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Na,K-ATPase structure/function relationships probed by the denaturant urea

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ABSTRACT

Urea interacts with the Na,K-ATPase, leading to reversible as well as irreversible inhibition of the hydrolytic activity. The enzyme purified from shark rectal glands is more sensitive to urea than Na,K-ATPase purified from pig kidney. An immediate and reversible inhibition under steady-state conditions of hydrolytic activity at 37 °C is demonstrated for the three reactions studied: the overall Na,K-ATPase activity, the Na-ATPase activity observed in the absence of K^+ as well as the K^+-dependent phosphatase reaction (K-pNPPase) seen in the absence of Na^+. Half-maximal inhibition is seen with about 1 M urea for shark enzyme and about 2 M urea for pig enzyme. In the presence of substrates there is also an irreversible inhibition in addition to the reversible process, and we show that ATP protects against the irreversible inhibition for both the Na,K-ATPase and Na-ATPase reaction, whereas the substrate paranitrophenylphosphate leads to a slight increase in the rate of irreversible inhibition of the K-pNPPase. The rate of the irreversible inactivation in the absence of substrates is much more rapid for shark enzyme than for pig enzyme. The larger number of potentially urea-sensitive hydrogen bonds in shark enzyme compared to pig enzyme suggests that interference with the extensive hydrogen bonding network might account for the higher urea sensitivity of shark enzyme. The reversible inactivation is interpreted in terms of domain interactions and domain accessibilities using as templates the available crystal structures of Na,K-ATPase. It is suggested that a few interdomain hydrogen bonds are those mainly affected by urea during reversible inactivation.

1. Introduction

Na,K-ATPase is the enzyme system which couples hydrolysis of ATP to a transport of Na^+ and K^+ across the plasma membrane against their electrochemical gradients. The energy stored in the Na^+ gradient is of vital importance for cellular functions such as nutrient uptake, volume regulation, action potentials in nerve and muscle cells and numerous other processes. Since the discovery of the Na,K-ATPase in 1957 [1] the enzyme (also known as the sodium pump) has been intensively characterised, both in terms of structure on the atomic level and of physiological function, including its multiple involvement in diseases. The enzyme has three protein components, the 112 kDa α-subunit bearing the phosphorylation site and cation binding sites, the 35 kDa glycosylated β-subunit, and (often) an 11 kDa subunit belonging to the FXYD-family of regulatory peptides. The α-subunit has also a binding site for the specific inhibitors cardiotonic steroids.

Structure–function relationships in Na,K-ATPase have been studied extensively [2], and are currently of considerable interest due to the recently described ouabain-signalling pathway [3,4]. It is suggested that analogues of ouabain act as extracellular messengers with Na,K-ATPase as a docking station for a number of intracellular molecules involved in these signalling processes [5]. Recently cardiotonic steroids have been screened for potential use in chemotherapy, where the properties of inhibition of growth of cancer cells were related to the inhibitory effect on Na,K-ATPase activity [4].

There has been a significant progress in crystallisation of P-type ATPases since the first high-resolution structure of the Ca-ATPase structure was published in 2000 [6]. For Na,K-ATPase the structures of both pig kidney and shark rectal gland enzymes are available [7–12]. The structures reveal the detailed architecture of K^+-bound conformations of the enzyme $(K_2)\cdot E_2\cdot MgF_x$ forms [7,8] as well as Na^+-bound conformations ($Na_3\cdot E_1P\cdot ADP\cdot AlF_4^-$ forms [11,12]). Domain movements are important bricks in the reorganisation of Na,K-ATPase structure which occurs in response to the ligands inducing the main kinetically distinguishable intermediates, and in the present study the interpretations are done mainly within the framework of the two crystallised major conformations [8,11,12] which are taken as representatives of the classical K^+-form ($E_2\cdot(K)$) and the Na^+-form ($E_1\cdot Na$).

A comparison of Na,K-ATPase properties for enzyme purified from pig kidney and shark rectal glands has given some clues to the factors

Abbreviations: Na,K-ATPase, Na^+- and K^+-activated ATPase; E_1, the protein conformation of Na,K-ATPase predominant in Na^+-containing media; E_2, the protein conformation of Na,K-ATPase predominant in K^+-containing media; K-pNPPase, K^+-*p*-nitrophenylphosphatase; $K_{0.5}$, denaturant concentration giving half-maximal effect; Na-ATPase, the ATPase activity in the absence of K^+; pNPP, *p*-nitrophenylphosphate

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influencing the stability of the protein. Thermal denaturation of these enzymes has been analysed using FTIR [13], DSC [14] and SRCD [15], and these techniques reveal a large difference between shark and pig enzymes for denaturation/unfolding induced by high temperature, with pig enzyme being more stable. Recently domain movements in the protein have been suggested from SRCD analysis of the ouabain-bound enzymes [16], which display a higher stability towards thermal denaturation.

One of the possibilities to study changes in the tertiary structure is investigation of the protein unfolding by denaturants (e.g. urea and guanidine) which are chaotropic agents [17,18]. In low to intermediate concentrations the effect of urea is reversible, i.e. removal of urea after a certain incubation time leads to refolding of the protein and enzymatic function reappears. At higher concentrations irreversible effects are observed, where function cannot be regained.

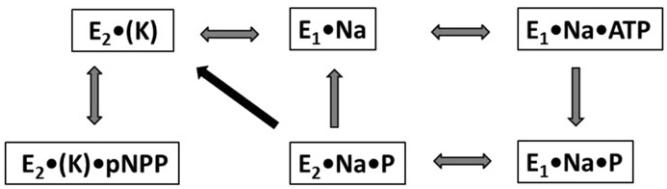
Perturbation of Na,K-ATPase protein structure by urea was earlier monitored by the spin-label ESR technique, and for both pig and shark Na,K-ATPase reversible as well as irreversible structural changes were observed [19,20]. Again a higher structural stability of pig enzyme was found relative to the shark enzyme.

The time- and concentration-dependence of the urea effect depends on the actual conformational state of the protein, which in the case of Na,K-ATPase depends on the nature of the ligands present (cations and substrates). In the present study we perturb the enzymatic reactions of the Na,K-ATPase activity, the Na-ATPase activity and the K-pNPPase activity. In experiments under hydrolysis conditions the substrates (ATP or pNPP) are also present, and the urea effects are compared with those in experiments under equilibrium conditions (absence of substrate, but all other ligands present). Scheme 1 is a simplified reaction scheme for the Post-Albers model (see [21,22]) depicting the relations between the three modes of hydrolytic activity studied here.

Under conditions of Na-ATPase activity (with Na^+, Mg^{2+} and ATP present, and absence of K^+) binding of ATP to the $E_1 \cdot Na$ form leads to phosphorylation. After the E_1P–E_2P conformational change the product of the reaction (inorganic phosphate, P_i) is released and the enzyme reverts to the $E_1 \cdot Na$ conformation. For the Na,K-ATPase activity (with Na^+, K^+, Mg^{2+} and ATP present) the initial steps are similar, but the presence of K^+ leads to a 30-fold increase in ATPase activity due to binding to the E_2P form and acceleration of the P_i-release (solid arrow in Scheme 1). The K-pNPPase activity is related to the $E_2 \cdot (K)$ form of the enzyme and is determined in the presence of K^+, Mg^{2+} and pNPP (and absence of Na^+).

The three reactions are studied in the presence of a large range of urea concentrations. We observe conformation-related differences in sensitivity to urea as well as stabilising effects of substrate. Both shark and pig enzymes become irreversibly inactivated in the presence of substrates, but, while ATP protects, pNPP appears to expose the protein towards urea. ATP protection is more marked with pig than with shark. Shark enzyme was also more sensitive under equilibrium conditions, i.e. the irreversible inactivation for shark enzyme occurs at lower urea concentrations than for pig enzyme.

Urea also exhibits a reversible inhibiting effect on both enzymes in the presence of substrates. It is observed on short time scales for shark enzyme and also on long time scales for pig enzyme, with shark enzyme being more sensitive to urea. For pig Na,K-ATPase the crystal structures of K^+- and Na^+-bound forms reveal a difference in the interdomain hydrogen bonding patterns. Since breaking and re-formation of these interdomain hydrogen bonds must occur on the same time-scale as the turnover of the enzyme (about 6 ms), it is plausible that the immediate and reversible inactivating effect of urea involves these transient hydrogen bonds in particular.



Scheme 1. A simplified reaction scheme for Na,K-ATPase activity (see text for details).

