_3$, KIO_3, KNO_3, KSCN, $KClO_4$ at different concentrations.

The values of the benchmark parameters τ and δ at different salt concentrations are listed in Tables S1 and S2 (see the Supplementary Material) and reported in Fig. 2. The dilute concentration trends of Fig. 2 are reported in Fig. S1 in the Supplementary Material. An inhibitory effect of all salt solutions is observed, typically with the increase in τ shown in Fig. 2a. For almost all salts the specific effects were detected in the 1 - 200 mM concentration range. The exceptions were for $H_2PO_4^-$ and IO_3^-. These showed a stronger inhibition. Below 1 mM the solutions show the same τ value of the blank. At approximately 200 mM the enzymatic activity is completely suppressed for all salts. This upper limit correlates with the activity of a restriction enzyme [40], with the physiological concentration of NaCl in mammals' blood [41,42] and with the critical salt concentration for inducing bubble-bubble coalescence [43]. It also coincides with the concentration at which change in conductivity occurs for each salt. This indicates the onset of nanobubble formation, itself connected to enzyme reaction mechanism [40].

We noticed also that the inhibitory effects are limited to a rather restricted concentration range (maximum ~2 orders of magnitude), above which the enzyme is completely deactivated.

Chloride is the most tolerated anion in terms of inhibition and attains the highest inhibition concentration of all. In passing we recall that Cl^- is the most abundant ions in extracellular fluids, and together with Na^+ and K^+ it helps to maintain the proper water distribution inside and outside the cell [44]. In the Hofmeister sequence, Cl^- is usually the break point between chaotropic (poorly hydrated) and kosmotropic (strongly hydrated) species [35,45]. In fact, Fig. 2a shows that moving from the mild chaotropic KCl (blue circles on the right hand side in the plot) to the strongly chaotropic KSCN (yellow circles on the left hand side in the plot), the inhibitory effect increases. This is also suggested by the largest feasible concentration that can be tested for each salt (except for KH_2PO_4 and KIO_3), before the enzyme is completely inhibited.

At 50 mM of $KClO_3$, the τ value shows a sudden increase unlike its δ value (Fig. 2). This divergence can be attributed to the narrow concentration range sensitivity of chaotropic ions. Additionally, δ values reflect the maximum enzymatic rate, while τ is influenced by the time to reach that rate. Therefore, at this concentration ClO_3^- keeps its maximum enzymatic rate but substantially inhibits the enzyme activity, even at lower pH, affecting the overall required time.

For the most kosmotropic ions, $H_2PO_4^-$ and IO_3^-, we observed a stronger effect (see the inset in Fig. 2a). The specific ion effects already emerge at 10 and 100 μM for iodate and dihydrogen phosphate, respectively.

Although kosmotropes inhibit the reaction at lower concentrations than chaotropes, a higher tolerance with respect to the complete inactivation of the enzyme was found for the kosmotropes. In fact, the highest values of concentration reported in the Fig. 2a do not correspond to the total inhibition of the activity, but relate to the longest clock time that was recorded. Interestingly, kosmotropic ions (silicates, sulfates, carbonates, magnesium, calcium and aluminum) represent the most abundant class of ions on the surface of Earth. That is, they are part of the general scenario in which life appeared and evolved on Earth [24,35,45,46].

Moreover, in the case of strongly kosmotropic ions, the clock time changes abruptly in a narrow concentration range similar to that of the most chaotropic ion, *e.g.* SCN^-. For example, $H_2PO_4^-$ has a change from 20 to 1200 s for concentrations spanning between 1 and 5 mM. The inhibition effect of $H_2PO_4^-$ is consistent with previous literature results [2,18].

We did not extend our investigation to higher concentrations because these would lead to very long clock times (above 30 min at $[SCN^-] = 15$ mM, above 45 min at $[IO_3^-] = 0.2$ mM and above 40 min at $[H_2PO_4^-] = 5$ mM). In addition, in the case of $H_2PO_4^-$, at a higher concentration the formation of the phosphate buffer is expected to affect the solution pH with a spurious effect on the clock time.

Overall, in all cases we detected a total inhibition at concentrations lower than 200 mM. This finding can pave the way to some biomedical applications such as the identification of a biocompatible inhibitor to the urease enzymatic activity for the treatment of infections caused by *Helicobacter pylori* [47]. Some known inhibitors include both organic and inorganic species, *e.g.*, thiols [48], quinones [49], transition metal ions [50], boric acid [51], 2-methylimidazole [52], and bismuth compounds [53] but regrettably most of them are not biocompatible. Instead, some of the ions investigated in this work are not harmful to humans and the maximum concentration is very close to the concentration of electrolytes in mammalian blood, equivalent to 0.17 M NaCl [41,42].

