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Specific Ion Effects on the Enzymatic Activity of Alcohol Dehydrogenase from *Saccharomyces Cervisiae*

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The enzymatic activity of alcohol dehydrogenase (ADH) in the presence of a range of electrolytes is investigated. In the presence of 150 and 200 mM cations a substantial increase in activity following the series GnCl < CsCl < KCl ~ NaCl < LiCl was observed with a 69% increase in the presence of KCl 200 mM respect to the salt-free solution. In the presence of 150 and 200 mM anions the increase in activity followed an ion specific trend NaF ~ NaCl ~ NaBr > no salt > $NaClO_4$ > NaSCN with a peak in activity increase of 75% in the presence of NaBr. The values of the Micaelis-Menten constant (K_m) did not show any significant ion specific effect, while the maximum rate (V_{max}) of ethanol oxidation to acetaldehyde was strongly ion specific. The changes in specific activity and V_{max} in the presence of anions likely arises from ion specific interactions with charged residues in the active site of ADH. The data indicate that the enzymatic activity of alcohol dehydrogenase can be modulated by the nature of electrolytes at physiological concentration.

1. Introduction

In living systems many proteins and enzymes are dissolved in aqueous solution and interact with a wide range of other biological molecules such as lipids, carbohydrates, nucleic acids, etc. These interactions are affected by the presence of weak and strong electrolytes.[1,2] The full range of the effects of electrolytes is only partially understood,[3,4] with weak electrolytes recognised as being important for pH regulation while strong electrolytes are utilised to maintain ionic strength and play active roles in protein stabilisation or as cofactors necessary for enzyme activity.[5] A majority of studies focus only on the charge of the ion and not its composition. For instance, it is assumed that the properties of Ca^{2+} and Na^{+} differ because the former is divalent while the latter is monovalent.[6] This view is based on conventional theories such as Debye-Hückel and its extensions[7] which relate average activity coefficients with ionic strength and ion charge only. However, a myriad of experimental evidence has shown that ions with the same charge behave very differently.[1,8] This was first reported by Hofmeister in 1888 with his studies on the effect of salts in protein solubility.[9] The 'Hofmeister series' lists ions in a sequence according to their ability to promote effects such as protein aggregation or solubilisation. Historically this effect was observed at medium to high salt concentrations of > 0.5 M, and typically in the range 1-2 M.[10] More recently, 'ion specific effects' have been found at much lower concentrations demonstrating that ion specificity occurs over a much wider concentration range.[11,12] Recent theoretical developments explain ion specificity as resulting from the interplay of electrostatic and ion dispersion forces,[13] which affect both physisorption and chemisorption at interfaces.[14,15] The former interaction is due to the ionic charge whereas the latter is mainly, but not solely, due to the polarizability of the ion.[16] The type and strength of the interactions depend both on the nature (charge, size and polarizability) of the ion and that (dielectric functions) of the interface.[16] Specific ion-protein interactions modulate different macroscopic phenomena, e.g. protein aggregation,[17] electrophoretic mobility,[18,19] Brownian motion,[20] adsorption,[21] etc. A detailed theoretical understanding of "Hofmeister effects" on enzymatic activities is not yet available. Nevertheless it has been reported in enzymes such as glucose oxidase, NADH oxidase, cytochrome c, and lipases.[22–27] Since enzymes present a complex system each work proposed a different mechanism tailored to the enzyme properties. For example for glucose oxidase an ions interaction with the catalytic site residues His and Glu was proposed.[23] Biocatalysis entails the use of enzymes as 'green' catalysts to avoid environmental hazards related to traditional chemical processes based on either organic synthesis or homogeneous catalysis.[28,29] As enzymes can be expensive and suffer from low stability, a range of methods to improve enzymatic activity and stability have been utilised.[30] Alcohol dehydrogenase (ADH) from yeast *Saccharomyces cerevisiae* (E.C. 1.1.1.1) is a tetrameric enzyme (347 residues, molecular mass 141 kDa) that contains four equal subunits in which the active site of each subunit binds a zinc ion.[31] The Zn^{2+} ion maintains a tetrahedral geometry due to three different ligands Cys-43, His-66 and Cys-153, whereas the fourth position is available for catalysis (Figure 1).[32] The cofactor NAD^{+} binds to the enzyme at the active site, resulting in a complex, E-NAD^{+}, that