2. Materials and methods

2.1. Protein purification

Na,K-ATPase from the salt gland of *Squalus acanthias* was prepared according to the method of Skou and Esmann [23], omitting the saponin treatment. The shark enzyme preparation was stored at a protein concentration of about 5 mg/ml in 20 mM histidine (pH 7.0) with 25% glycerol. Na,K-ATPase from pig kidney microsomal membranes was prepared by treatment with SDS and purified by differential centrifugation [24]. Pig enzyme was stored in 20 mM histidine (pH 7.0) with 250 mM sucrose. The specific activity of both preparations – containing fully membrane-embedded enzyme – was approximately 30 μmol ATP hydrolysed/mg protein per min at 37 °C [25]. The Na,K-ATPase reaction medium contained 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 3 mM ATP in 30 mM histidine (pH 7.4), the Na-ATPase reaction medium contained 150 mM NaCl, 4 mM $MgCl_2$, 3 mM ATP in 30 mM histidine (pH 7.4), and the K-pNPPase reaction medium contained 150 mM KCl, 20 mM $MgCl_2$, 10 mM p-nitrophenylphosphate in 30 mM histidine (pH 7.4).

2.2. Denaturation experiments with urea at 37 °C

In steady-state experiments enzyme was incubated with the appropriate ligands together with urea in varying concentrations. The protein concentration was typically about 0.001 mg/ml for determinations of Na,K-ATPase and K-pNPPase activities and about 0.04 mg/ml for Na-ATPase activity measurements. After the desired incubation period the hydrolysis of the substrate was terminated by addition of 5% TCA, and the amount of product formed was determined by colorimetric methods as described [25]. In the experiments designed to test reversibility of the urea-inhibition (Fig. 5) the enzyme was first incubated with all ligands present at a high urea concentration (eg. 1.5 M for shark enzyme experiments), and after the desired time interval the solution was diluted threefold, leading to a reduction in the urea concentration as well as the enzyme concentration. The diluting solution contained the same concentrations of other ligands, and it is therefore only the urea and enzyme concentrations which are affected by the dilution.

Irreversible inactivation by urea was measured by incubation of enzyme with urea and the ligands relevant for a given enzyme activity, but omitting the substrate (ATP or pNPP). After the desired time periods the solution was diluted 10-fold into reaction medium containing the same ligands, but now with substrate present, and the reaction was allowed to proceed for 5 min before termination with TCA.

2.3. Structure analysis

The probability of the hydrogen bond formation between the three major domains of the α-subunit was analysed using the LigPlot+ software [26] applied to known crystal structures [8,11,12]. For Na,K-ATPase from pig kidney the N domain is defined by residues 377–588, the P domain by residues 348–376 and 589–747, and the A domain by residues 19–81 and 158–275. Domain definitions for Na,K-ATPase from shark rectal gland are shifted by +7 relative to the pig kidney enzyme. The hydrogen bonds between the three domains are listed in Table 2. The total number of hydrogen bonds in the α-subunits were analysed using the WHAT IF molecular modelling and drug design programme [27].

2.4. Data analysis

Data analysis was performed using the Origin 6.0 software (Microcal Inc).

3. Results

3.1. Effect of urea on steady-state hydrolysis reactions

Fig. 1 shows the time dependence of product release under conditions of steady-state hydrolysis, in the presence of different concentrations of urea. In these experiments, the substrate (ATP or pNPP) and other assay ligands are present during the whole time that the enzyme is exposed to urea. Under conditions of steady-state Na,K-ATPase activity there is a marked – and immediate – inhibitory effect of urea on both shark and pig enzymes (Fig. 1, panels A and D). In the absence of urea the release of P_i from ATP is almost linear in the time

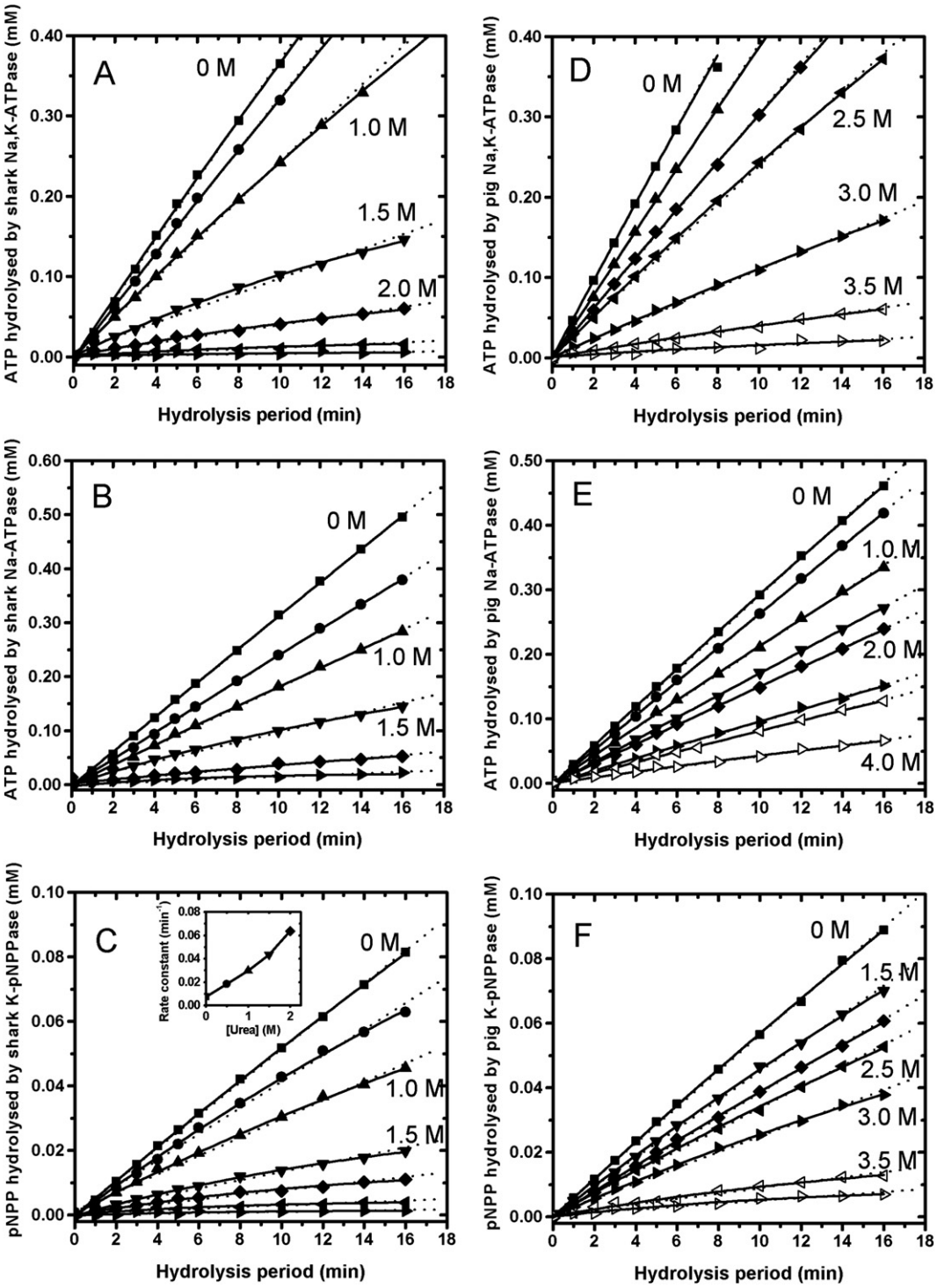


Fig. 1. Steady-state inhibition at 37 °C by urea of overall and partial reactions of the sodium pump. Na,K-ATPase protein from shark (panels A–C) or pig tissues (panels D–F) was incubated for the indicated periods with substrate under conditions of Na,K-ATPase (A, D), Na-ATPase (B,E) and K-pNPPase activity (C, F). The product formation is given as a function of the incubation time. The concentrations of urea during hydrolysis were 0 M (filled squares), 0.6 M (filled circles), 1 M (filled up-triangles), 1.5 M (filled down-triangles), 2 M (filled diamonds), 2.5 M (filled left-triangles), 3 M (filled right-triangles), 3.5 M (open left-triangles) and 4 M (open right-triangles). The dotted lines are linear fits of the data, and the full lines are single-exponential fits of the form $P_t = P_\infty \cdot [1 - \exp(-t/t_1)]$, characterised by a rate constant for inactivation of the enzyme of $1/t_1$ (min^{-1}). The rate constant was 0.046 min^{-1} for shark Na,K-ATPase in 1.5 M urea (panel A) and 0.04 min^{-1} for shark Na-ATPase in 1.5 M urea (panel B). For shark K-pNPPase we observed a concentration dependence as indicated in the inset to panel C, and for pig K-pNPPase at 3 M urea the single-exponential fit reflects a rate constant of 0.077 min^{-1}.

range studied (up to 16 min), and the inhibition by urea is seen as a reduction of the slope of the curves.

The dotted lines are linear fits of the data, the slope of which is proportional to the Na,K-ATPase activity. Since a deviation from linearity is clearly discernible at some urea concentrations, the data were also subjected to an exponential function analysis. The full lines represent single-exponential fits of phosphate-release with time (product formation, P_t):

$$P_t = P_\infty \cdot [1 - \exp(-t/t_1)] \quad (1)$$

The enzyme activity calculated from this type of fit is $A_t = dP/dt$, and the initial activity is $A_{t=0} = P_\infty/t_1$, where P_∞ and t_1 are obtained from the fitting procedure. In the absence of urea the difference between the linear and the exponential fits is very small, and the time constant t_1 is very large compared to the 16 min of reaction time monitored here. At intermediate urea concentrations – see for example the 1.5 M urea curve for shark enzyme (down-triangles in Fig. 1, panel A) – there is a clear but small difference, which indicates that inactivation by urea is a two-step process, with both an immediate reduction in the initial slope of the curves (equal to $A_{t=0} = P_\infty/t_1$) and also an inactivation manifesting itself as a time-dependent reduction of the slope with time constant t_1.