Besides the case of chlorate, similar insights can be proposed for the maximum rate values, δ, as they follow the same trend of τ, including IO_3^- and $H_2PO_4^-$ (see Fig. 2b). The overall effect is the decreasing maximum rate of the reaction with the addition of electrolytes.

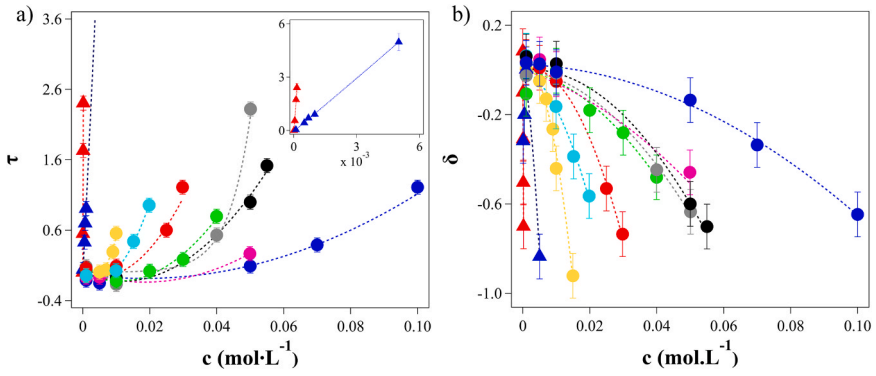

Fig. 2. (a) τ values for the anion series as a function of the salt concentration at 25 °C; inset: magnification of τ values for iodate and dihydrogen phosphate salt; (b) δ values for anions as a function of the salt concentration at 25 °C. Blue circles: KCl, pink circles: KBr, black circles: $KBrO_3$, gray circles: $KClO_3$, green circles: KNO_3, red circles: KI, cyan circles: $KClO_4$, yellow circles: KSCN, blue triangles: KH_2PO_4 and red triangles: KIO_3. The dotted lines are guides for the eye.

Fig. 3 shows the concentration (in Log scale) needed to obtain a value of δ of -0.5 for each salt as a function of the surface tension molar increment at the air/water interface ($k_{\text{air-water}}$, panel a), and the lyotropic number (N, panel b), in order to describe the specific effect of each anion in terms of these descriptors. These parameters were chosen because $k_{\text{air-water}}$ reflects the interfacial properties of the ion, while N reflects the specific interactions established between biological macromolecules and water [24]. The correlation between these parameters and the experimental parameters is of great help to envisage the mechanisms that lead to the observed results [24].

A non-monotonic trend, with the strong kosmotropic ions IO_3^- $H_2PO_4^-$ and strong chaotropic ions showing similar behaviors create what is usually referred to as a "V" plot [54]. This behavior is quite common in Hofmeister phenomena, for example in the phase separation of lysozyme dispersions [11].

Spanning from Cl^- to strong chaotropes (*e.g.*, SCN^-) a linear correlation with $k_{\text{air-water}}$ is observed, as shown in Fig. 3a. This result can be explained considering that chaotropes strongly adsorb at the enzyme interface [11]. Instead in the case of kosmotropic ions (including the mild chloride ion), there is no correlation. We observe a slight change in the slope, suggesting that the introduction of kosmotropes has a minimal impact on the interfacial properties of urease.

The lyotropic number provides a quantitative measure to the lyotropic activity of electrolytes [24]. This parameter is derived from colloidal systems such as gelatin and agar dispersions in water. As a matter of fact, the case of amylose ion absorption parallels our findings. This observation implies that the interactions occurring between urease and electrolytes are of a complex nature and share a resemblance to those observed in the case of starch.

As we already anticipated, in complex systems different competing processes may occur simultaneously. This may result in the nonlinear behavior of some observed variables, *i.e.* in a "V" plot [24,54]. The reason of such peculiar behavior has been ascribed to the onset of more concurrent mechanisms, for example adsorption of ions at interfaces, non electrostatic interactions, hydration, ion binding and bridging, and more [11]. They all participate in the global measured effect, but each mechanism is ion specific and can produce a different ranking for the same ions.