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binds the alcohol substrate and subsequently enables a hydride transfer.[33] This causes the formation of a new complex E-NADH-aldehyde. Finally, the aldehyde product is released and NADH can dissociate from the enzyme. During catalysis a water molecule bound to Zn^{2+} is displaced by the oxygen moiety of the alcohol substrate. In this way zinc stabilises the intermediate alkoxide maintaining its tetrahedral coordination.[32,33]

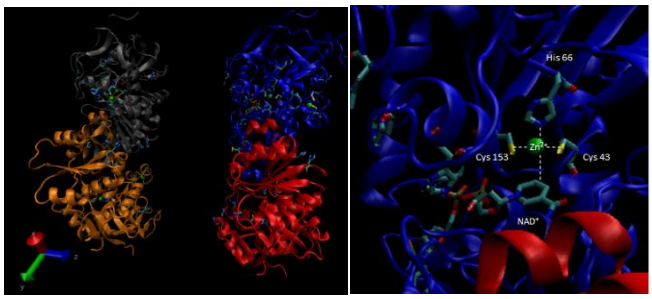


Figure 1. Representation of alcohol dehydrogenase (ADH) from yeast *Saccharomyces cerevisiae* PDB:4W6Z) (A) whole enzyme; (B) representative zoom on the cleft for the active site of ADH without substrate.

In the human body ADH catalyses the oxidation of ethanol to acetaldehyde which is then rapidly converted to acetate by acetaldehyde dehydrogenase and eventually metabolised in the muscle to carbon dioxide and water via the Krebs cycle.[34] ADH has recently attracted much interest, in particular for the preparation of chiral alcohols that are used as intermediates for the synthesis of pharmaceutical compounds.[35] The aim of this work is the investigation of the specific effect of salts to modulate the enzymatic activity of ADH. Models have been developed to quantify the effect of pH and ionic strength on the rate of enzymatic catalysis for ADH.[32] The models are primarily based on electrostatic theories that do not take into account the specific nature of ions of the same charge. Here, the specific activity and the Michaelis-Menten parameters (K_m and V_{max}) of ADH enzyme were determined.

2. Experimental

Chemicals. Alcohol dehydrogenase (ADH) from yeast *Saccharomices cervisiae* was purchased from Sigma Aldrich (product number A3263) and used without further purification. Lithium chloride (LiCl, 98%), sodium chloride (NaCl, 98%), potassium chloride (KCl, 98%), guanidinium chloride (GnCl, 98%), caesium chloride (CsCl, 98%), sodium fluoride (NaF, 98%), sodium bromide (NaBr, 98%), sodium perchlorate (NaClO4, 98%), and sodium thiocyanate (NaSCN, 98 %) and the cofactor β-Nicotinamide were purchased from Sigma Aldrich. Ethanol 99.9 % v/v was purchased Merck-Sigma.

Alcohol dehydrogenase activity measurements. For the activity test a spectrophotometric protocol from Sigma was followed. The total volume of the cuvette solution was 1.5 mL. The assay was started by adding 100 µL of ADH 1 mg/mL (diluted 1:50) to the reaction solution containing NAD+ 5 mM (250 µL), ethanol standard solutions (10 µL) from 2 to 40 mM and 10 mM (pH 8.3) Tris-HCl buffer (1.140 µL without salt or 940 µL with the addition of salt solutions). ADH activities were determined by UV-Vis spectroscopy by measuring the increase in absorbance due to the production of NADH at 340 nm.

Enzymatic activity measurement. Specific ion effects on ADH specific activity were investigated by adding to the reaction mixture containing the dissolved enzyme in a 10 mM Tris-HCl buffer solution at pH 8.30 - different sodium (NaF, NaCl, NaBr, NaClO4, NaSCN) or chloride (LiCl, NaCl, KCl, GnCl, CsCl) salts at 100 mM, 150 mM, and 200 mM, respectively. The experimental procedure was based on the analysis of NAD^+ formation monitored through UV-Vis spectrophotometry (λ = 340 nm).

