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Mutagenesis of glutamate 820 of the gastric H^+,K^+-ATPase α-subunit to aspartate decreases the apparent ATP affinity

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Abstract

Mutagenesis of Glu^{820}, present in the catalytic subunit of gastric H^+,K^+-ATPase, into an Asp hardly affects K^+-stimulated ATPase and K^+-stimulated dephosphorylation of the enzyme. The ATP phosphorylation rate of the E820D mutant, however, is rather low and the apparent affinity for ATP in the phosphorylation process of this mutant is 2–3 times lower than that of the wild type enzyme. The reduction in the ATP phosphorylation rate of the E820D mutant has only an effect on the ATPase activity at low temperature. These findings suggest that Glu^{820} might play a role in H^+ stimulation of the phosphorylation process. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Gastric H^+,K^+-ATPase; Mutagenesis; Glutamate 820; Baculovirus expression system; E820D

1. Introduction

Gastric H^+,K^+-ATPase belongs to the subfamily of P_2-type ATPases which also includes Na^+,K^+-ATPase and Ca^{2+}-ATPases [1,2]. The H^+,K^+-ATPase, involved in gastric acid secretion, exchanges K^+ ions from the lumenal side of the membrane against H^+ ions originating from the cytosol of the parietal cell. The energy required to perform this cation translocation is provided by ATP hydrolysis. During the catalytic cycle the enzyme is phosphorylated on a conserved aspartyl residue present in the large intracellular loop of the α-subunit. The mechanism involved in coupling of cation transport to (de)phosphorylation is not explained on a molecular scale. The Post-Albers scheme (Fig. 1) provides a framework to study the reaction mechanism of these kinds of enzymes [3].

Within the P_2-type ATPases it is accepted that negatively charged amino acids located in the transmembrane domains are involved in the binding and transport of cations [4–6]. Site directed mutagenesis has revealed that glutamate 820, present in the catalytic subunit, plays a crucial role in the K^+ activation of gastric H^+,K^+-ATPase [4–7].

In the latter studies an E820D mutant was expressed in Sf9 cells using baculoviruses encoding the gastric H^+,K^+-ATPase. The E820D mutant was still able to form a phosphorylated intermediate, and showed like the wild type enzyme a K^+ activation of

Abbreviations: SERCA, sarcoplasmic and endoplasmic reticulum; E-P, phosphorylated intermediate; SCH 28080, 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)imidazo[1,2a]pyridine; E_{act}, activation energy

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the ATPase activity, as well as a K^+-stimulated dephosphorylation [5,7]. However, the maximal phosphorylation level of this mutant after 10 s at pH 6.0 and 0°C, was only 29% of that of the wild type enzyme. When the ATP phosphorylation was performed at 21°C the phosphorylation level increased to 80% of that of the wild type enzyme [7].

In the present paper we study the reason for the low phosphorylation level of the E820D mutant at 0°C. It is demonstrated that this mutant has a decreased apparent ATP affinity, which depends on the pH. This suggests that glutamate 820 is not only involved in K^+ binding but might also play a role in H^+ binding during the phosphorylation process.

2. Materials and methods

2.1. Mutagenesis and protein expression

All DNA manipulations were carried out according to standard molecular biology techniques described by Sambrook et al. [8]. The E820D mutant was constructed with aid of the Unique Site Elimination (Pharmacia Biotech) procedure developed by Deng and Nickoloff [9] as described before [5]. Sf9 cells were grown at 27°C in 100 ml spinner flask cultures as described by Klaassen et al. [10]. For production of H^+,K^+-ATPase the cells were infected at a multiplicity of infection of 1–3 in the presence of 1% (v/v) ethanol [11] and incubated for 3 days using Xpress medium (Biowhittaker, Walkersville, MD) containing 0.1% (v/v) pluronic F-68 (Sigma Bornem, Belgium) as described by Swarts et al. [7]. Sf9 cells were harvested and membranes were isolated as described before [7]. Protein concentrations were determined with the modified Lowry method described by Peterson [12] using bovine serum albumin as a standard.

2.2. ATP phosphorylation and K^+-ATPase activity

ATP phosphorylation was determined as described before [5,7,11]. Different phosphorylation conditions were used with respect to temperature (0 or 21°C), pH (6.0 or 7.0) phosphorylation time (0–30 s), and ATP concentration. The SCH 28080 sensitive K^+-activated ATPase activity was determined with a radiochemical method [13] as described by Swarts et al. [7].