For most of the data sets shown for Na,K-ATPase the linear fit appears to be an adequate description of the urea effect, but since we at some urea concentrations observe the time-dependent reduction of activity we have analysed all the curves by the exponential relationship (Eq. (1)). The initial Na,K-ATPase activities calculated from this equation are shown in Fig. 2 as a function of the urea concentration in the reaction medium.

The effect of urea on steady-state hydrolysis of ATP in the absence of K^+ (the Na-ATPase activity) is also shown in Fig. 1 (panels B and E), and urea inhibition of the K^+-dependent phosphatase reaction (the K-pNPPase activity) is given in panels C and F. Inhibition of the shark K-pNPPase activity by urea is less linear compared to other partial reactions, and the rate constants for the inhibition of activity obtained from the exponential fitting are given in the inset to Fig. 1, panel C. With a rate constant (equal to $1/t_1$) for inactivation of about 0.04 min^{-1} at 1.5 M urea the half-time of inactivation is about 17 min, in good agreement with the time-course found below (Fig. 6, panel C). The initial Na-ATPase and K-pNPPase activities for pig and shark as a function of the urea concentration are given in Fig. 2.

The shark data in Fig. 2 are fitted by a Boltzmann relationship of the form:

$$y = A0 + (A1 - A0)/(1 + \exp[\{\Delta G - m_G \cdot [Urea]\}/(R \cdot T)]) \quad (2)$$

and the parameters derived are shown in Table 1 (see [19] for a detailed description of the Boltzmann analysis). The effective free energy of unfolding, ΔG is within a range -5.5 to -7.1 $kJ \cdot mol^{-1}$, and the parameter m_G, which reflects the steepness of the transition from active to inactive enzyme in the two-state model, is in the range -5.1 to -6.1 $kJ \cdot mol^{-1} \cdot M^{-1}$. Urea thus gives 50% inhibition at $K_{0.5} = \Delta G/m_G$ which is 1.15 M for Na,K-ATPase, 1.07 M for Na-ATPase and 1.14 M for K-pNPPase.

For pig enzyme (Fig. 2, panel B) a slightly different analysis of was adopted, since we observed a clear two-component effect of urea on the Na-ATPase reaction (down-triangles). The data were fitted by a double Boltzmann function:

$$y = A1/(1 + \exp[\{\Delta G_1 - m_{G1} \cdot [Urea]\}/(R \cdot T)]) + A2/(1 + \exp[\{\Delta G_2 - m_{G2} \cdot [Urea]\}/(R \cdot T)]) \quad (3)$$

For pig Na,K-ATPase and K-pNPPase a single-term Boltzmann analysis was adequate (the parameter A2 is set to 0), and the fitted value ΔG_1 of the single-term Boltzmann analysis is about -11 $kJ \cdot mol^{-1}$ (see Table 1), i.e. much more urea is required to inhibit pig enzyme than shark enzyme for these two activities. The terms m_{G1} in the Boltzmann analyses for pig Na,K-ATPase and K-pNPPase are smaller than for the shark activities, about -4.3 $kJ \cdot mol^{-1} \cdot M^{-1}$ compared to about -5.5 $kJ \cdot mol^{-1} \cdot M^{-1}$ for shark, corresponding to $K_{0.5} = 2.46$ M for Na,K-ATPase and 2.89 M for K-pNPPase.

For the pig Na-ATPase the parameters for the two Boltzmann terms are also given in Table 1. The two components are of almost equal size (A1 ≈ A2), but with very different sensitivities towards urea,

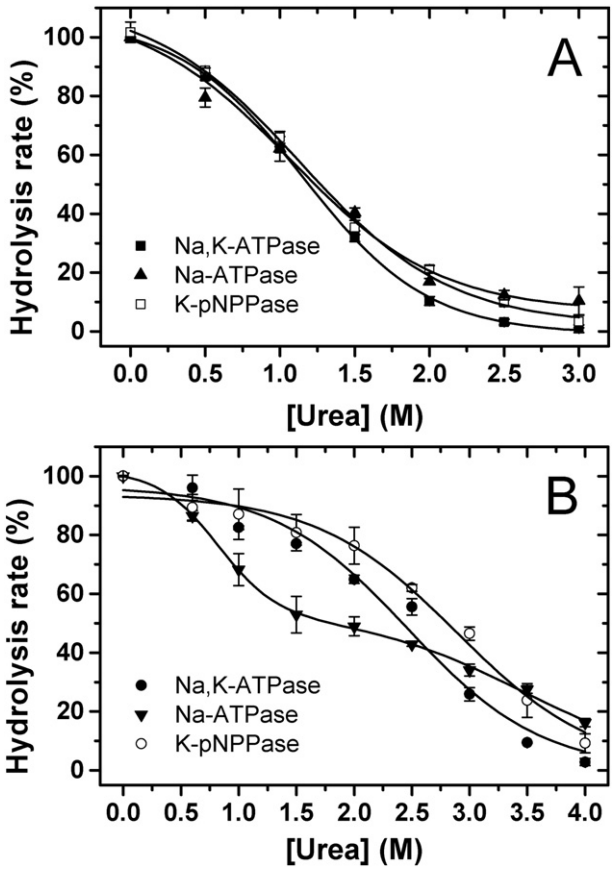


Fig. 2. Concentration dependence of immediate urea-inhibition of the sodium pump. Panel A: The initial activity (P_∞/t_1) of the curves shown in Fig. 1 for shark (Fig. 1 panels A, B and C) are given for each urea-concentration in percent of the initial activity in the absence of urea. Data are fitted by a single Boltzmann-function (Eq. (2)). Na,K-ATPase activity is represented by filled squares, Na-ATPase by filled up-triangles and K-pNPPase by open squares. Panel B: Similar analysis is given for pig enzyme, except that the loss of Na-ATPase activity here is fitted by a two-component Boltzmann equation (Eq. (3)). Na,K-ATPase activity is represented by filled circles, Na-ATPase by filled down-triangles and K-pNPPase by open circles. The data are given as the mean ± standard deviation for three separate experiments.

Table 1
Boltzmann parameters obtained from fit of data in Fig. 2 using Eqs. (2) (for shark enzyme) and (3) (for kidney enzyme). ΔG has the unit $J \cdot mol^{-1}$ and m_G has the unit $J \cdot mol^{-1} \cdot M^{-1}$. T = 310 K.

Shark enzyme	A_1	A_0	ΔG	m_G
Na,K-ATPase	106	−0.91	−7060	−6134
Na-ATPase	110	6.1	−5519	−5173
K-pNPPase	112	2.2	−5857	−5122

Pig enzyme	A_1	ΔG_1	m_{G1}	A_2	ΔG_2	m_{G2}
Na,K-ATPase	96.6	−10,889	−4426	0		
Na-ATPase	48.9	−8219	−10,024	53.4	−12,918	−3723
K-pNPPase	93.7	−12,265	−4244	0		

$\Delta G_1 = -8.2$ kJ·mol^{-1} and $\Delta G_2 = -12.9$ kJ·mol^{-1}, corresponding to $K_{0.5}$ values of 0.82 M and 3.47 M, respectively.

3.2. Inhibition by urea in the absence of substrate

The data presented in Fig. 1 reveals two effects of urea under steady-state conditions: an immediate inactivation (initial slopes are reduced) and in addition a time-dependent decrease of the slopes. In order to elucidate the kinetics of urea inhibition, we study the rates of inactivation of enzyme activities in the absence of substrates (Figs. 3 and 4) and in the following Section 3.3 we compare these rates with those in the presence of substrate in order to establish the presence of a reversible component of inactivation by urea.