3. Results and discussion

Figure 2 shows the effect of anions on the specific activity of ADH at two different ethanol (10 mM and 40 mM) concentrations. ADH activity is in general higher at 40 mM than at 10 mM ethanol concentration as expected according to the Michaelis-Menten plot. The addition of 100 mM NaF, NaCl, and NaBr results in an increase in specific activity (+46% for NaCl) in comparison to the absence of salt. The addition of $NaClO_4$ resulted in no net change in activity, whereas the addition of NaSCN caused a strong inactivation of ADH. A similar inactivating effect due to the highly chaotropic SCN^- anion was observed in previous works.[27] A non-monotonic (bell-shaped) anion specific trend such as that observed in Figure 2 was also observed at higher salt concentrations. The addition of NaBr at 150 mM and 200 mM resulted in increases in specific activity of 52% (Figure 2B) and 75% (Figure 2C), respectively. An activating effect due NaBr addition was previously observed with lipase (*Aspergillus niger*) in the hydrolysis of p-nitrophenylacetate.[36]

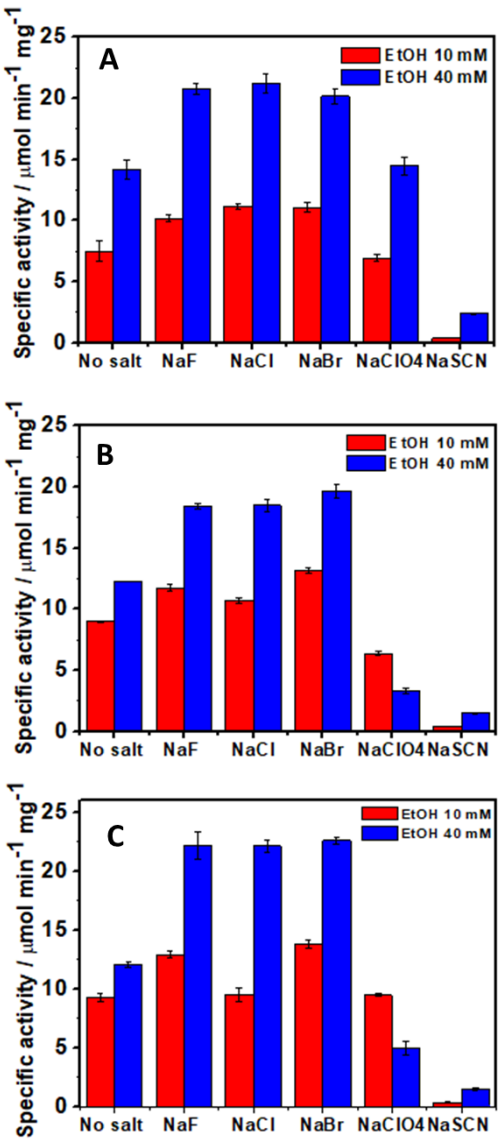


Figure 2. Specific effects of anions on the specific activity of ADH enzyme at ethanol concentrations of 10 and 40 mM, A) 100 mM, B) 150 mM, and C) 200 mM sodium salts.

Figure 3 shows the effect of cations (LiCl, NaCl, KCl, CsCl, GnCl), on the activity of ADH at concentrations of 150 and 200 mM. As with the anion series, the presence of cations has a general activating effect on the activity (+57% and +69% with LiCl at 150 and 200 mM, respectively) in comparison to that with no addition of salt. Nonetheless, at a salt concentration of 150 mM, cation specificity was not strongly evident with a slightly higher activity obtained with LiCl in comparison to other salts. At a concentration of 200 mM, cation specificity was more evident, with specific activity decreasing according to the sequence: LiCl ~ NaCl ~ KCl > CsCl > GnCl > No Salt.

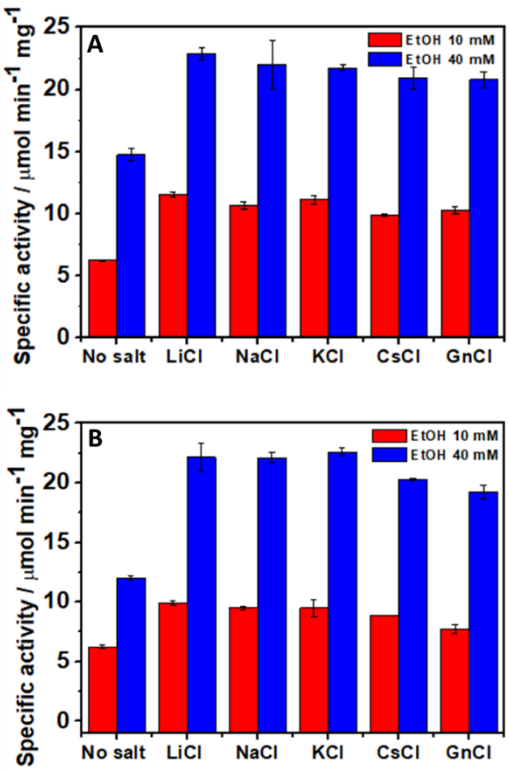


Figure 3. Specific effects of cations on the specific activity of ADH enzyme at ethanol concentrations of 10 and 40 mM, A) 150 mM, and B) 200 mM sodium salts.