2.3. Chemicals

[γ-^{32}P]ATP (3000 Ci mmol^{-1}, Amersham, Buckinghamshire, UK) was diluted with non-radioactive Tris-ATP (pH 6.0 or 7.0) to a specific radioactivity of 20–100 Ci mmol^{-1}. SCH 28080, kindly provided by Dr. A. Barnett, Schering-Plough, Kenilworth, NJ, was dissolved in ethanol and diluted to its final concentration of 0.1 mM in 0.2% (v/v) ethanol.

3. Results

Expression of the E820D mutant and the wild type gastric H^+,K^+-ATPase in Sf9 cells revealed a significant lower ATP phosphorylation level of the E820D mutant as compared to that of the wild type [7]. The difference in phosphorylation level between mutant and wild type enzyme could not be explained by a lower expression level of the mutant [5]. In this report we focus on the differences between the wild type enzyme and the E820D mutant in order to obtain additional information about the role of Glu^{820}.

3.1. ATP phosphorylation rates and levels

Fig. 2 shows the results of ATP phosphorylation

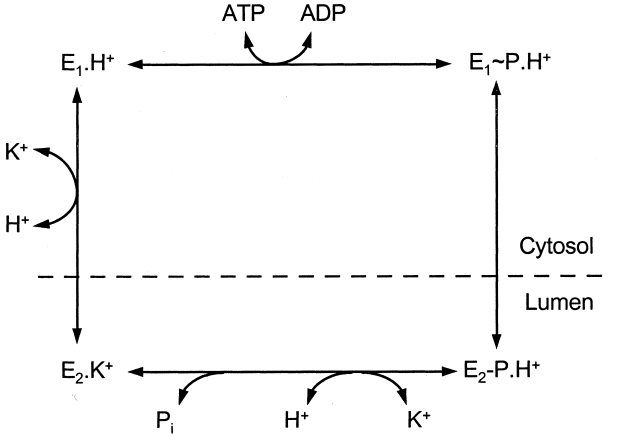

Fig. 1. Post-Albers scheme, describing the reaction cycle of the gastric H^+,K^+-ATPase.

