

Glucose Regulation of Pre-steady State Kinetics of ATP Hydrolysis by Na,K-ATPase

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Abstract The effect of glucose and 2-deoxy-*D*-glucose on pre-steady state kinetics of ATP hydrolysis by Na,K-ATPase has been investigated by following pH transients in a stopped-flow spectrophotometer. A typical pre-steady state signal showed an initial decrease then subsequent increase in acidity. Under optimal Na⁺ (120 mM) and K⁺ (30 mM) concentrations, magnitudes of both H⁺ release and H⁺ absorption were found to be approximately 1.0/ATPase molecule. The presence of 1 mM glucose significantly decreased H⁺ absorption at high Na⁺ concentrations, whereas it was ineffective at low Na⁺. H⁺ release was decreased significantly in the presence of 1 mM glucose at Na⁺ concentrations ranging from 30 mM to 120 mM. Similar to the control, K⁺ did not show any effect on either H⁺ release or H⁺ absorption at all tested combinations of Na⁺ and K⁺ concentrations. Pre-steady state H⁺ signal obtained in the presence of 2-deoxy-*D*-glucose did not vary significantly as compared with glucose. Delayed addition of K⁺ (by 30 ms) to the mixture (enzyme+120 mM Na⁺+ATP+glucose) showed that only small fractions of population absorb H⁺ in the absence of K⁺. No H⁺ absorption was observed in the absence of Na⁺. Delayed mixing of Na⁺ or K⁺ did not have any effect on H⁺ release. Effect of 2-deoxy-*D*-glucose on H⁺ absorption and release was almost the same as that of glucose at all combinations of Na⁺ and K⁺ concentrations. Results obtained have been discussed in terms of an extended kinetic scheme which shows that, in the presence of either glucose or 2-deoxy-*D*-glucose, significantly fewer enzyme molecules reach the E~P(3Na⁺) stage and that K⁺ plays an important role in the conversion of E1.ADP.P(3Na⁺) to H⁺.E1~(3Na⁺) complex.

Keywords Na,K-ATPase; pre-steady state kinetics; proton absorption; ATP hydrolysis; stopped-flow

Na,K-ATPase (or Na,K-pump) is an integral membrane protein found in most mammalian cells and is responsible for translocating Na⁺ and K⁺ across the cell membrane using ATP as a driving force. The transport is electrogenic. For each ATP molecule hydrolyzed, three Na⁺ are transported out of and two K⁺ are transported into the cell [1]. Na,K-ATPase is a member of the P-type class of ATPases which include Ca²⁺-ATPase and H⁺,K⁺-ATPase and several prokaryotic transport enzymes [2]. Glucose profoundly affects activities of several P-type ATPases: H⁺-ATPase of yeast [3,4], Ca²⁺-ATPase [5] and Na,K-ATPase [6,7]. Currently the most accepted model of Na,

K-ATPase functioning, known as the Albers-Post model, is based on accumulated results of steady state kinetics [8]. H⁺ is intimately associated with the function of several ATPases: myosin-ATPase [9,10], F₀F₁-ATPase [11] and H⁺-ATPase [12]. The kinetic model of Albers-Post does not reveal anything about the absorption and release of H⁺ during reaction or the nature of transition between the E₁ and E₂ states of Na,K-ATPase. Skou [13] has dealt with the role of protonation-deprotonation on the transition of enzymes between Na⁺ and K⁺ forms. Amino groups involved are hidden in the presence of K⁺ but exposed in the presence of Na⁺. In the protonated form, the enzyme's affinity for Na⁺ and ATP is reported to be low, but it is high for K⁺. In the deprotonated form, the enzyme's affinity for Na⁺ and ATP is high, but is low for K⁺. The reaction

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cycle of Skou (**Fig. 1**) suggests unknown numbers of H^+ are absorbed or released by the enzyme. Two additional conformational states of the enzyme are suggested, one each for E_1 and E_2 depending on whether protons are bound or not.

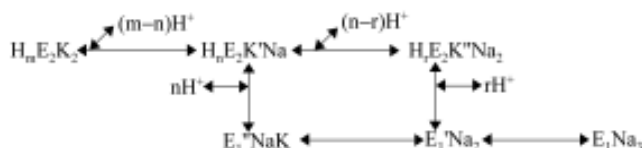


Fig. 1 Na,K-ATPase reaction cycle

m , n , and r represent successively decreasing but unknown numbers of H^+ . Reproduced from Skou [13]

In the present study we have investigated fast kinetics of ATP hydrolysis using the stopped-flow technique. Transient pH changes have been recorded in the absence and presence of glucose and its non-metabolizable analog 2-deoxy-*D*-glucose. Results have been discussed in terms of an extended kinetic scheme for Na,K-ATPase.