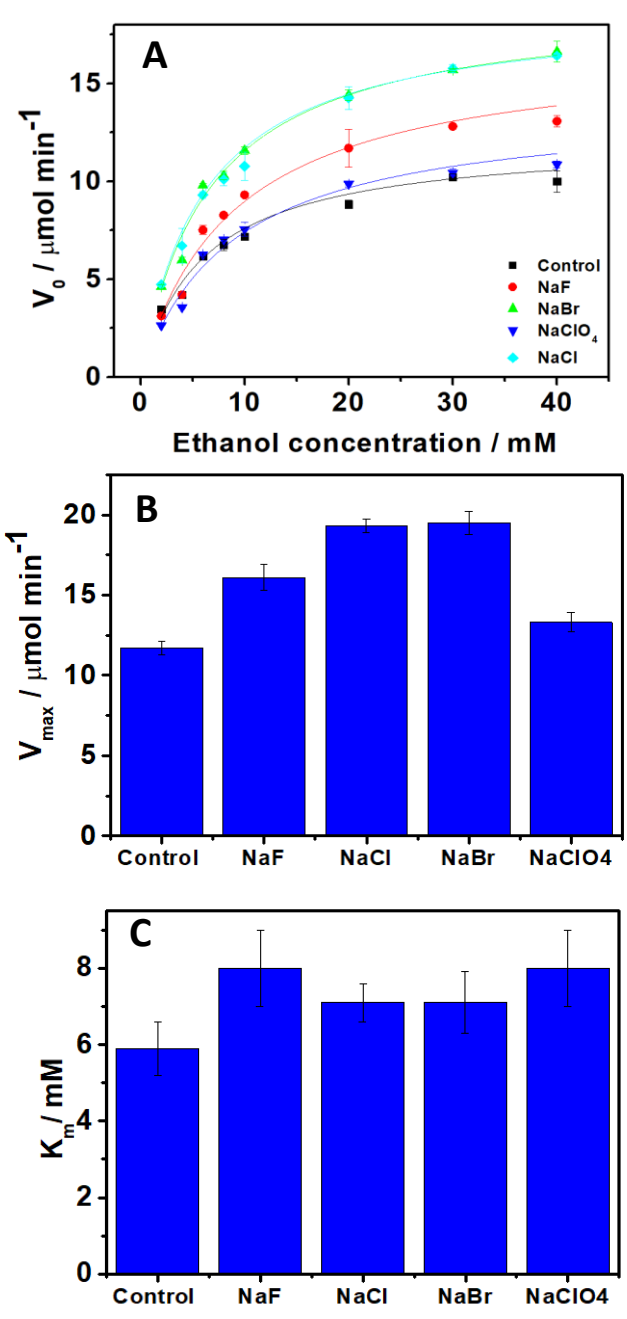
The Michaelis Menten kinetic model describes the dependence of the initial reaction rate (V_0) by the substrate concentration [S],

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

Where, V_{max} represents the maximum rate achieved by the system, and K_m (Michaelis Menten constant) is equal to the substrate concentration at which the reaction rate is half of V_{max}. The kinetic parameters, K_m and V_{max}, of ADH at pH 8.30 (10 mM Tris buffer) in the absence of any background electrolyte were measured initially. Figure 4A shows the Michaelis-Menten plot of the initial reaction rate, V_0, as a function of ethanol concentration. Values of 5.5 mM and 11.3 μmol mL^{-1} min^{-1} were obtained for K_m and V_{max}, respectively (Figure 4B-C). Hofmeister effects on the kinetic parameters of ADH were investigated. Michaelis-Menten plots in the presence of different 150 mM sodium salts are shown in Figure 4. In agreement with the results obtained in Figure 2, the addition of salts resulted in an increased rate of reaction in comparison with the data obtained with no added salt. Moreover, the nature of the salt modulated the initial reaction rate in a specific manner. The kinetic parameters, K_m and V_{max}, obtained in the presence of different 150 mM sodium salts are plotted in Figure 4B-C. On the addition of salt, K_m increased up to

8 mM, indicating that the presence of the salts can decrease the enzyme-substrate affinity. A lower affinity between enzyme and substrate indicates that the ions interfere with substrate binding at the active site. However, the results in Figure 4C show that K_m is not ion specific, as the differences obtained in the presence of NaF, $NaClO_4$, or NaCl and NaBr are within experimental error. In contrast, anions strongly affect V_{max}, following a bell-shaped (non-monotonic) specific ion effect series: NaBr ~ NaCl > NaF > $NaClO_4$ > no salt.