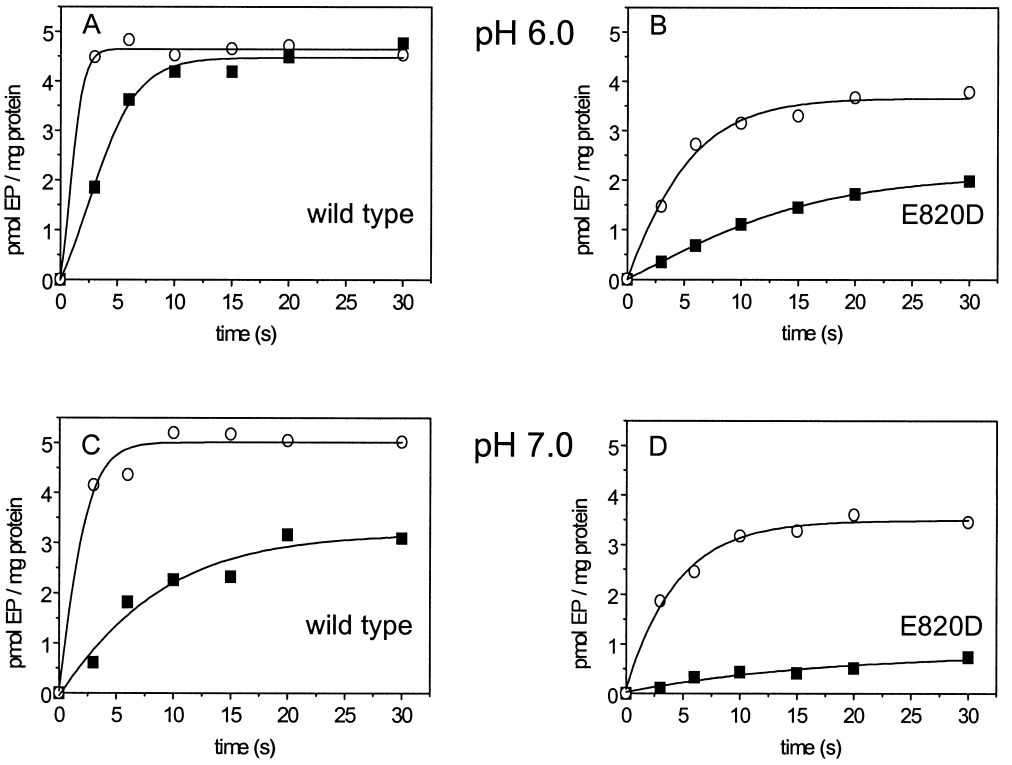

Fig. 2. Time dependence of ATP phosphorylation of H^+,K^+-ATPase and its mutant E820D. Sf9 membranes (1 μg) were preincubated at 21 (open circles) or 0°C (closed squares) with 0.2 mM EDTA, 1.2 mM $MgCl_2$ and 50 mM Tris-acetic acid (pH 6.0 or 7.0) with and without 100 μM SCH 28080. After phosphorylation with 0.1 μM [γ^{32}-P]ATP at the indicated temperature, pH and time, the SCH 28080 sensitive phosphorylation level (EP) was determined and expressed as pmol mg^{-1} protein and plotted as function of time (0–30 s). (A) and (C) represent phosphorylation of the wild type at pH 6.0 and 7.0, respectively, whereas (B) and (D) represent phosphorylation of the E820D mutant also at pH 6.0 and 7.0, respectively. Representative for three experiments.

experiments carried out with both the wild type enzyme and the E820D mutant at two pH values (6.0 and 7.0) and two temperatures (0 and 21°C). It is clear that under the standard conditions, previously used [4,10] (10 s, pH 6.0 and 0°C), the steady-state phosphorylation level was reached for the wild type enzyme but not for the E820D mutant. Even after 30 s no maximal steady-state phosphorylation level was reached at 0°C. Moreover, Fig. 2 shows that both the phosphorylation rate and the obtained phosphorylation level were lower for the E820D mutant than for the wild type enzyme. An increase of the pH resulted in a decrease of the phosphorylation rate, in particular at 0°C. Raising the temperature from 0 to 21°C increased the phosphorylation rate both at pH 6.0 and 7.0. At 21°C a steady-state level was reached after 10 s at both pH values. The latter conditions were chosen to measure the effect of mutation on ATP affinity.

3.2. ATP affinity

Scatchard plots for the ATP dependence of the 10 s phosphorylation level of the wild type enzyme and the mutant E820D measured at 21°C and pH 6.0 or 7.0 are shown in Fig. 3. The maximal phosphorylation levels correspond with those shown in Fig. 2 (3.5–4.3 pmol mg^{-1} protein). The maximal phosphorylation levels were independent of the pH. The apparent ATP affinity of the wild type enzyme was 9 ± 2 and 20 ± 2 nM at pH 6.0 and 7.0, respectively, compared to 22 ± 5 and 56 ± 14 nM of the E820D mutant. The apparent ATP affinity of the E820D mutant was thus about 2–3 times reduced at both pH values. Comparison of the ATP affinities at pH 6.0 and 7.0, indicates that a 10 times decrease in the H^+ ion concentration resulted in a 2–3 times decrease in ATP affinity of both wild type and E820D mutant.