It is not unusual to find systems and phenomena with a V-shaped trend [55]. An interesting example is the association rate constants of cyanide binding into cytochrome C for different anions [13]. A similar result was found in a study on the effect of salts on amyloid [56], in a study of the activity and stability of alkaline phosphatase [29]. In the case of enzymes, the non-monotonic effect of ions in the modulation of properties with respect to their position in the Hofmeister series is relatively common [17].

This explains the peculiar trend found in our studies, and especially the behavior of the strong kosmotropes, $H_2PO_4^-$ and IO_3^-. The strong deviation of these ions suggests that these ions interact through different mechanisms with the enzyme with respect to other ions. These interaction mechanisms will be discussed in detail in the following subsections.

3.2. Ion specific β coefficients

It has been shown that some experimental physico-parameters, including equivalent conductivity, viscosity, apparent molar volume, formation of supramolecular adducts, optical rotation, solubility of scarcely soluble molecules in water, critical micellar concentration of a phospholipid and others follow a semi-empirical equation (see Eq. 3) that, in the case of viscosity, is named after Jones and Dole [35,57–62]:

$$Y = Y_0\left(1 + a\sqrt{c} + bc\right) \quad (3)$$

Here Y_0 and Y are the parameters measured in pure water and in the presence of a salt solution at concentration c at the same temperature, respectively. The $a\sqrt{c}$ term reflects the non-specific electrostatic interactions and dominates when c is very low, while the bc term is related to non-electrostatic interactions, is ion specific and dominates when the concentration of the salt is moderately high. In the case of the Jones-Dole formula, $\frac{\eta}{\eta_0} - 1 = A\sqrt{c} + B_{JD}c$, the coefficient of the linear term (B_{JD}) is positive for kosmotropic, and negative for chaotropic species [63,64].

In the case of the urease activity, the δ values obtained with the different anions were fitted according to the following equation (Eq. 4):

$$\delta = \alpha\sqrt{c} + \beta c \quad (4)$$

Table S3 (Supplementary Material) shows the extracted α and β fitting coefficients. The α values do not change considerably, confirming that the $\alpha\sqrt{c}$ term is nonspecific and thus accounts for electrostatic interactions.

On the other hand, the β coefficients, that reflect ion specificity, are similar for kosmotropes and strong chaotropes, in line with our observations. In particular the β coefficients follow the trend:

$$Cl^- > Br^- > BrO_3^- \sim ClO_3^- \sim NO_3^- > I^- > ClO_4^- > SCN^- > H_2PO_4^- > IO_3^-$$

In spite of their strong kosmotropic nature, dihydrogen phosphate and iodate are located on the right-hand side, after the chaotropes. Fig. S2 (Supplementary Material) shows the β coefficients for the different potassium salts as a function of the surface tension molar increment at the air/water interface, $k_{\text{air-water}}$. This plot mirrors the case of the $k_{\text{air-water}}$ *vs.* Log(c_δ) (see Fig. 3a) supporting the conclusion that these empirical coefficients do in fact reflect ion specificity [24,65]. With the exception of iodate and dihydrogen phosphate that induce strong perturbations in the enzyme activity already at low concentrations, the value of β is very low for the most chaotropic species, *e.g.*

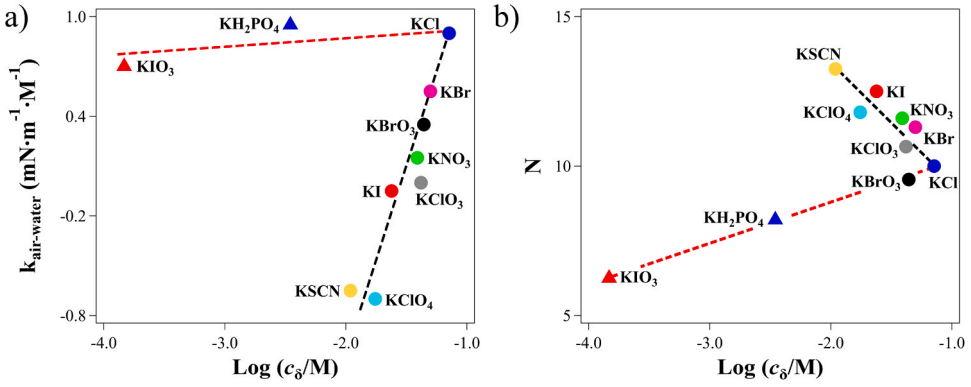

Fig. 3. Plots of (a) $k_{\text{air-water}}$, (b) lyotropic number (N) *vs.* Log(c_δ). The dotted lines are guides for the eye: the red line is for the kosmotropes and the black line is for the chaotropes.

perchlorate and thiocyanate.