Michaelis-Menten plots obtained in the presence of different 150 mM chloride salts are shown in Figure 5A. As with the anion series, K_m increases on addition of salt (Figure 5C). As for the anion series, a clear cation specific effect was not observed, indicating that the cations do not specifically affect enzyme-substrate binding affinity. Similarly, V_{max} increased on addition of 150 mM chloride salts, e.g. from 11.3 ± 0.4 µmol mL^{-1} min^{-1} (no salt) to 20.1 ± 0.8 µmol mL^{-1} min^{-1} (150 mM).



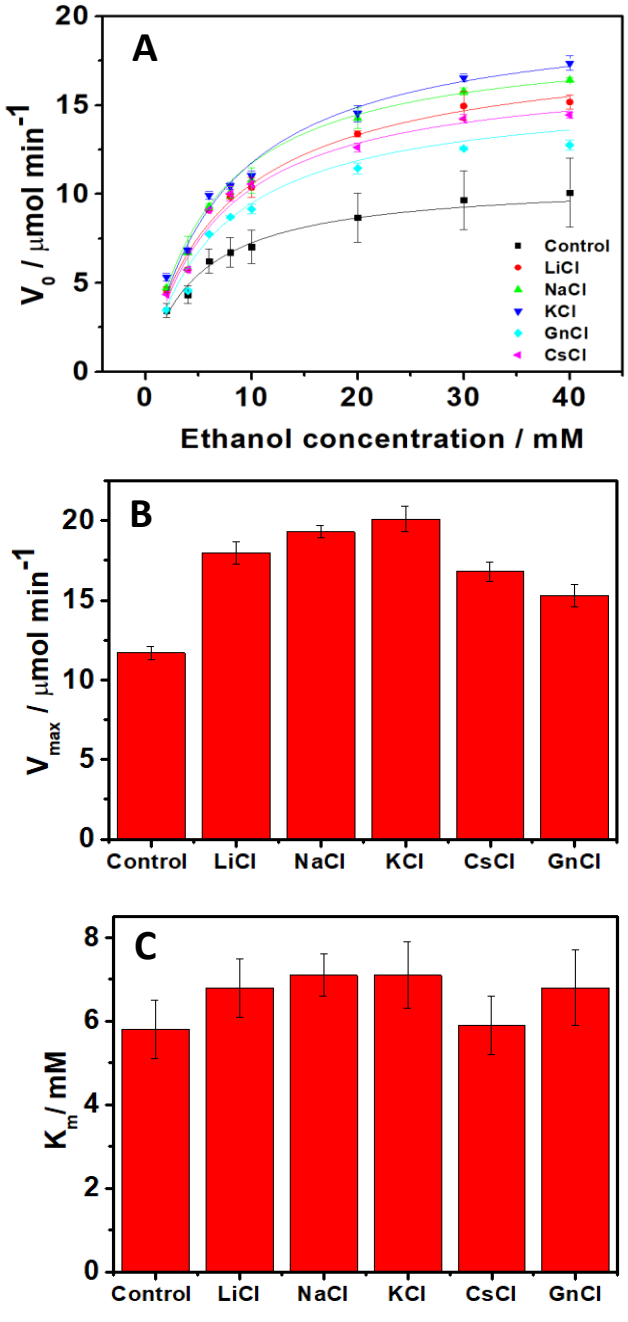


Figure 4. Specific anion effects on A) ADH enzyme Michaelis-Menten kinetics B) K_m Michaelis-Menten model) C) V_0 vs [ethanol] (Michaelis-Menten model). Salt concentration 150 mM, pH = 8.30; T= 298 K.

Figure 5. Specific cation effects on A) ADH enzyme Michaelis-Menten kinetics B) K_m Michaelis-Menten model) C) V_0 vs [ethanol] (Michaelis-Menten model). Salt concentration 150 mM, pH = 8.30; T= 298 K.