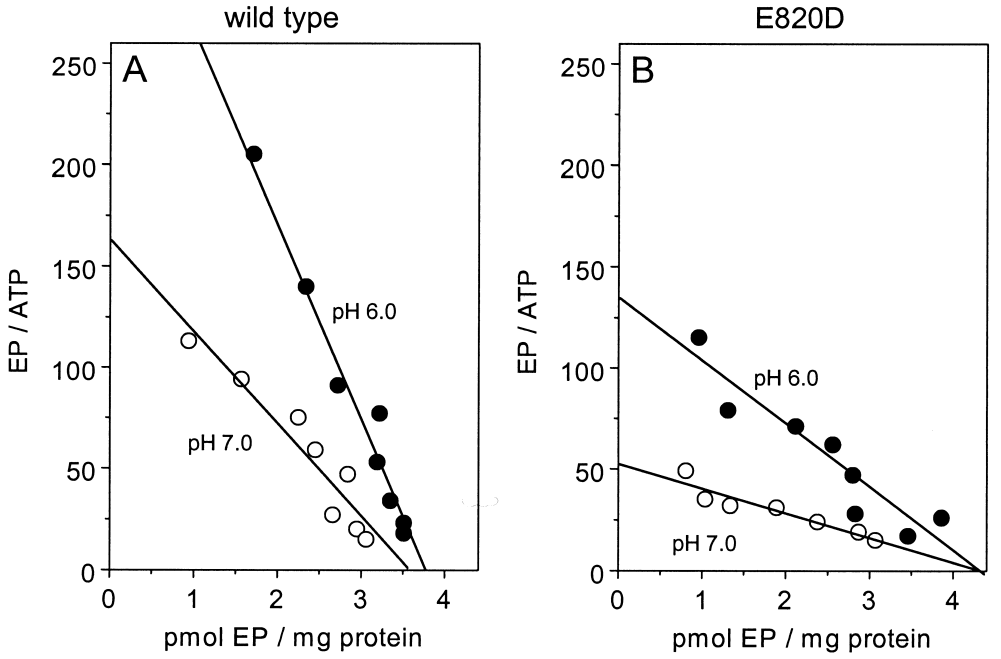

Fig. 3. Scatchard plot of the ATP dependence of the wild type and the E820D mutant. A 10 s phosphorylation was performed, with 1 μg protein, in the presence of 0.2 mM EDTA, 1.2 mM $MgCl_2$ and 50 mM Tris-acetic acid (pH 6.0 closed circles or pH 7.0 open circles) at 21°C, for both the wild type enzyme (A) and the E820D mutant (B). After phosphorylation with 8–200 nM [γ^{32}-P]ATP the SCH 28080 sensitive phosphorylation level (EP) was determined and expressed as pmol mg^{-1} protein, plotted on the *x*-axis, whereas on the *y*-axis the ratio EP over free ATP is plotted. Representative for three experiments.

3.3. *Effects on K^+-ATPase activity*

We showed a different temperature effect on the phosphorylation rate and steady-state phosphorylation level of the mutant compared to the wild type. However, only small effects on K^+-stimulated ATPase activity, determined at 37°C, were observed [5]. At 37°C the maximal ATPase activity of the mutant is about 78% of that of the wild type activity (Fig. 4). Lowering the temperature leads to a stepwise decrease of the K^+-ATPase activity, but more for the E820D mutant than for the wild type enzyme. This results in an ATPase activity of the E820D mutant which is only 36% of that of the wild type when measured at 0°C. Apparently, a different step in the ATPase cycle determines the velocity of the wild type and the mutant E820D.

These data were used to calculate the activation energy (E_{act}) of the total catalytic cycle, which of course is not necessarily identical to the activation energy for a particular reaction. From the Arrhenius plot equation the activation energy was calculated and plotted against the KCl concentration as shown in Fig. 5. This figure shows, both for the wild type enzyme and the E820D mutant, that the calculated activation energy was independent of the K^+ concentration, if higher than 0.1 mM. In addition, Fig. 5 shows that the activation energy for the E820D mutant ($\sim$77 kJ mol^{-1}) is higher than that of the wild type enzyme ($\sim$63 kJ mol^{-1}).

4. Discussion

In the present study we show that the mutant E820D had a lower phosphorylation rate and steady state phosphorylation level compared to that of the wild type enzyme, at 0°C and pH 6.0. However, at 21°C the phosphorylation rate increased and the maximal phosphorylation levels were already obtained after 10 s. Furthermore, we noticed that increasing the pH to 7.0 also reduced the phosphorylation rate and steady state phosphorylation level of the wild type enzyme at 0°C. This might suggest that the lower phosphorylation rate and level of the E820D mutant was due to a reduction in H^+ affinity.