3.3. Interaction mechanism

Although ureases obtained from different sources possess structural differences, they always present a high degree of homology, and their active sites are always located in the α-subunits as depicted in Fig. 4 [2]. The quaternary structure of plant ureases is made of dimers of homotrimers $(\alpha_3)_2$ [2].

The urease active site bears two Ni^{2+} ions bridged by a hydroxide group (see Fig. 4a). A water molecule is coordinated to each Ni^{2+} ion and together with a third water molecule they form the cluster that fills the active site entrance [66]. These water molecules interact with each other mainly by H-bonding and the water cluster forms a tetrahedral template for the catalytic transition step. The cavity is occupied by a molecule of urea during the hydrolysis reaction. The structure is further stabilized by a mobile flap that contains a catalytic histidine residue [66].

In the past, several attempts were made to elucidate the mechanism that rules over the urease catalytic activity [67]. In this work, we will refer to the mechanism proposed by Benini [67,68].

The observed specific ion effects can be discussed in terms of the interactions between the ions and the enzyme, that can result in a lower access of urea to the active site. The correlation of $\mathrm{Log}(c_\delta)$ values with $k_{\text{air-water}}$ suggests that the interfacial properties of the ions are at play. In fact, chaotropic ions are expected to interact directly and adsorb at the enzyme interface, leading to at least a partial perturbation of the hydration layer of the enzyme [11].

The NH groups that belong to the amide residues of the enzyme backbone offer suitable docking points for chaotropes [69,70]. At acidic pH, amide NH groups bear a positive charge that promotes the interaction between the enzyme and anions also *via* electrostatic forces. Rembert *et al.* showed that beside the amide NHs, also the α-carbons (that carry a partial positive charge) adjacent to the carboxylate units of the protein backbone offers another site of interaction for chaotropic anions [69].

In the active site of urease four His residues coordinate the Ni^{2+} ions. Sidechains containing Arg, Lys and His residues (weakly hydrated and positively charged) represent further adsorption sites for chaotropic anions [71]. Therefore, the His residues actively participate in the enzyme-anion interactions and in changes in the flexibility of the active site [12]. Considering that water molecules are directly involved in the active site of the enzyme (Fig. 4a) we reasonably expect that the presence of differently hydrated anions can perturb the stability of the catalytic cavity in different manners.

Besides a direct interaction with the active site, we expect that the ions affect also the mobile flap (Fig. 4b) and modify its flexibility and therefore the enzyme performance [12]. The flap itself possesses an His residue that is essential for the catalytic activity. The blockage of this residue can result in the inhibition of the enzyme activity. Previous reports show that hydroquinone inhibit the urease activity due to the covalent binding on the mobile flap [72].

Another possible mechanism for enzyme-ion interactions comprises the formation of salt bridges between subunits. In addition to neutralizing interfacial positive charges, a chaotropic ion can also form bridges between nonpolar surface groups belonging to two separate enzyme units. Salt bridges are extremely important and have various effects on the structure and function of proteins [11]. Moreover, the adsorption of chaotropic ions can modify the outer surface and the hydration layer of the macromolecule. This sandwich effect typical of chaotropic species can modify the structural features of the enzyme, such as its conformation and flexibility. Moreover, the formation of dimers and higher oligomers can decrease the solvent-accessible area and therefore block the substrate entrance [11].

In any case, it is evident that water-enzyme interactions dominate the enzyme activity, and that specific ion effects on the enzyme are mediated by water through the solvation of the ions and of the enzyme.

Our results indicate that the inhibition of the enzymatic activity is stronger with the increasing ion size for chaotropes. The bigger the ion, the lower its concentration to suppress the enzymatic activity. This is particularly notable in case of SCN^-, that has a very restricted tolerable concentration range. This observation may lead to the conclusion that large anions that easily adsorb at the enzyme surface perform their inhibiting action more efficiently than small species. In passing, we recall that sodium perchlorate and thiocyanate salts reduce the partitioning of the anesthetic tetracaine into zwitterionic micelles [73].

Our results confirm that chaotropic ions possess high toxicity for urease, in line with what was found for enzymes and in general for living organisms [11,74].

In case of the strong kosmotropic ions, *i.e.* $H_2PO_4^-$ and IO_3^-, the different behavior can be assigned to different interaction mechanism (s).