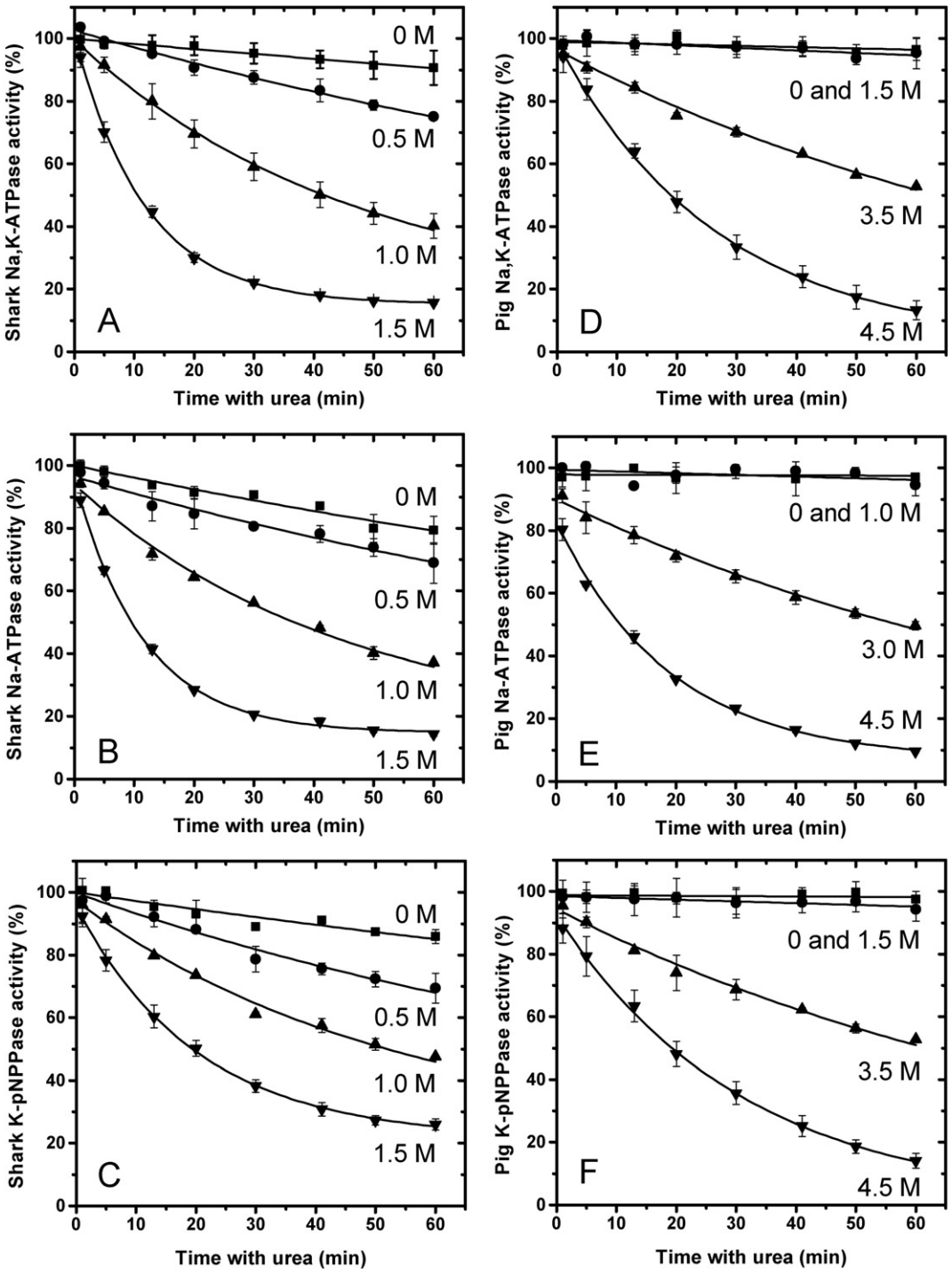


Fig. 3. Time course for inactivation of the sodium pump by urea in the absence of substrates (37 °C). Na,K-ATPase protein from shark (panels A–C) or pig tissue (panels D–F) was incubated with urea and ligands supporting Na,K-ATPase (A, D), Na-ATPase (B, E) and K-pNPPase activities (C, F), but omitting the substrates ATP or pNPP. At the indicated times an aliquot was diluted 10-fold into a urea-free solution containing the same ligands together with substrate (ATP or pNPP), and the activity was determined from the rate of hydrolysis. The data are given as the mean ± sd for three separate experiments, with the activity at t = 0 in the absence of urea is taken as 100%. For shark enzyme the urea concentrations were 0 M (squares), 0.5 M (circles), 1.0 M (up-triangles) and 1.5 M (down-triangles). For pig enzyme the concentrations were 0 M (squares), 1.5 M (circles), 3.5 M (up-triangles) and 4.5 M (down-triangles) for Na,K-ATPase and K-pNPPase activities and 0 M (squares), 1.0 M (circles), 3.0 M (up-triangles) and 4.5 M (down-triangles) for the Na-ATPase activity shown in panel E. The lines represent single-exponential fits of the form $A_t = A_0 \cdot \exp(-k \cdot t) + A_{t=\infty}$, (Eq. (4), see also text).

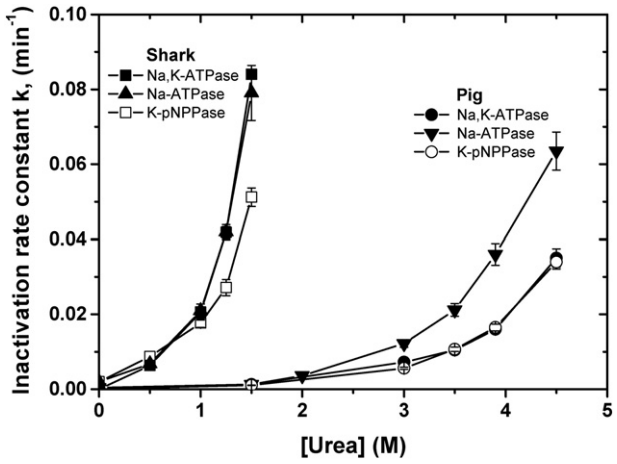


Fig. 4. Concentration dependence of the rate-constant (k) for inactivation by urea for shark and pig enzymes. Shark Na,K-ATPase activity is represented by filled squares, Na-ATPase by filled up-triangles and K-pNPPase by open squares. Pig Na,K-ATPase activity is represented by filled circles, Na-ATPase by filled down-triangles and K-pNPPase by open circles. The data of Fig. 3 were analysed by single-exponential fitting (Eq. (4)) for the loss of Na,K-ATPase activity (■, ●), Na-ATPase (▲,▼) and pNPPase (□,○). For shark there was a very slow inactivation of 18% of activity, and for pig this was about 4% ($A_{t=\infty} \approx$ 18% and 4%, respectively).

Inhibition of the pig and shark enzymes by urea under equilibrium conditions (absence of the substrates ATP or pNPP) was studied by incubating the enzyme with urea and the appropriate ligands for a given period of time at 37 °C (up to 60 min, see Fig. 3), followed by a 10-fold dilution of this solution into reaction medium containing the substrate, but no urea. The dilution of urea was so large, that the concentration of urea under conditions of activity assay was sufficiently small as to affect the hydrolysis reaction very little (see Figs. 1 and 2). At the highest urea concentrations used (1.5 M for shark and 4.5 M for pig, before the 10-fold dilution) there is an about 10% reduction of activity due to the presence of urea during steady-state (see Fig. 2), which explains that some of the time courses in Fig. 3 do not extrapolate to 100% at time = 0, but rather to 85–90%. The hydrolysis reaction period was 5 min. Linearity of product formation with time was tested in the 1 to 10 min range, and there was no deviation of linearity (data not shown). In addition we tested reversibility of the urea inactivation in the following manner: after a given time with urea, the enzyme-urea solution was diluted 5-fold and kept for 30 min. The activity was then tested for 5 min as above. There was no effect of this dilution and incubation on the activity, showing that dilution of urea stopped the inactivation process and that no re-activation was occurring after the dilution of the urea. The reduction in activities shown in Fig. 3 is therefore referred to as irreversible inactivation by urea.

Shark enzyme activities are shown in panels A–C of Fig. 3. In the absence of urea there is a small but significant loss of activity with time in the absence of urea for all three sets of incubation media studied, which indicates that at 37 °C the shark enzyme as such is unstable for prolonged periods of incubation. The rate of inactivation is increased markedly by urea, and with 1.5 M urea in the incubation medium half of the initial activity is lost in less than 10 min (a little longer for the K-pNPPase condition). The time-dependencies are fitted by single exponential functions of the form:

$$A_t = A_0 \cdot \exp(-k \cdot t) + A_{t=\infty} \quad (4)$$

where $A_{t=\infty}$ represents a fraction of activity of about 18% not sensitive to urea at the concentrations used here. The rate constants (k) for inactivation of the major fraction (about 82%) of activity are shown in Fig. 4, with markedly higher rate constants for inactivation for the Na,K-ATPase and Na-ATPase conditions than for the K-pNPPase condition.

Pig enzyme is less sensitive to urea, as was also observed for the steady-state experiments (Figs. 1 and 2). In Fig. 3, panels D–F, the losses of activities are shown, and half-time for inactivation is in the range 10–20 min at 4.5 M urea. The data are fitted by an exponential function (as for shark enzyme) with a residual urea-insensitive activity $A_{t=\infty}$ of about 4%. The rate constants are shown in Fig. 4, and for pig enzyme the Na-ATPase condition is more sensitive to urea than the Na,K-ATPase or K-pNPPase conditions.