Materials and Methods

Materials

All biochemicals were obtained from Sigma-Aldrich (St. Louis, USA). All inorganic chemicals were of analytical grade and procured from Merck (Mumbai, India).

Preparation of Na,K-ATPase

Na,K-ATPase containing membrane fragments were prepared and purified from the outer medulla of goat kidney according to a previously described procedure [14]. The specific activity of Na,K-ATPase was measured according to a standard protocol [14,15]. For demasking of latent Na,K-ATPase activity, the membranes (0.5 mg protein/ml) were incubated with 0.065% sodium deoxycholate in 50 mM imidazole-HCl and 2 mM EDTA, pH 7.0. After incubation for 30 min at 20 °C, 25 μ l of aliquots was transferred to test tubes containing 1 ml of 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 25 mM imidazole-HCl, pH 7.5, and 1 mM ouabain (for control). After incubation for 3, 5 and 10 min at 37 °C, the reaction was stopped with 1 ml ice-cold 0.5 M HCl containing 30 mg ascorbic acid, 5 mg ammonium heptamolybdate and

10 mg sodium dodecylsulfate. The tubes were transferred to an ice-bath. For color development, 1.5 ml solution containing 30 mg sodium metaarsenite, 30 mg sodium citrate and 30 μ l acetic acid was added. The tubes were incubated for 10 min at 37 °C and absorbance was measured at 850 nm in a spectrophotometer. Steady state activity was routinely found to be 60 $nmol \cdot min^{-1} \cdot (mg \text{ protein})^{-1}$.

Pre-steady state kinetics

The absorption/release of H^+ from Na,K-ATPase system was measured by the change of the optical density of o-cresol red at 574 nm [9,16,17] in an SFM3 stopped-flow spectrophotometer (Bio-Logic, Claix, France). In all the sets of experiments, three syringes were used. ATP and Na,K-ATPase were placed in two different syringes. For experiments with varying concentrations of Na^+ and K^+ (keeping ATP constant) three syringes (S_{1-3}) were used in the stopped-flow spectrophotometer. S_1 contained 3 mM ATP, 3 mM $MgCl_2$ and o-cresol (50 μ M). S_2 contained 3 mM ATP, 3 mM $MgCl_2$, o-cresol (50 μ M) and KCl, and S_3 contained desired concentrations of NaCl, Na,K-ATPase (0.02 μ M) along with all the constituents of S_1 (except for ATP). Different amounts of solutions were mixed from syringes 1 and 2 to vary concentrations of K^+ whereas a constant volume of 50 μ M was always transferred from S_3 so that enzymes were not diluted. For delay experiments, S_1 contained ATP, o-cresol and $MgCl_2$ whereas S_2 contained the desired concentration of NaCl (for K^+ delay experiments) or KCl (for Na^+ delay experiments) and 0.02 μ M Na,K-ATPase along with all constituents of S_1 (except ATP). S_3 contained ATP, $MgCl_2$, o-cresol and the desired concentration of KCl or NaCl. In this set of experiments, solutions from S_3 were delivered after 30 ms or 60 ms as required. A similar procedure was used for experiments in the presence of glucose and 2-deoxy-*D*-glucose, and all three syringes in the stopped-flow spectrophotometer contained 1 mM glucose or 2-deoxy-*D*-glucose.

Prior to loading, the solutions were kept under reduced pressure for 12 h to remove dissolved gases, especially CO_2 . The pH of solutions was fixed under N_2 atmosphere. The syringe compartment was flushed with CO_2 -free N_2 gas, which was also streamed across the exit port to avoid any CO_2 contact of the cuvette or syringe compartment during experiments. Glucose or 2-deoxy-*D*-glucose was added to solutions just prior to loading to avoid any non-enzymatic glycation of enzyme, then mixed immediately after syringe loading to avoid pH drift or glycation [18]. Time of exposure of enzyme to glucose or 2-deoxy-*D*-glucose was indeterminate but in no case exceeded 5 min. Quantification of H^+ absorption/release was done using