In our system $H_2PO_4^-$ is the only ion with an acid/base character, with a pK_a value of 7.2 ($H_2PO_4^-/HPO_4^{2-}$). In our experimental setup, the enzymatic reaction proceeds in a pH range from 3.6 to 9.2. Referring to Fig. 1 it is clear that the maximum acceleration of the process occurs around pH 7, and the clock time is in fact taken at this pH, suggesting that $H_2PO_4^-$ ions dominate in this part of the reaction. Therefore we assume that the effects we obtain with this anion are not attributed to the effect of pH changes, *i.e.* to the presence of HPO_4^{2-} and PO_4^{3-}. We confirmed this assumption by plotting the reaction rates *vs.* pH for $H_2PO_4^-$ and for the other anions that are insensitive to pH (Fig. S3, Supplementary Material). If the phosphate ion were to form its conjugated species (HPO_4^{2-} and PO_4^{3-}), the shape of the curve of the activity *vs.* pH should be significantly different than that given by other anions. Instead, no appreciable difference was detected. This result is in line with previous findings on the effect of phosphate buffers on the urease activity, which indicated $H_2PO_4^-$ as the only species responsible for the inhibition [2,18].

According to the proposed mechanism the $H_2PO_4^-$ ion enters into the cavity of the enzyme, interacts with the tetrahedral water cluster template, and blocks the access of the urea substrate to the active site [2,18]. As illustrated in Fig. S4 (Supplementary Material) the anion coordinates

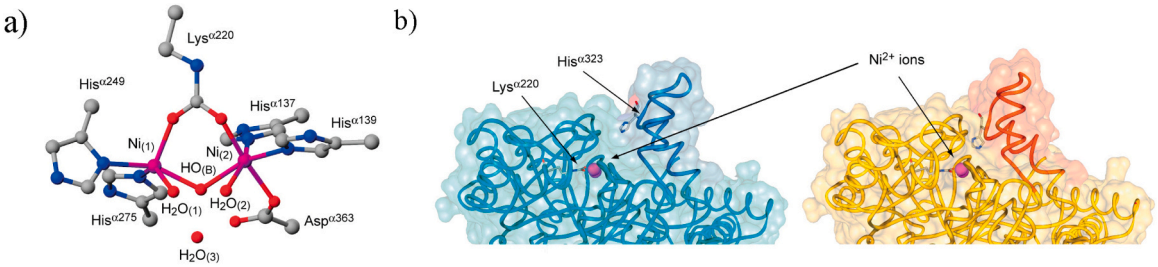

Fig. 4. (a) Active center of urease found in α-subunits. Color code: nickel, purple; carbon, gray; nitrogen, blue; oxygen, red. (b) Catalytically essential mobile flap in open (left) and closed (right) conformations.
Adapted with permission from Ref. [66], Copyright 2011, American Chemical Society.

the Ni^{2+} ions.

A similar mechanism that implies the de-hydration of the active site can be envisaged also for the iodate ion, another strong kosmotrope. Indeed, both iodate and dihydrogen phosphate are able to make strong H-bonds. We argue that strong kosmotropic, de-hydrating species deplete water molecules from the enzymatic cavity and bring about a strong inhibition of the catalytic activity even at very low salt concentrations.

We recall that we did not include fluoride (another strong kosmotropic ion) in this study because of its relatively strong nature as a base in the considered pH range (the pK_a of HF is 3.17) [75]. The inhibition of F^- ions on urease is a well-known phenomenon [19,67]. Apparently F^- binds to the Ni(1), replaces $H_2O_{(1)}$ and the bridging hydroxide ($OH_{(B)}$) residue in the absence of urea (Fig. S5(a) in Supplementary Material) [19,67].

Moreover, in the presence of urea a ternary enzyme-F^--urea complex is formed in the active site [67]. The study of the crystal structure revealed the presence of F^- in the Ni-bridging position (*i.e.*, replacing the $OH_{(B)}$ residue) while the cluster of three water molecules were replaced by urea and the mobile flap was left in the closed conformation (Fig. S5 (b), Supplementary Material). This study revealed that even with the urea in place and the mobile flap in a closed conformation, the presence of F^- in the bridging hydroxide site inhibits the reaction [67].

In conclusion, we argue that the interaction mechanism of kosmotropes with the urease does not involve a change in the flap conformation or flexibility, instead the anions are supposed to interact directly with the active site, through de-hydration and coordination to the Ni^{2+} ions.

Notably, the inhibition constant (K_i), *i.e.* the concentration required to induce a half maximum inhibition, for F^- is reported as 30 μM, very close to the range we detected for iodate [76]. The inhibition constant for $H_2PO_4^-$ is about 0.53 mM, confirming the effect induced by strong kosmotropes [18].