The cation specificity follows a non-monotonic specific ion effect, decreasing in the sequence: KCl > NaCl > LiCl > CsCl > GnCl > no salt (Figure 5B). Note that these effects occur at a concentration range that is similar to that obtained in biological systems. V_{max} is strongly ion specific whereas K_m is not affected by the nature of the ion. ADH follows an ordered sequential bi-bi mechanism, where NAD^+ binds to the enzyme to form an intermediate ternary complex, *EAB*:[37]

$$E + A \overset{K^A_s}{\rightleftharpoons} EA + B \overset{K^{AB}}{\rightleftharpoons} EAB \overset{k_{cat}}{\rightarrow} E + P + Q \qquad (2)$$

where *E* is enzyme, *A* is the first substrate (NAD^+), *B* the second substrate (ethanol), *P* the first product (acetaldehyde) and *Q* the second product (NADH). Since the concentration of [A] is constant ([NAD^+] = 5 mM) for all measurements, *EA* will predominantly exist as *EAB*. Therefore, it is possible to consider the overall reaction as a single-substrate reaction and utilise a simplified Michaelis-Menten model.[38] It has been reported that in catalysis by yeast and liver ADH, release of NADH is the slow step in the reaction when using ethanol as substrate.[39] As the concentration of *A* is constant, dissociation of the ternary complex *EAB* can be considered to be the slow step of the reaction.[40,41] Hence, k_{cat}, the rate constant for dissociation of the *EAB* complex is low. V_{max} is then given by:[37]

$$V_{max} = k_{\mathrm{cat}}[E_{\mathrm{T}}] \qquad (3)$$

Where [E_T] (=[E]+ [EA] +[EAB]) is the total enzyme concentration. The data show an increase in V_{max} at salt concentrations of 150 mM, indicating that the addition of salt has the general effect of increasing the value of k_2. Yeast ADH and horse liver ADH differ by 21 amino acid residues in the catalytic domain. In particular His-66 in yeast ADH plays a crucial role in the catalytic activity of the enzyme.[33,42] According to recent theories a delicate interplay between electrostatic, involving charge screening, and non-electrostatic forces are likely at work resulting in the trend for V_{max} of Br^-> Cl^-> F^-> ClO_4^- > No Salt.[14,16] That is, bromide optimal interaction with His-66 can enhances k_{cat}, whereas a too strong interaction, likely occurring with perchlorate or the fully inactivating thiocyanate (Figure 2), may be the cause of ADH inactivation. The fact that the specific effect of cations is less pronounced than that of anions supports the involvement of the positively charged His-66 residue in the mechanism. Cation specificity is likely an indirect (coion) effect which modulates Cl^---His^+ interactions. The effect of salts on K_m (Figure 4C and 5C) is much less significant than on V_{max}. In the ordered sequential bi-bi mechanism K_m, (also named K') is given by:[37]

$$K_m = \frac{K_s^A + K^{AB}}{[A]} + K^{AB} \qquad (4)$$

Where, K_s^A and K^{AB} are the formation constants of *EA* and *EAB*, respectively. K_m is not affected by the nature of the ions because depends on the formation constants of *EA* and *EAB* complexes, thus confirming that the dissociation of the ternary complex *EAB* is the rate determining step of the reaction. The fact that NAD^+ binding to ADH is not rate limiting is also confirmed by the data in Figure 3. Binding of NAD^+ occurs through the interaction with Asp-223 and Glu-68 residues of ADH. It may be the case that specific cation binding to carboxylate sites affects this mechanism.[43] Since the data in Figure 3 indicate that cations only slightly affect the specific activity of ADH, it can be assumed that cofactor-enzyme binding does not play a significant role on the overall rate of reaction. However, recent work has shown that the electrochemical properties of the NAD^+/NADH couple are ion specific, [44] therefore the role of the ions with respect to the cofactor cannot be excluded a priori.

4. Conclusions

In summary, we have found that the specific activity of ADH is ion specific in a range of salt concentration (100-200 mM) which is relevant for living organisms. Kinetic parameters obtained at 150 mM salt concentration help to shed light on ion specificity in the catalytic mechanism. Anions likely interact, at the level of the active site, with the histidine involved in the proton-relay mechanism, generally increasing the rate constant (k_{cat}) of *EAB* complex dissociation. Cations, which have a lower effect than anions, are likely indirectly involved in this mechanism or, alternatively, may affect NAD^+/ADH binding. Overall, ADH specific activity can be modulated with a suitable choice of background electrolyte at physiological concentration

Conflicts of interest

There are no conflicts to declare.

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