the titration curve of o-cresol red prepared under conditions similar to that of test experiments. The optimum pH of mammalian kidney Na,K-ATPase is approximately 7.2 [14,19]. pKa of the dye under our experimental conditions was 7.8, and transient pH changes therefore were on the linear portion of the dye titration curve. With respect to the pre-steady state signal, where no systematic change was observed with variations in concentration of Na⁺ or K⁺, the data have been averaged for various concentrations. Variation within $\pm 5\%$ has not been considered a change and has been averaged. Experiments for each concentration has been carried out on at least two enzyme samples on different days, which had its own variation. To obtain a final value, the day 1 average of one concentration was averaged with the day 2 average of that concentration. For this reason experimental variation could not be shown with results. Maximum possible variations in these results are, however, less than $\pm 5\%$.

Results

Effects of glucose on H⁺ absorption and release at various Na⁺ and K⁺ concentrations

Table 1 shows H⁺ absorption and H⁺ release at various Na⁺ and K⁺ concentrations in the presence of 1 mM glucose. A typical recording of such experiments is shown in **Fig. 2**. At 120 mM Na⁺ with K⁺ varying from 30 mM to 120 mM, H⁺ absorption did not show any significant systematic change. The average value of H⁺ absorption was approximately 0.61 H⁺ per Na,K-ATPase molecule. The value for control H⁺ absorption was 1.15 in the absence of glucose. With the decrease in Na⁺ concentration to 90 mM, H⁺ absorption (at 0.59) changed little and was very close to the observed value of H⁺ absorption (0.63) in the absence of glucose under identical Na⁺ and K⁺ concentrations. The

Table 1 Moles of H⁺ absorbed and released per mole of Na,K-ATPase in the presence of different Na⁺ and K⁺ concentrations following mixing of enzyme with 1.5 mM ATP in the presence of 1 mM glucose

Na ⁺ (mM)	K ⁺ (mM)	H ⁺ absorbed	H ⁺ released	Ratio of H ⁺ (released/absorbed)
120	30	0.60	0.30	0.50
120	60	0.61	0.29	0.47
120	90	0.62	0.30	0.48
120	120	0.60	0.29	0.48
NA	Average	0.61	0.29	0.47
	(control)	(1.15)	(1.08)	(0.94)
90	30	0.60	0.31	0.52
90	60	0.59	0.31	0.53
90	90	0.60	0.29	0.48
90	120	0.59	0.29	0.49
NA	Average	0.59	0.30	0.51
	(control)	(0.63)	(0.54)	(0.86)
60	30	0.57	0.30	0.53
60	60	0.57	0.29	0.51
60	90	0.56	0.29	0.52
60	120	0.58	0.30	0.52
NA	Average	0.57	0.29	0.51
	(control)	(0.53)	(0.34)	(0.64)
30	30	0.54	0.29	0.54
30	60	0.52	0.29	0.56
30	90	0.53	0.29	0.55
30	120	0.52	0.31	0.59
NA	Average	0.53	0.29	0.55
	(control)	(0.32)	(no release)	—

Values in parentheses are averages for the control carried out in the absence of glucose. NA means non-available.

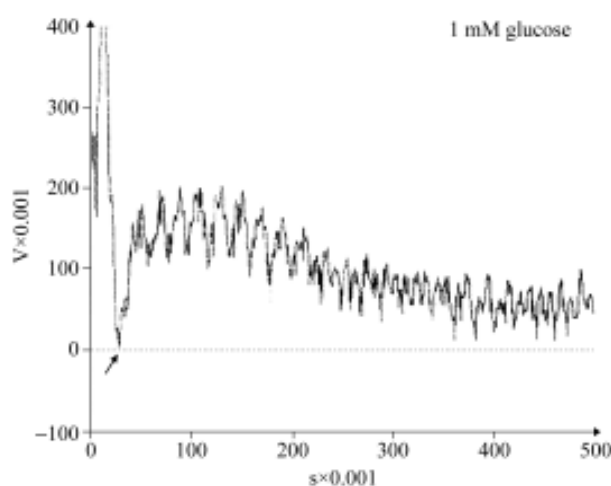


Fig. 2 Time-course of H^+ absorption/liberation at 574 nm in the presence of 1 mM glucose