3.3. Reversibility of the urea inhibition

In Fig. 1 we observe that with for example 1 M urea in the hydrolysis reaction medium about one third of the Na,K-ATPase activity of shark enzyme is immediately inhibited (the effect is accomplished before the first time point at 1 min, Fig. 1 panel A, filled up-triangles). In contrast to this we find that 1 M urea requires about 30 min to give one-third irreversible inhibition as seen from Fig. 3 (panel A, up-triangles). The Na,K-ATPase activity of the enzyme is thus much more sensitive to urea in the presence of the substrate (Fig. 1) than in the absence (Fig. 3).

In order to elucidate the origin of this discrepancy we have studied the reversibility of the effect of urea under hydrolysis conditions, Fig. 5. The results are given for shark and pig enzymes. For the Na,K-ATPase reaction the data in Fig. 5 panel A shows the hydrolysis of ATP in the presence of 0.5 M urea (filled circles) as well as the hydrolysis in the presence of 1.5 M urea (open symbols, see legend to Fig. 5). The experiments are designed to show the reversibility of the urea inhibition after 4, 7 or 10 min of incubation with substrate and 1.5 M urea, followed by three-fold dilution of enzyme into Na,K-ATPase reaction medium, now with only 0.5 M urea present. The filled triangles and diamonds in Fig. 5 reveal this recovery of a large fraction of Na,K-ATPase activity, relative to the activity present with 1.5 M urea (open symbols in Fig. 5 panel A). In Fig. 5 similar experiments for re-activation of Na-ATPase (panel B) and K-pNPPase (panel C) activities for shark enzyme by dilution of urea from 1.5 to 0.5 M concentrations are shown. Fig. 5 also shows reversibility of the inhibition of the reactions of the pig enzyme by dilution from 4.5 M to 1.5 M urea (panels D–F).

It is clear from Fig. 5 that the immediate urea inhibition under hydrolysis conditions for all the reactions is reversible, and it is also evident that the recovery of for example shark Na,K-ATPase activity (panel A) is smaller at the longer times of incubation with the high urea concentration, compare for example 4 and 10 min of incubation with 1.5 urea (filled triangles and diamonds, respectively).

The data of the type shown in Fig. 5 are analysed as described in the following example: the Na,K-ATPase activity of shark enzyme incubated with 0.5 M urea (filled circles in panel A) is calculated from the slope of the straight line, and this is taken as 100% activity. The slopes of the straight lines after urea-dilution are given in percent of this as a function of the time spent in 1.5 M urea prior to dilution. These data are shown in Fig. 6 (filled squares in panel A) with periods of exposure to 1.5 M urea ranging between 4 and 60 min. Similar analyses were made for shark Na-ATPase and K-pNPPase (also with urea dilutions 1.5 M to 0.5 M) and for pig activities with urea dilutions from 4.5 M to 1.5 M (Fig. 6 panels D–F). The observed decrease in slope (see Fig. 5) indicates irreversible inactivation of the enzyme under the conditions of hydrolysis. These data (filled squares) are in Fig. 6 compared with the loss of activities observed in the absence of substrates (data from Fig. 3 are here shown as open circles) at 1.5 M (shark, panels A–C) and 4.5 M urea (pig, panels D–F).

For shark enzyme as well as pig enzyme there is a clear protection by ATP against irreversible inhibition by urea of the Na,K-ATPase as well as the Na-ATPase activity (filled squares in Fig. 6, panels A, B, D and E, are well above the open circles). In contrast to this pNPP, the substrate for the K-pNPPase reaction, appears to expose both shark and pig enzymes towards irreversible inactivation by urea (filled squares in Fig. 6 panels C and F are below the open circles).

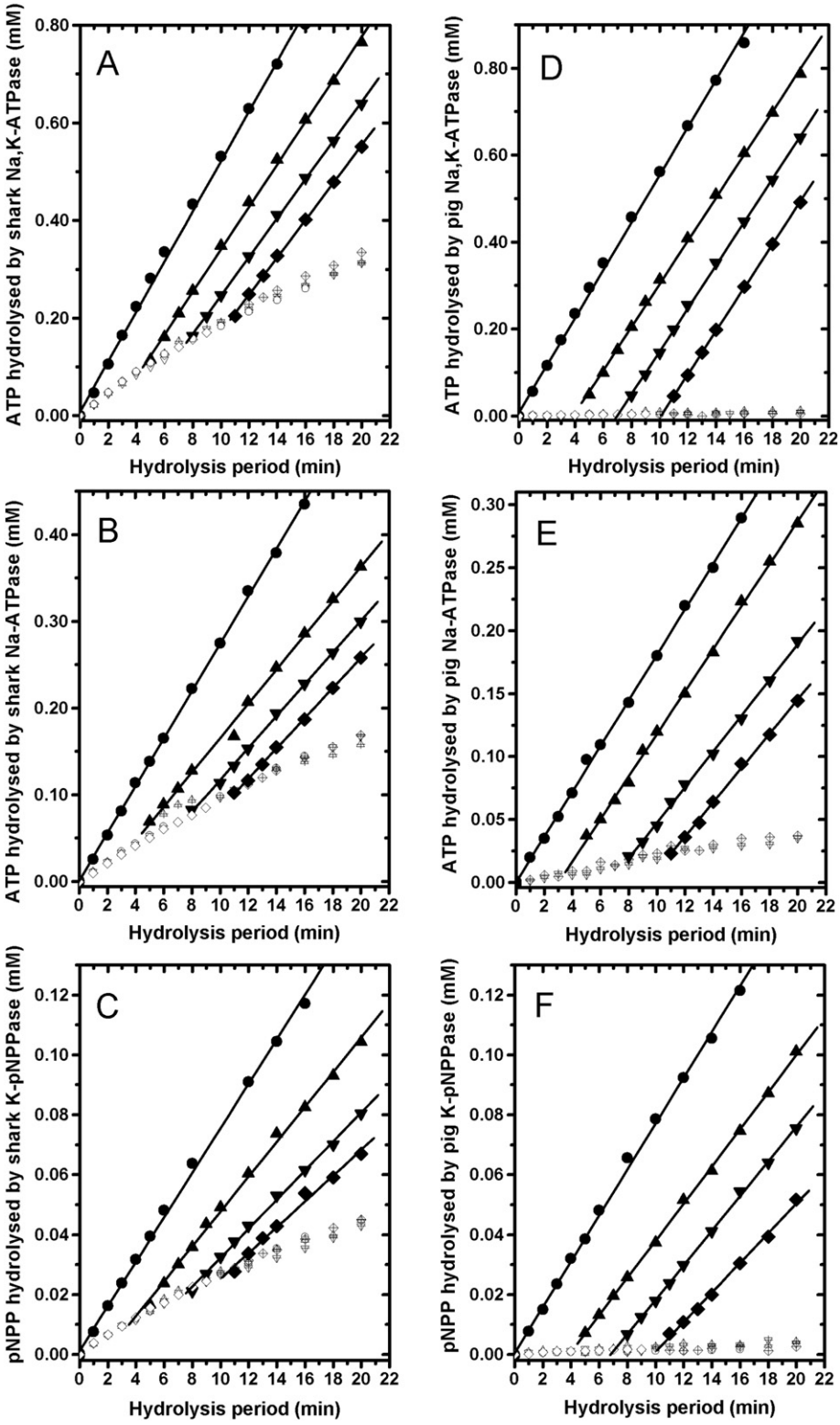


Fig. 5. Reversibility of urea-inhibition of the sodium pump during steady-state hydrolysis (37 °C). Enzyme from shark (panels A–C) or pig tissue (panels D–F) was incubated for the indicated periods with substrate under conditions of Na,K-ATPase (A, D), Na-ATPase (B, E) and K-pNPPase activity (C, F). The product formation is given as a function of the incubation time. Panel A shows a Na,K-ATPase progress curve for shark enzyme at 0.5 M urea (filled circles). The open triangles and open diamonds in panel A are similar experiments in 1.5 M urea. The filled triangles and diamonds represent samples which initially were in 1.5 M urea and then diluted to 0.5 M urea after 4 min (filled up-triangles), 7 min (filled down-triangles) or 10 min (filled diamonds). The protein concentration is the same in the samples represented by the filled symbols, and it is three-fold higher for 1.5 M urea samples (open symbols). In panels B and C similar experiments are shown for Na-ATPase and K-pNPPase hydrolysis. For pig enzyme the initial urea concentration was 4.5 M (open triangles and diamonds) which at 4, 7 or 10 min was diluted to 1.5 M (filled symbols). Symbols for panels B–F are as for panel A.