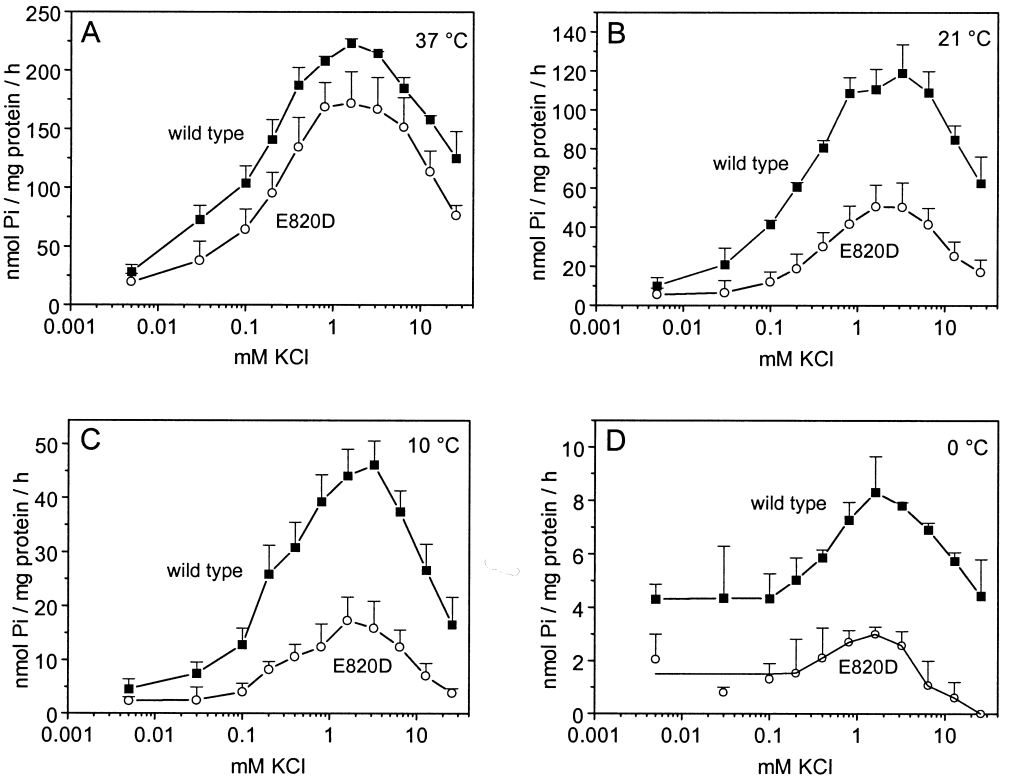

Fig. 4. Effect of the temperature on the K^+-stimulated ATPase activity in Sf9 membranes infected with recombinant baculoviruses expressing the wild type (closed squares) or the E820D mutant (open circles) H^+,K^+-ATPase. The assay was performed at 37 (A), 21 (B), 10 (C) and 0°C (D). The SCH 28080 sensitive part of the ATPase activity, in the presence of the indicated K^+ concentration, 10 μM ATP, 1.2 mM $MgCl_2$ and 50 mM Tris-acetic acid pH 7.0 was measured. Mean values for three experiments with S.E.M. are presented.

It remains difficult to pinpoint the changed phosphorylation characteristics of the mutant entirely on changed binding properties of H^+ ions. We demonstrated that a 10 times higher H^+ concentration resulted in a 2–3 times increased apparent ATP affinity. At both pH 6.0 and 7.0 the apparent ATP affinity of the E820D mutant was 2–3 times lower than of the wild type enzyme. The apparent ATP affinity of the wild type enzyme at pH 7.0 was comparable with the affinity of the E820D mutant at pH 6.0.

We show that the activation energy for the E820D mutant is higher than that of the wild type enzyme. This means that at least one of the partial reactions is influenced in the mutant resulting in an enzyme which needs more energy to become activated. Moreover, the K^+-stimulated ATPase activity of the E820D mutant at 37°C is similar to that of wild type. However, the K^+-induced dephosphorylation of the mutant is similar to that of the wild type enzyme [5,7]. It is therefore likely that the increased activation energy of the mutant might be due to the slower phosphorylation rate of the E820D mutant.

The above resulted in the hypothesis that mutagenesis of Glu^{820} to an Asp decreases both the apparent H^+ affinity as well as the ATP affinity resulting in a higher activation energy of this E820D mutant. Most workers locate this Glu^{820} [5] or its counterpart in other P-type ATPases, Asp^{804} (Na^+,K^+-ATPase) [14], Asn^{796} (SERCA1a Ca^{2+}-ATPase) [15] and Asn^{879} (plasma membrane Ca^{2+}-ATPase) [16], in the sixth transmembrane domain of the catalytic subunit. On the other hand, the ATP binding site in these P-type ATPases is located in the large intracellular loop between transmembrane domains 4 and 5 [17–20]. Pedersen et al. [21] showed that for the opposite D804E mutant of Na^+,K^+-ATPase, [^{3}H]ATP binding was similar as compared to the