Mix of $0.01 \mu\text{M}$ Na,K-ATPase (reaction chamber concentration) with 3 mM ATP along with 90 mM Na^+ and 30 mM K^+ at 25 °C. Increase in transmission indicates decrease in acidity. Three syringes (S_{1-3}) were used: S_1 contained 3 mM ATP, 50 μM o-cresol and 3 mM MgCl_2 , pH 7.5; S_2 contained 3 mM MgCl_2 , 50 μM o-cresol, 3 mM ATP, 1 mM glucose and the desired concentration of KCl; and S_3 contained the desired concentration of NaCl and $0.02 \mu\text{M}$ Na,K-ATPase along with all constituents of S_1 (except ATP). The initial portion of the trace shows syringe movement and the signal starts from 30 ms (\uparrow). All traces are the average of four or five recordings.

negative effect of glucose observed at the optimal Na^+ concentration to a large extent vanished with the drop in Na^+ concentration. The negative effect of low Na^+ therefore appears to be overriding the negative effect of glucose. This was further reinforced by the fact that H^+ absorption was almost the same in the presence of glucose (0.57) as in the absence of glucose (0.53) at 60 mM Na^+ concentration. At 30 mM Na^+ , glucose provided a small positive effect on H^+ absorption, up from 0.32 in the absence of glucose to 0.53 in the presence of glucose.

H^+ release is more significantly affected by glucose. At 120 mM, 90 mM and 60 mM Na^+ , H^+ release was of a similar magnitude of 0.29 H^+ per molecule of Na,K-ATPase, which is in contrast to a systematic decline observed in H^+ release with decrease in Na^+ concentrations in the absence of glucose. At 30 mM Na^+ , in the presence of glucose, H^+ release of 0.29 was in stark contrast with similar experiments in the absence of glucose where no H^+ release was observed. The H^+ release/absorbed ratio was an almost constant value of approximately 0.5 in the presence of glucose at all Na^+ concentrations. This ratio decreased rapidly with decreased Na^+ in the absence of glucose. Therefore, it appears that in the presence of glucose the population of ATPase molecule, which undergoes a

complete hydrolytic cycle, is affected by neither Na^+ nor K^+ . Compared to glucose absent situations, these populations are less for all tested Na^+ concentrations except 30 mM. It appears, therefore, that glucose negatively affects the overall catalytic cycle at Na^+ concentrations of 120 mM, 90 mM and 60 mM. Variation of K^+ at all Na^+ concentrations did not produce any significant change either in H^+ absorption or release. Na^+ (30 mM) in the presence of glucose is an exception because it promotes ATP hydrolysis.

Effects of 2-deoxy-D-glucose on H^+ absorption and release at various Na^+ and K^+ concentrations

Table 2 gives a recording of H^+ absorption and release at varying Na^+ and K^+ concentrations in the presence of 1 mM 2-deoxy-D-glucose, a non-metabolizable analog of glucose, following mixing of $0.01 \mu\text{M}$ Na,K-ATPase with 3 mM ATP. The results obtained were almost identical with those obtained in the presence of 1 mM glucose.

Effects of glucose on H^+ absorption and release with delayed addition of Na^+ and K^+ concentrations

Three typical original recordings for this set of experiments under various conditions, which are mentioned with the traces, are shown in Figs. 3–5. Table 3 presents a record of H^+ absorbed and released in the presence of 1 mM glucose when varying concentrations of K^+ and Na^+ were added with a delay of 30 ms and 60 ms, where the other ion had a fixed concentration of 120 mM. Thirty milliseconds was the average time elapse observed for 50% signal of H^+ absorption in the absence and presence of glucose at varying Na^+ and K^+ concentrations. The average H^+ absorption of 0.53 per enzyme molecule was observed when the ATPase was already incubated with 120 mM Na^+ and 3 mM ATP, and K^+ was mixed after a delay of 30 ms. Recording of the time-course of H^+ absorption or release was done after addition of either cation. This meant that part of the signal would be missed if the enzyme started the ATP hydrolytic cycle in the absence of either cation. The objective of these sets of experiments was to estimate the signal lost and captured before and after mixing of either cation. The average value of H^+ absorption was 0.53 when K^+ was added after a delay against 0.52 when there was no delay. This indicates that only a very small fraction of enzyme population absorb H^+ in the absence of K^+ . The average value of H^+ absorption when Na^+ was added after a delay of 30 ms was 0.62, which did not significantly differ from H^+ absorption when Na^+ was provided from time zero. This indicates that no H^+ absorption takes place in the absence of Na^+ and virtually all the H^+ absorption takes place only when Na^+ is provided.