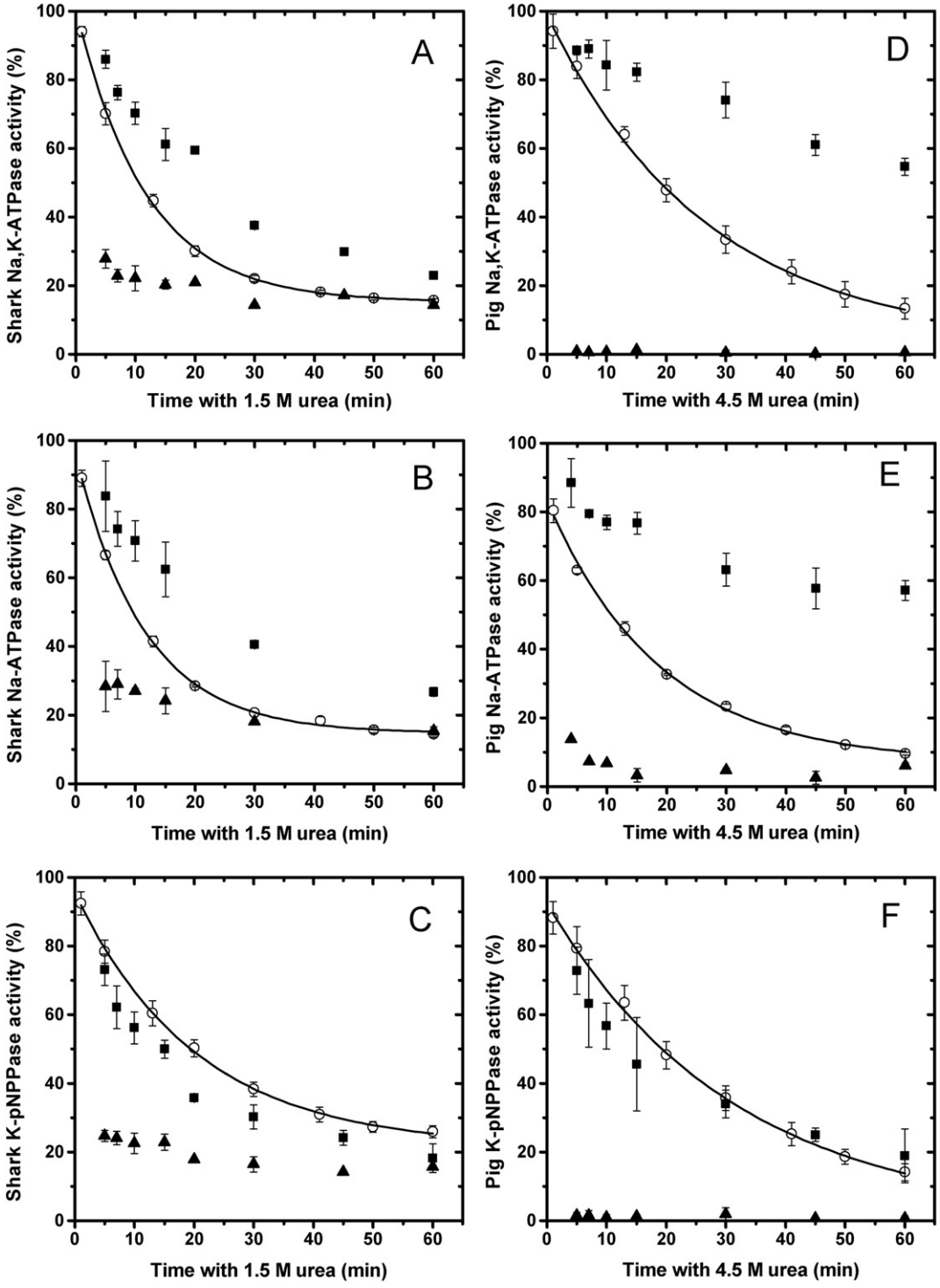


Fig. 6. Irreversible inactivation of enzymatic activities during steady-state hydrolysis at 37 °C. The slopes of the straight lines for data such as those shown in Fig. 5 are given in percent of the slope obtained with enzyme exposed to the low urea-concentration only (filled circles of Fig. 5), and the enzymatic activities remaining after incubation at the high urea concentrations are shown for shark (panel A, B, and C) and pig (panels D, E and F). The triangles represent the activity at the high urea concentration (1.5 M for shark and 4.5 M for pig), and the squares represent the activity after 3-fold dilution at times between 4 and 60 min. The data are given as the mean ± sd for three separate experiments. For comparison the data from Fig. 3 for the inactivation of shark enzyme at 1.5 M and pig at 4.5 M urea in the absence of substrates are also shown (open circles).

4. Discussion

Urea-induced inactivation was studied for Na,K-ATPase from two different sources both at equilibrium and under steady-state conditions of hydrolysis. Both reversible and irreversible types of inactivation are found under steady-state conditions. Since the urea effect was estimated from the hydrolytic activity measurements, the results obtained under equilibrium conditions reflect only irreversible effects.

4.1. Steady-state hydrolysis experiments

Urea inhibits all three reactions studied under steady-state conditions, Figs. 1 and 2. For shark enzyme all three reactions have about

the same sensitivity to urea (Fig. 2 and Table 1). The sensitivity towards urea of the pig enzyme is markedly smaller than that of the shark enzyme for the Na,K-ATPase and K-pNPPase reactions.

The Na-ATPase of pig enzyme is inhibited in a more complicated fashion (see Fig. 2 and Table 1), and the two-state model (cf. Eq. (2)) used to analyse the Na,K-ATPase and pNPPase inactivation is not sufficient. The analysis using Eq. (3) of the reversible Na-ATPase inactivation suggests the presence of an additional intermediate with only about half of the molar activity of the native enzyme (see [19] for a discussion of multi-intermediate analysis).

The free energies of unfolding deduced from the steady-state experiments (Table 1) are comparable with those deduced from structural unfolding of maleimide spin-labelled Na,K-ATPase [19], which at 37 °C are $\Delta G \sim -6700\ J \cdot mol^{-1}$ for shark enzyme and $\Delta G \sim -9700\ J \cdot mol^{-1}$ for pig enzymes (cf. Fig. 5 in Ref. [19]).

4.2. Urea effects under equilibrium conditions (in the absence of ATP or pNPP)

Incubation with urea and the same ligands as during steady-state hydrolysis, but omitting the substrates ATP or pNPP, gives the time-courses shown in Fig. 3 for the three reactions at different concentrations of urea, and the analysis of the inactivation rates is shown in Fig. 4. This inhibition is considered to be irreversible since no reappearance of activity was observed in a 30 min period after a 5-fold dilution of urea (see Section 3.2).

4.3. Reversible inactivation by urea

Fig. 5 shows a set of representative experiments designed to test reversibility of the urea effect observed under hydrolysis conditions. For all three reactions studied, and for both pig and shark enzymes, a reappearance of activity occurred immediately after a three-fold dilution of the urea concentration. Almost all activity could be recovered after dilution provided that the period with the higher urea concentration was short, which suggests that this immediate effect of urea during hydrolysis (Fig. 1) is completely reversible. However, extending the period with the high concentration of urea up to 60 min led to a progressive reduction in the fraction of activity which could be recovered by dilution, Fig. 6. The reversibility for pig enzyme at long incubation times is far greater than that of shark enzyme, compare filled squares with open circles in Fig. 6.

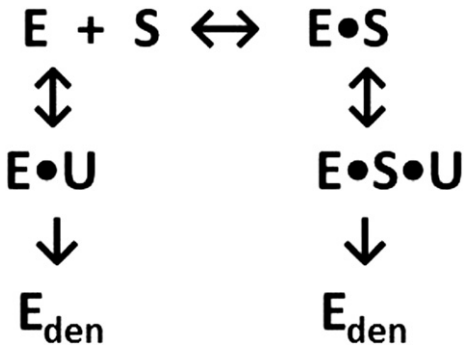
4.4. Reversible versus irreversible inactivation

The effect of urea under hydrolysis conditions is thus partly reversible, and there is a time dependent irreversible inactivation in addition to the reversible effect, Figs. 5 and 6. Comparison of the time course of irreversible inactivation in the presence of substrate (Fig. 6) with the time course in the absence of substrates (data from Fig. 3) shows that ATP as substrate offers significant protection to pig and to some extent to shark (open symbols compared with the closed symbols in Fig. 6, panels A, B, D and E), but that pNPP actually leads to an increased irreversible inactivation for both shark and pig (Fig. 6, panels C and F).

The rates of irreversible inactivation in the absence of substrate (Fig. 4) show a higher sensitivity towards urea for shark enzyme than pig enzyme (the same type of behaviour seen in Fig. 2 with reversible inactivation in the steady-state situation).

4.5. Model for urea effects on the enzyme

A minimal model for the effect of urea (U) and substrates ATP and pNPP (S) includes reversible binding of substrates and of urea (cf. Scheme 2). Urea binds reversibly to the equilibrium conformation (E) in the absence of substrate, and the intermediate E·U may be irreversibly inactivated (vertical single-direction arrow). E thus represents



Scheme 2. A minimal model for urea interactions with the Na,K-ATPase enzyme (see text for details).

either conformations of the E_1 type (in Na^+) or the E_2-type (in K^+) or a mixture of these (in 130 mM Na^+ + 20 mM K^+). In the presence of substrate, urea interacts with the steady-state conformation(s) E·S in a reversible and irreversible manner through the intermediate E·S·U. The E·S intermediate comprises among other forms the E_1P and E_2P states, see Scheme 1. E_{den} denotes irreversibly denatured inactive enzyme.