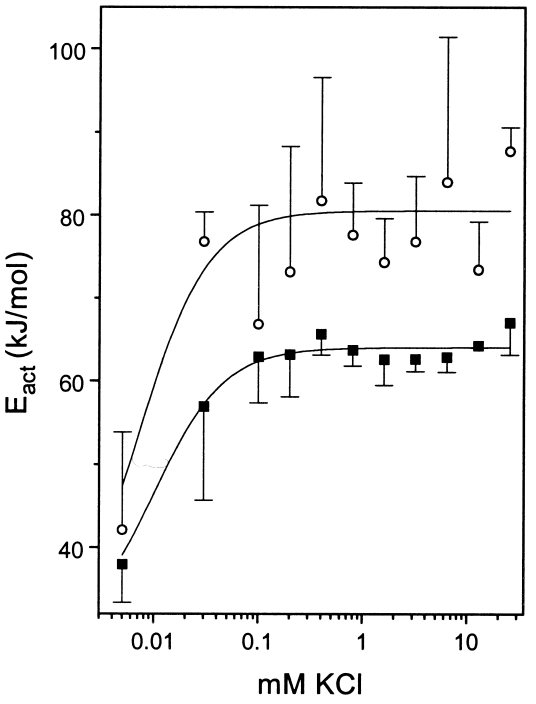

Fig. 5. Activation energy (E_{act}), calculated from the Arrhenius plot equation, as a function of the KCl concentration. The data presented in Fig. 4 were used to calculate the activation energy of the E820D mutant (open circles) and the wild type H^+,K^+-ATPase (closed squares). Mean values for three experiments with S.E.M. are presented.

wild type Na^+,K^+-ATPase, but that K^+-induced displacement of [^{3}H]ATP did not occur with the mutant. In conclusion, they stated that this mutant did not directly affect the ATP binding site. Expression of the H4H5 cytoplasmic loop of Na^+,K^+-ATPase in *Escherichia coli* had no effect on ATP selectivity compared with a fully expressed wild type enzyme [22,23]. This indicates that it is unlikely that mutagenesis of the 820 residue in the gastric H^+,K^+-ATPase affects ATP binding in our experiments directly. When we consider the Post-Albers scheme in Fig. 1, it is clear that the ratio between H^+ and K^+ ions determines the equilibrium between the E_1 and E_2 conformation of the enzyme. The results obtained above led to the hypothesis that a decreased H^+ affinity might result in a shift of the conformation to the E_2 form, which could explain the lower apparent ATP affinity as well as the increased activation energy.

In the Post-Albers scheme for P-type ATPases outward H^+ transport is coupled to the E_1-P$\leftrightarrow E_2$-P conversion and inward K^+ transport to the $E_2 \leftrightarrow E_1$ step. The equivalent residue for Glu^{820} in both SERCA- and PM-Ca^{2+}-ATPase are likely to be involved in Ca^{2+}-ATPase binding and transport [19,24–26]. In Na^+,K^+-ATPase D804 fulfils a role in both Na^+ and K^+ dependent steps [14,21,27]. This suggests that the same binding pocket, although in a different conformation, might be involved in both outward and inward transport. For H^+,K^+-ATPase we previously presented evidence for a role of Glu^{820} in K^+-dependent processes [5,7]. The present study suggests that this residue might also be involved in H^+ binding and transport.

If mutagenesis of the Glu^{820} into an Asp indeed affects H^+ affinity, why has this mutation no effect on the K^+-ATPase activity? At 37°C the phosphorylation reaction is apparently not rate limiting. Moreover, we show that upon decreasing the temperature of the ATPase reaction, the activity of the E820D mutant decreases more than that of the wild type, suggesting that at lower temperature the phosphorylation reaction rate plays a significant role in the total reaction rate.

To summarize our hypothesis: mutagenesis of Glu^{820} to Asp leads to a decreased apparent ATP affinity, which might be caused by a decreased apparent H^+ affinity. These changes mainly affect phosphorylation kinetics, which indicates that Glu^{820} is not only involved in K^+ binding, but might also play a role in H^+ binding.

Acknowledgements

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