Table 2 Moles of H⁺ absorbed and released per mole of Na,K-ATPase in the presence of different Na⁺ and K⁺ concentrations following mixing of enzyme with 1.5 mM ATP in the presence of 1 mM 2-deoxy-D-glucose

Na ⁺ (mM)	K ⁺ (mM)	H ⁺ absorbed	H ⁺ released	Ratio of H ⁺ (released/absorbed)
120	30	0.62	0.25	0.40
120	60	0.62	0.25	0.40
120	90	0.63	0.24	0.38
120	120	0.62	0.24	0.39
NA	Average	0.62	0.24	0.39
90	30	0.60	0.25	0.42
90	60	0.61	0.24	0.39
90	90	0.61	0.24	0.42
90	120	0.60	0.25	0.42
NA	Average	0.60	0.24	0.40
60	30	0.57	0.25	0.44
60	60	0.58	0.25	0.43
60	90	0.57	0.24	0.42
60	120	0.59	0.26	0.44
NA	Average	0.58	0.25	0.43
30	30	0.52	0.25	0.48
30	60	0.53	0.24	0.45
30	90	0.53	0.25	0.47
30	120	0.54	0.24	0.44
	Average	0.53	0.24	0.45

NA means non-available.

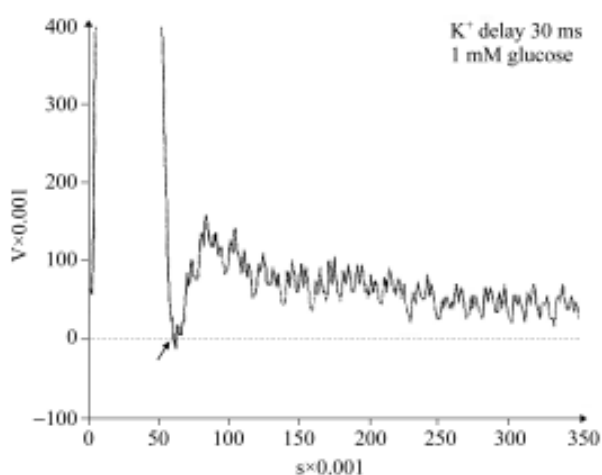


Fig. 3 Time-course of H⁺ absorption/liberation at 574 nm in the presence of 1 mM glucose after K⁺ delay of 30 ms

Mix of 0.01 μM Na,K-ATPase (reaction chamber concentration) with 3 mM ATP along with 120 mM Na⁺ and 30 mM K⁺ (mixed after a delay of 30 ms through syringe S₂) at 25 °C. Increase in transmission indicates decrease in acidity. Three syringes were used: S₁ contained 3 mM ATP, 50 μM o-cresol, 3 mM MgCl₂, 1 mM glucose pH 7.5; S₂ contained the desired concentration of NaCl and 0.02 μM Na,K-ATPase along with all constituents of S₁ (except ATP); and S₃ contained the desired concentration of KCl, 50 μM o-cresol, 3 mM MgCl₂ and 1 mM glucose. The initial portion of the trace shows syringe movement and the signal starts from 60 ms. All traces are the average of two or three recordings.

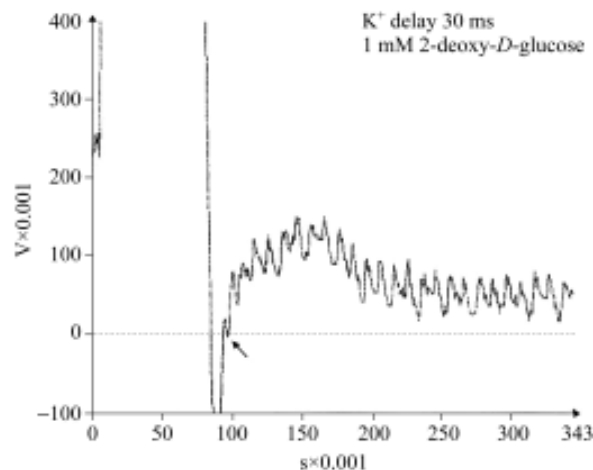


Fig. 4 Trace shows time-course of H⁺ absorption/liberation at 574 nm in the presence of 1 mM 2-deoxy-D-glucose following mixing of 0.01 μM Na,K-ATPase (reaction chamber concentration) with 3 mM ATP along with 120 mM Na⁺ and 30 mM K⁺ (mixed after a delay of 60 ms through syringe S₂) at 25 °C