The equilibrium constants for urea binding and the rate constants for denaturation are species-dependent as well as "activity dependent", i.e. shark enzyme requires less urea than pig enzyme for a given effect, and irreversible inhibition of for example pig Na,K-ATPase is much slower in the presence of ATP (E·S·U) than in the absence (the E·U form — see data in Fig. 6 panel D).

4.6. Urea–protein interactions

Urea interacts with proteins through polar interactions (disruption of native hydrogen bonds of the protein) as well as apolar interactions (solvation of apolar groups of the protein) [28,29]. Both types of interaction can lead to reversible as well as irreversible denaturation of the protein. Here the immediate and reversible effects of urea will be interpreted as results of disruption of the tertiary protein structure due to breaking of the relatively few hydrogen bonds between the domains of the α-subunit. The irreversible effects of urea most probably involve a larger fraction of the intramolecular hydrogen bonds. The total number of hydrogen bonds in the crystal structures of the different forms are 878 for the shark E_2·(K) form (PDB ID: 2ZXE), 567 for the pig E_2·K) form (PDB ID: 3KDP) and 730 for the pig E_1·Na form (PDB ID: 3WGU).

4.7. Structural interpretation of the reversible urea effects

In Fig. 7 we compare the overall structures of the E_2·(K)-form of the protein [7,8] with the E_1·Na-structure [12]. All structures include phosphate analogues AlF_4^- or MgF_4^{2-} offering significant stabilisation of the extramembranous cytoplasmic part of the enzyme. The structures represent forms either right before formation of acyl-phosphate covalent bond, or just after its hydrolysis. The major difference between these forms is the association of the A-domain with the N-domain (which occurs upon nucleotide binding and phosphorylation) and the simultaneous movement of the P-domain.

In the lower panels of Fig. 7 the hydrogen bonds formed between the three domains are highlighted in both conformations. In the E_2·(K)-form of pig kidney Na,K-ATPase we find 6 hydrogen bonds, 4 of which are between the N and P domains. In the E_1·Na-form there are 12 hydrogen bonds. Interestingly 3 of the hydrogen bonds are preserved in both conformations, all between the N and P domains. The residues involved are listed in Table 2. In the E_2·(K)-form of shark rectal gland Na,K-ATPase we find 5 hydrogen bonds, 2 of which are between the N and P domains and 3 between the A and P domains.

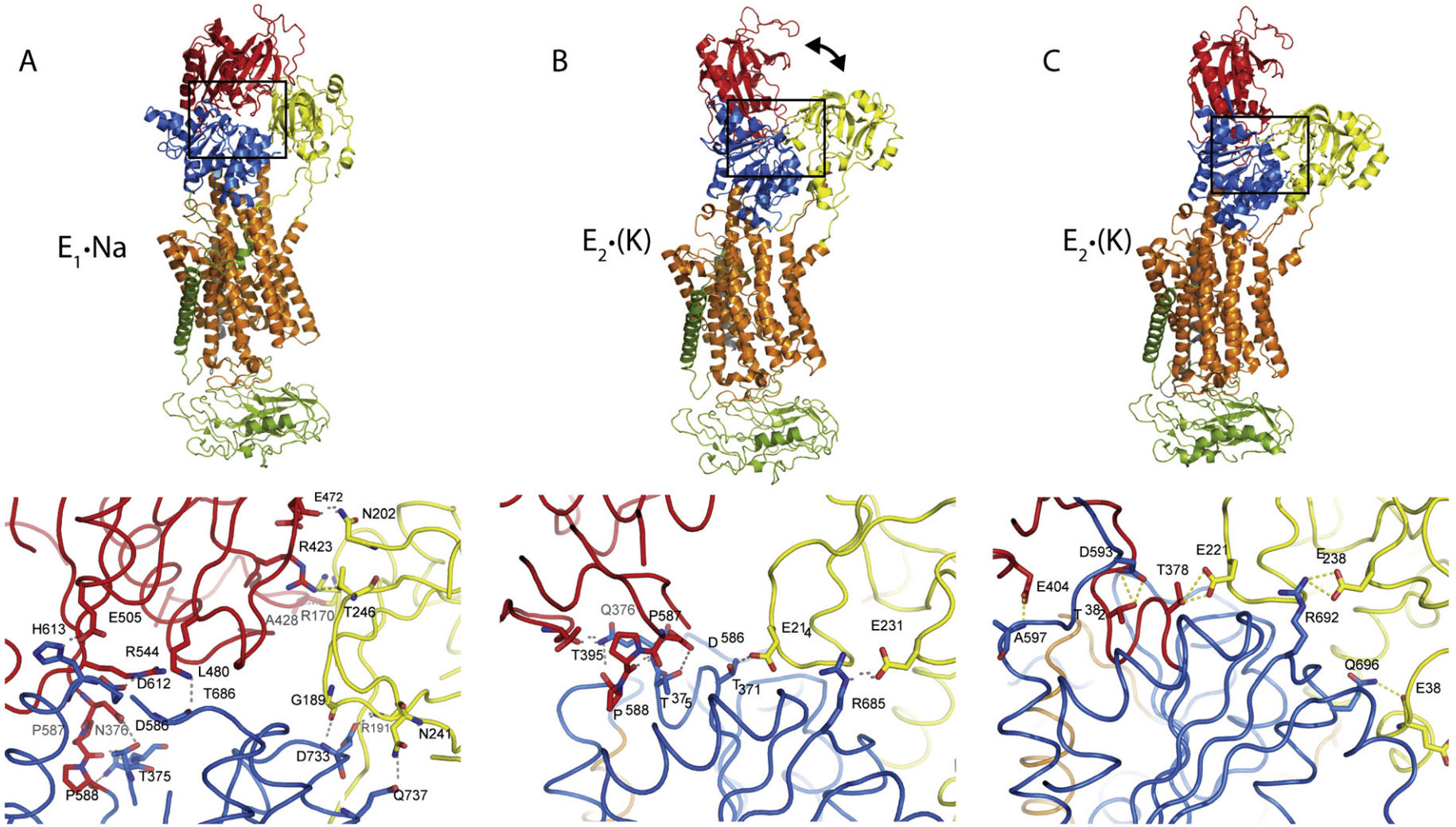


Fig. 7. Structural models of Na,K-ATPase conformations. Crystal structures of Na,K-ATPase reveal the detailed architecture of the Na^+-bound conformation $Na_3 \cdot E_1P \cdot ADP \cdot AlF_4^-$ of pig kidney Na,K-ATPase (left panel A, PDB ID: 3WGU [12]) and the K^+-bound conformation $(K_2) \cdot E_2 \cdot MgF_x$ of pig kidney (middle panel B, PDB ID: 3KDP [8]) and the shark rectal gland (right panel C, PDB ID: 2ZXE [7]). The α subunit contains three cytoplasmic domains, the phosphorylation domain (P, blue), the nucleotide binding domain (N, red) and the actuator domain (A, yellow) in addition to the transmembrane part (orange). The β subunit is shown in green and the γ subunit in grey. The movements of the three cytoplasmic domains are indicated by the arrow in the right panel. This figure was prepared with PyMOL [35]. In the lower part the location of the interdomain hydrogen bonds (see Table 2) are shown as dotted lines between the labelled residues.

Table 2
Hydrogen bonds between domains of Na,K-ATPase in the $E_1 \cdot Na$ and $E_2 \cdot (K)$ forms of pig kidney Na,K-ATPase (PDB ID: 3WGU and 3KDP, respectively) and the $E_2 \cdot (K)$ form of shark rectal gland Na,K-ATPase (PDB ID: 2ZXE). Conserved hydrogen bond pairs are indicated by "===". Note that the shark enzyme sequence is shifted by 7 amino acids relative to the pig enzyme.

PDB ID: 3WGU			3KDP			2ZXE	
Form: $E_1 \cdot Na$			$E_2 \cdot (K)$			$E_2 \cdot (K)$	
A + P domain							
GLY 189	ASP 733		GLU 214	THR 371	===	GLU 221	THR 378
ARG 191	ASP 733		GLU 231	ARG 685	===	GLU 238	ARG 692
ASN 241	GLN 737					GLU 38	GLN 696
A + N domain							
LYS 170	ALA 428		NONE			NONE	
ASN 202	GLU 472						
THR 246	ARG 423						
N + P domain							
ASP 586	THR 375	===	ASP 586	THR 375	===	ASP 593	THR 382
PRO 587	GLN 376	===	PRO 587	GLN 376		GLU 404	ALA 597
PRO 588	GLN 376	===	PRO 588	GLN 376			
LYS 480	THR 696		THR 395	GLN 376			
GLU 505	HIS 613						
ARG 544	ASP 612						

Table 3
Predominant conformations of the sodium pump at equilibrium and during steady-state hydrolysis.