This conclusion is also supported by the of Law of Matching Water Affinities [71]. In fact the strong kosmotropes, Ni^{2+}, $H_2PO_4^-$ and IO_3^- can form stable ion pairs, as suggested by the large (in absolute value) Gibbs free energy of hydration ($\Delta_{hydr}G$) − 1980, − 465 and − 400 kJ/mol they possess [77]. In conclusion, the results indicate that kosmotropes act with a different mechanism with respect to chaotropes.

All these interactions can bring about changes in the structure, flexibility and conformation of the active site, that in turn modify the enzymatic activity [18,78]. The investigated anions, with the exception of $H_2PO_4^-$, do not perturb the pH of the solution, thus, based on the experimental results we obtained, we can exclude the presence of pH effects on the active site function or overall structure of urease.

3.4. Simulations

Following the same approach used in Ref. 32, we supported the data analysis and interpretation with a kinetic approach which identifies the reaction steps that are affected by the presence and concentration of different background salts. In fact, by identifying the kinetic parameters that are related to changes in τ and δ, we can indirectly infer that ions can influence these specific parameters and the interaction they account for. Although this approach cannot give a microscopic view of the molecular interactions between the intervening species (ions, enzyme, substrate and solvent), it provides a related macroscopic information. Simulations and their discussion can be found in the Supporting Information, Section 3.

4. Conclusions

The kinetics of urease in the presence of different aqueous salt solutions was investigated by calculating two experimental parameters, *i.e.* the clock time and the maximum rate in the pH *vs.* time plot. The addition of salts does not change the final pH. Interestingly at low salt concentrations no specificity appears, with a minimum threshold of 1 mM for chaotropes and a much lower concentration for kosmotropes lower than 100 μM).

At moderately high concentrations the experiments show a significant ion specificity that apparently depends mainly on the interfacial features of the individual ions. The most prominent salt effect is the increase of the clock time and the decrease of the maximum rate during the enzymatic reaction. In the case of kosmotropes specific ion effects appear starting from 10 μM for iodate and 100 μM for dihydrogen phosphate. However, kosmotropes do not completely inhibit the enzymatic activity in the concentration range investigated, suggesting a better tolerance for kosmotropes rather than for chaotropes at the same concentration. In fact in the case of chaotropes the highest concentration achievable before total inhibition is below 200 mM. This antagonistic effect is stronger in the case of chaotropes, for example in the case of KSCN the enzymatic activity stops when the salt reaches a concentration of just 20 mM. These effects may represent an interesting option for the treatment of *Helicobacter pylori* infections since the bacterium will not survive in the stomach without the help of its urease activity. Furthermore, some of the salts that we investigated in the present work are not harmful to humans and the concentration needed to induce inhibition in the enzyme is about the same concentration as that of NaCl in mammalian blood, *ca.* 0.17 M [41,42]. Based on these premises we expect that our findings be useful in the design of innovative materials.

The data analysis suggests a direct interaction of the chaotropic anions with the enzyme interface, with a significant perturbation of the conformation and structure of the enzyme active site and the mobile flap. On the other hand, kosmotropes act in a different mechanism.

A variety of different interaction mechanisms are at play, including the adsorption on the enzyme interface and possible formation of salt bridges for chaotropes, hydration and a direct interaction of kosmotropes with the Ni^{2+} centers and the water cluster.

In conclusion, the results pinpoint and underline the specific and unique interaction of each ion in the considered system and show the non-negligible presence of the specific ion effects even at low-to-moderate concentrations. Understanding specific ion effects in the urea-urease system will certainly provide deeper insights on the enzymatic activity and better control of the system performances.

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CSGI (Sesto Fiorentino, Italy).

CRediT authorship contribution statement

Rossi Federico: Writing – original draft, Supervision, Resources, Project administration, Investigation, Conceptualization. **Lo Nostro Pierandrea:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Acar Mert:** Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Tatini Duccio:** Writing – original draft, Validation, Investigation. **Budroni Marcello A.:** Writing – original draft, Validation, Software, Investigation, Formal analysis, Data curation. **Ninham Barry W.:** Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Rustici Mauro:** Validation, Supervision, Project administration, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary Material

Comparison of Jones-Dole coefficients *vs.* surface tension increment values, rate *vs*. pH plots, inhibition schematics, additional experimental details, fitting and simulation constants, tables presenting obtained values (PDF).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2024.113789.

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