Increase in transmission indicates decrease in acidity. Three syringes were used: S₁ contained 3 mM ATP, 50 μM o-cresol, 3 mM MgCl₂, 1 mM 2-deox-D-glucose, pH 7.5; syringe S₂ contained the desired concentration of NaCl and 0.02 μM Na,K-ATPase along with all constituents of S₁ (except ATP); and S₃ contained the desired concentration of KCl, 50 μM o-cresol, 3 mM MgCl₂ and 1 mM 2-deoxy-D-glucose. The initial portion of the trace shows syringe movement and the signal starts from 90 ms. All traces are the average of two or three recordings.

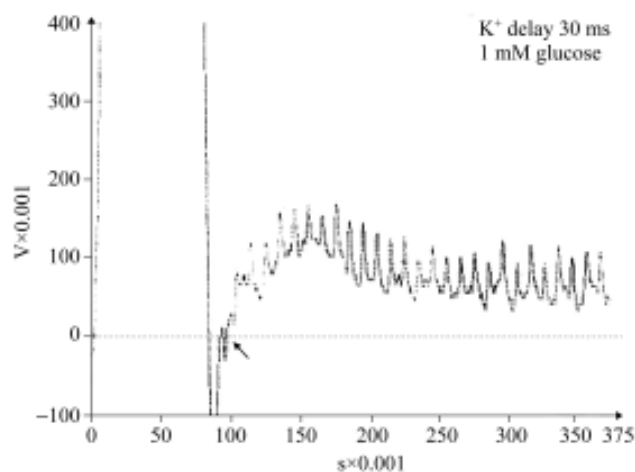


Fig. 5 Trace shows time-course of H^+ absorption/liberation at 574 nm in the presence of 1 mM glucose following mixing of 0.01 μ M Na,K-ATPase (reaction chamber concentration) with 3 mM ATP along with 120 mM Na^+ and 30 mM K^+ (mixed after a delay of 60 ms through syringe S_3) at 25 °C

Increase in transmission indicates decrease in acidity. Three syringes were used: S_1 contained 3 mM ATP, 50 μ M o-cresol, 3 mM $MgCl_2$, 1 mM glucose pH 7.5; S_2 contained the desired concentration of NaCl and 0.02 μ M Na,K-ATPase along with all constituents of S_1 (except ATP); and S_3 contained the desired concentration of KCl, 50 μ M o-cresol, 3 mM $MgCl_2$ and 1 mM glucose. The initial portion of the trace shows syringe movement and the signal starts from 90 ms. All traces are the average of two or three recordings.

Delayed mixing of Na^+ or K^+ did not significantly affect the H^+ release. Average time for attainment of peak alkalinity was 60 ms. Magnitude of H^+ absorption after 60 ms delayed mixing of K^+ was almost similar to K^+ 30 ms delay. As compared to Na^+ 30 ms delay experiments, no change in H^+ absorption was observed in 60 ms delayed addition of Na^+ . H^+ release was slightly higher when K^+ was mixed with a delay of 60 ms, whereas no significant change in H^+ release was observed when Na^+ was mixed with a delay of 60 ms.

Effects of 2-deoxy-D-glucose on H^+ absorption and release with delayed addition of Na^+ and K^+ concentrations

Table 4 gives a record of H^+ absorbed and released in the presence of 1 mM 2-deoxy-D-glucose. K^+ or Na^+ were added after a delay of 30 ms or 60 ms. The following results were obtained in the presence of 1 mM 2-deoxy-D-glucose: (1) K^+ (delay 30 ms), no change in H^+ absorption; (2) K^+ (delay 60 ms), no change in H^+ absorption; (3) Na^+ (delay 30 ms), no change in H^+ absorption; (4) Na^+ (delay 60 ms), small decrease in H^+ absorption (indicating that a small population of enzyme

Table 3 Moles of H^+ absorbed and released per mole of Na, K-ATPase in the presence of varying concentrations of Na^+ and K^+ following mixing of enzyme with 1.5 mM ATP in the presence of 1 mM glucose