	Shark		Pig	
	Equilibrium[a)]	Steady-state[b)]	Equilibrium[a)]	Steady-state[b)]
Na,K-ATPase	20% E_1, 80% E_2	Low EP-level	5% E_1, 95% E_2	Low EP-level
Na-ATPase	100% E_1	35% E_1P + 65% E_2P	100% E_1	94% E_2P
K-pNPPase	100% E_2	not EP	100% E_2	not EP

[a)] The protein conformation is taken as E_1 in the presence of Na^+ and E_2 in the presence of K^+. Using the fluorescent dye eosin [30] it is estimated that in the presence of 130 mM Na^+ and 20 mM K^+ (Na,K-ATPase conditions) the conformation is about 20% E_1 for shark enzyme [30] and less than 5% for pig enzyme (experiments not shown).
[b)] During steady-state hydrolysis in the K-pNPPase reaction very little acid stable phosphate intermediate is observed [31]. For the Na-ATPase reaction the dominant form is E_2P for pig enzyme [32] and a mixture of E_1P and E_2P for shark enzyme [33].

It is assumed that the reversible effect of urea –which is also very rapid, see Fig. 1 –is not due to major rearrangements of protein structure. From an enzyme kinetics point of view the reversible urea effect could be due to a simple competition with the substrates ATP or pNPP or with the activating cations at their binding sites. Since all three reactions (at least for shark enzyme) are equally sensitive to urea this is probably not the case. A more plausible explanation could be that urea interferes with domain–domain interactions of electrostatic nature, and that these interactions are of importance of the steady-state enzymatic cycle.

If urea interferes with rapid rearrangements of domains between E_1 and E_2 (rapid because it occurs during each Na,K-ATPase cycle, which takes about 6 ms to complete at 37 °C) a reversible inactivation would occur. In terms of Fig. 7, the formation of $E_1 \cdot Na$ from $E_2 \cdot (K)$ would require formation of 9 hydrogen bonds when the domains are rearranged as part of the normal Na,K-ATPase cycle, and urea could interfere with this process, slowing the overall hydrolysis rate (see Table 2). In the second half of the enzymatic cycle 3 hydrogen bonds (Glu214-Thr371, Glu231-Arg685 and Thr395-Gln376) need to be established for $E_2 \cdot (K)$ to be formed, and urea could slow or prevent ATP hydrolysis by interference with this process. When urea is removed, the domain transitions return to normal and the hydrolysis rate is fully restored.

For the reversible inactivation of the Na-ATPase reaction a similar mechanism can be envisaged. Some of the many interdomain hydrogen bonds in the $E_1 \cdot Na$ form could be prone to perturbation by urea and thus explain the reversible inhibition as for the Na,K-ATPase activity. Alternatively the protein conformations of the Na-ATPase cycle (see Scheme 1) could differ in interdomain interactions in the same manner as $E_1 \cdot Na$ and $E_2 \cdot (K)$, and therefore vulnerable to urea attack.

The pNPPase activity associated with the $E_2 \cdot (K)$ conformation is also reversibly inhibited by urea. Some of the hydrogen bonds between the domains in the $E_2 \cdot (K)$ conformation could in analogy with the other two reactions be susceptible to urea interactions (but at present only little is known of "sub-conformations" related to pNPP hydrolysis). However, binding of the substrate pNPP has an effect on urea-sensitivity (see below) and a conformational rearrangement during pNPP hydrolysis is therefore also conceivable, leading to reversible urea inhibition.

4.8. Structural interpretation of the irreversible urea effects

Denaturation of protein structure due to unfolding and thus irreversible inhibition by urea can be considered to be a secondary process to the perturbation of domain–domain interactions described above.

Bennion and Daggett [28] suggested that urea promotes unfolding by both direct and indirect mechanisms. The first is due to screening of intramolecular hydrogen bonds when urea makes hydrogen bonds to polar moieties of the protein, as hypothesised above for the reversible inactivation. Then water and urea can reach the hydrophobic interior. The indirect mechanism involved altering (increasing) water interaction with the nonpolar groups leading to exposure of the hydrophobic core residues. Stumpe and Grubmüller [29] suggested that the denaturation power of urea rests on a trade-off between two features: urea is apolar enough to solvate apolar groups and it is polar enough to form weak hydrogen bonds to the backbone and to disrupt the water hydrogen bond network. Urea thus interfaces between water and natively buried parts of the protein, allowing for irreversible inhibition.

The irreversible inhibition observed in Fig. 3 and analysed in Fig. 4 reveals a high sensitivity towards urea for shark enzyme in 150 mM Na^+ (100% E_1-form) and 130 mM Na^+ + 20 mM K^+ (a mixture of E_1 and E_2-forms with about 20% E_1, see Table 3) and less sensitivity in 150 mM K^+ ("pure" E_2-form). The sensitivity towards urea for pig enzyme in 150 mM Na^+ (100% E1-form) is higher than in 130 mM Na^+ + 20 mM K^+ (95% in the E_2-form) and in 150 mM K+ ("pure" E_2-form), see Fig. 4 and Table 3.

The observed rate constants for shark enzyme inactivation (Fig. 4) are thus larger in the conformations with large fractions of E_1-form (100% for Na-ATPase and 20% for Na,K-ATPase) and in accordance with this, the rate constants for pig enzyme are smaller in the conformations with large fractions of E_2 (100% for K-pNPPase and 95% for Na,K-ATPase). In pig enzyme there are 567 hydrogen bonds in the $E_2 \cdot (K)$ form and 730 in the $E_1 \cdot Na$ form, and the larger sensitivity to urea of the latter form (cf. Fig. 4) could be due to the presence of more potentially urea-sensitive hydrogen bonds in shark.

4.9. Equilibrium versus steady-state sensitivity towards urea: effects of ATP and pNPP

Fig. 6 shows that addition of ATP protects against irreversible inactivation (upper 4 panels) and the effect is very prominent for the pig enzyme. In terms of Scheme 2, the E·ATP intermediates (which during the hydrolytic cycle will include phosphorylated intermediates) in the presence of Na^+ or Na^+ + K^+ are protected relative to the free enzyme forms (E). For the pNPPase reaction (lower panels in Fig. 6) the opposite effect of substrate is observed, namely a larger sensitivity towards urea under pNPP-hydrolysis conditions, which correlates well with the absence of phosphorylated intermediate during pNPP hydrolysis.

4.10. Comparison of pig and shark enzyme stabilities towards urea

The reversible inactivation by urea observed under steady-state conditions is markedly more pronounced for shark enzyme than for pig enzyme, Table 1. For the $E_2 \cdot (K)$-form there are 5 interdomain hydrogen bonds in the shark enzyme (3 of these are the same as in pig enzyme,

see Table 2), whereas there is a total of 6 interdomain hydrogen bonds in the pig enzyme. This difference is probably too small to account for the difference in urea sensitivity. The A–P domain interaction is stabilised by 3 hydrogen bonds in shark, and only two of these are present in the pig structure. Since pig enzyme is less sensitive to urea than shark enzyme under steady-state conditions (see Fig. 2) it is suggested that the N–P domain interactions play an stabilising role. More urea is thus required to disrupt the 4 hydrogen bonds between the N- and P-domains of the pig enzyme than the corresponding 2 hydrogen bonds of the shark enzyme. As the crystal structure of the $E_1 \cdot Na$ form of the shark enzyme is not yet available, a comparison cannot be made and a stabilising role for the 12 hydrogen bonds of the pig enzyme in the $E_1 \cdot Na$ form remains to be elucidated.

Shark enzyme is more sensitive to irreversible inactivation by urea than pig enzyme both under equilibrium (Fig. 4) and steady-state conditions (Fig. 6). This could be related to the total number of hydrogen bonds. The shark $E_2 \cdot (K)$-form contains 878 hydrogen bonds whereas the corresponding pig structure contains 567 hydrogen bonds, and the larger number of potentially urea-sensitive bonds in shark could be the reason for the larger sensitivity to urea. An analysis of hydrogen bonds in the intracellular A-, N- and P-domains of the α-subunit show that the shark $E_2 \cdot (K)$-form contains 389 hydrogen bonds whereas the corresponding pig structure contains 261 hydrogen bonds. The difference in sensitivity towards the irreversible inactivation by urea could thus be related to the intracellular domains of the α-subunit alone. With spin-labelled preparations of shark and pig Na,K-ATPase it was shown that the pig enzyme is structurally more stable than the shark enzyme towards unfolding by urea [19,20]. It is also possible that the oligomeric structures of the enzymes (cf. [34] for a review) influence the sensitivity towards urea. Interactions between neighbouring α-subunits, as well as with the β- and γ-subunits, which are different in shark and pig Na,K-ATPase, could also play a role in the stability towards urea.

In conclusion, we find that urea interaction with Na,K-ATPase has two components: a reversible component, seen here under steady-state conditions, and an irreversible component, which leads to a completely inactive enzyme. Using urea sensitivity as an analytical tool we can discriminate between at least 4 protein conformations for both shark and pig enzymes. The total number of hydrogen bonds appears to correlate with the pattern of the irreversible inactivation of the defined conformations. The few transient interdomain hydrogen bonds are suggested to be involved in the rapid and reversible urea interactions.

Conflict of interest

There are no conflicts of interests in relation to this paper.

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