Na^+ (mM)	K^+ (mM)	H^+ absorbed	H^+ released
120	30	0.52	0.35
120	60	0.53	0.31
120	90	0.54	0.32
120	120	0.53	0.34
NA	Average	0.53	0.33
	(control)	(0.52)	(0.97)
K^+ (mM)	Na^+ (mM)	H^+ absorbed	H^+ released
	(30 ms delay)		
120	30	0.63	0.26
120	60	0.61	0.21
120	90	0.64	0.25
120	120	0.61	0.23
NA	Average	0.62	0.24
Na^+ (mM)	K^+ (mM)	H^+ absorbed	H^+ released
	(60 ms delay)		
120	30	0.61	0.31
120	60	0.63	0.33
120	90	0.65	0.25
120	120	0.52	0.42
NA	Average	0.52	0.41
	(control)	(0.07)	(0.92)
K^+ (mM)	Na^+ (mM)	H^+ absorbed	H^+ released
	(60 ms delay)		
120	30	0.61	0.32
120	60	0.63	0.21
120	90	0.65	0.33
120	120	0.60	0.32
	Average	0.62	0.32

Na^+ and K^+ were added after a delay of 30 ms and 60 ms. Values in parentheses are the averages for the control carried out in the absence of glucose. NA means non-available.

molecules start giving signal); (5) K^+ (delay 30 ms), no change in H^+ release; (6) K^+ (delay 60 ms), small decrease in H^+ release; (7) Na^+ (delay 30 ms), small decrease in H^+ release; and (8) Na^+ (delay 60 ms), small decrease in H^+ release. It appears that these cations can independently affect the two legs of the ATP hydrolytic cycle. The structural distortions produced by glucose and 2-deoxy-D-glucose do not appear to be exactly the same, although the net effect of the two on the pre-steady state ATP hydrolysis is similar.

Table 4 Moles of H⁺ absorbed and released per mole of Na, K-ATPase in the presence of varying concentrations of Na⁺ and K⁺ following mixing of enzyme with 1.5 mM ATP in the presence of 1 mM 2-deoxy-D-glucose

Na ⁺ (mM)	K ⁺ (mM) (30 ms delay)	H ⁺ absorbed	H ⁺ released
120	30	0.61	0.31
120	60	0.63	0.33
120	90	0.65	0.25
NA	Average	0.53	0.30
Na ⁺ (mM)	K ⁺ (mM) (30 ms delay)	H ⁺ absorbed	H ⁺ released
120	60	0.63	0.22
120	90	0.64	0.20
120	120	0.58	0.20
NA	Average	0.62	0.20
Na ⁺ (mM)	K ⁺ (mM) (60 ms delay)	H ⁺ absorbed	H ⁺ released
120	30	0.61	0.20
120	60	0.63	0.21
120	90	0.58	0.21
120	120	0.60	0.22
NA	Average	0.60	0.21
Na ⁺ (mM)	K ⁺ (mM) (60 ms delay)	H ⁺ absorbed	H ⁺ released
120	30	0.51	0.21
120	60	0.52	0.23
120	90	0.53	0.24
120	120	0.58	0.21
NA	Average	0.52	0.22

Na⁺ and K⁺ were added after a delay of 30 ms and 60 ms. See Table 3 for control. NA means non-available.

Discussion

Results obtained can be discussed in terms of an extended kinetic scheme for Na,K-ATPase (Fig. 6). No proton release or absorption was observed after mixing of ADP with Na,K-ATPase under conditions similar to that of ATP mixing experiments at all tested concentrations of Na⁺ and K⁺. This implies that all H⁺ release/absorption signals obtained are because of ATP hydrolysis and not ATP binding. The H⁺ release might be coming from transphosphorylation of the active site Asp in the P-domain. Two new steps of H⁺ absorption (step 4) and H⁺ release (step 9) are proposed over the Albers-Post model. Our finding that glucose significantly decreases H⁺ absorption at high Na⁺ concentrations and also its ineffectiveness (or slightly positive

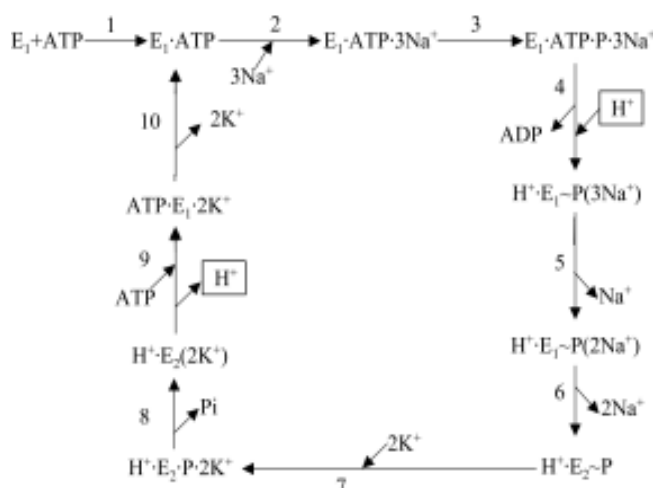


Fig. 6 Proposed kinetic scheme of ATP hydrolysis by Na,K-ATPase, showing steps 1–10

effect) can be understood in terms of glucose interacting with the enzyme leading to a conformational change. In this new state, E₁^{*}, the enzyme appears to be more sensitive towards both Na⁺ binding and its release. The E₁^{*}.ATP complex has a higher affinity for Na⁺, therefore promoting step 2. Release of Na⁺ (steps 5 and 6) is inhibited. In the presence of glucose at any given Na⁺ concentration, net absorption and release emanate from two counter-balancing tendencies of Na⁺ and release. Higher and faster Na⁺ binding, which is not offset by lower and slow exit of Na⁺, results in increased H⁺ absorption. At high Na⁺ concentration, there is promotion of step 2. It appears that inhibition of steps 5 and 6 is greater, compared to promotion of step 2, leading to an observed decrease in H⁺ absorption under these experimental conditions. Increased H⁺ absorption, compared to the control, at low Na⁺ concentration, can be explained as a result of greater promotion of step 2 compared to inhibition of steps 5 and 6. Variation with respect to K⁺, in the presence of glucose, could be due to a higher rate constant of the steps involving K⁺ binding and release (step 7 onwards) compared to Na⁺ binding and release steps. Decrease in H⁺ release at all Na⁺ concentrations, in the presence of glucose, could be understood in terms of inhibition of the steps of Na⁺ release.

Almost identical effects on H⁺ absorption and release observed in the presence of 2-deoxy-D-glucose at all tested Na⁺ and K⁺ concentrations indicate that the mode of effect of this analog is the same as that of glucose. Decrease in H⁺ absorption and comparatively greater decrease in H⁺ release in the presence of glucose and its analog indicate that less of the enzyme population reaches the E~P(3Na⁺)

complex and still less of the population is able to eject Na^+ and bind K^+ . Under steady state conditions, this would translate into meaning that the enzyme's activity would be negatively affected in the presence of glucose. These results are in consonance with the overall effect of glucose on $\text{Na}_2\text{K-ATPase}$. Exposure of enzyme and glucose/2-deoxy-*D*-glucose in the syringe was less than 5 min in the absence of ATP. In the presence of ATP, this exposure was less than 1 s. Three classes of amino groups known to be glycosylated [18] have $t_{1/2}$ of 9 min, 53 min and 9 h. The effects of glucose observed in this study can not be attributed to the non-enzymatic glycosylation of the amino groups shown by Garner *et al.* [18], who observed that $t_{1/2}$ of the glucose effect was below 9 min. Our results, however, do permit us to identify the groups with which glucose reacts.

Results of Na^+ delay experiments in the presence of glucose or 2-deoxy-*D*-glucose were broadly similar to control delay results, which indicates that enzymes do not start the ATP hydrolytic cycle in the absence of Na^+ . Results of K^+ delay experiments in the presence of glucose and 2-deoxy-*D*-glucose are in contrast with control delay experiments. It is thus suggested that the enzyme, even when mixed with ATP and Na^+ , does not start the ATP hydrolytic cycle and waits for K^+ . It was expected that at least part of the cycle would be operational. It appears that in the presence of glucose and 2-deoxy-*D*-glucose, K^+ plays a very significant role in the transformation at step 4 of the kinetic scheme, that is, $\text{E}_1\cdot\text{ADP}\cdot\text{P}(3\text{Na}^+)$ to $\text{H}^+\cdot\text{E}_1\sim(3\text{Na}^+)$. Furthermore, this transformation must have a low requirement of K^+ as no effect of K^+ is observed when its concentration is varied from 30 mM to 120 mM. Small variations in magnitude of H^+ release between glucose and 2-deox-*D*-glucose could be attributed to slight differences in structural distortion following their binding, which would result in minor alterations in steps 2–6 of the suggested kinetic scheme for $\text{Na}_2\text{K-ATPase}$.

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