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Free protons do not substitute for sodium ions in buffer-mediated phosphorylation of $(Na^+ + K^+)$-ATPase *

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(1) In view of our recent finding of imidazole-activation of the phosphorylation of $(Na^+ + K^+)$-ATPase and the suggestion by others of an activating role of protons, in lieu of sodium ions, in the overall hydrolytic and phosphorylation processes of the enzyme, we have investigated the effect of pH on the phosphorylation process. (2) No indication of proton activation is found. Rather, phosphorylation at low pH in the absence of Na^+ is dependent on the buffer concentration. Imidazole-H^+ stimulated phosphorylation at pH 5 reaches the same maximal steady-state level as Na^+-stimulated phosphorylation. (3) Low pH also elicits Tris-H^+ stimulated phosphorylation, but due to a simultaneous inhibitory effect of this buffer the maximal steady-state level is no more than 50% of the Na^+-stimulated phosphorylation level. (4) Protons inhibit rather than activate phosphorylation. Upon decreasing the pH from 7 to 5, we observe for all ligands, whether activating or inhibiting phosphorylation (ATP, Na^+, protonated imidazole, Mg^{2+} and K^+), a decrease in affinity (largest for Mg^{2+}) and a decrease in the maximal steady-state phosphorylation capacity. (5) The effects of Na^+ and imidazole-H^+ on the phosphorylation step have been compared with those on the $E_2 \rightarrow E_1$ conformational change, which leads to the phosphorylation step. The different pH-dependence of the affinities for Na^+ and protonated buffer in the $E_2 \rightarrow E_1$ transition suggests that there are separate activation sites with different pK values for Na^+ and the buffer cation. (6) The above findings rule out a role of free protons as a substitution for Na^+ in the phosphorylation process.

Introduction

Recently we have detected ATP-dependent phosphorylation of $(Na^+ + K^+)$-ATPase in imidazole-buffered medium in the absence of added Na^+, indicating that imidazole-H^+ can replace Na^+ in the phosphorylation process [1]. Low (0.1 mM) Mg^{2+} concentrations stimulate this buffer-activated phosphorylation, while higher Mg^{2+} concentrations antagonize the imidazole effect. On the other hand, Tris (pH 6–8) hardly stimulates phosphorylation, and inhibits the imidazole-stimulated activity. We have attributed this to the induction of a non-phosphorylating E_1 conformation by Tris and of a phosphorylating E_1 conformation by imidazole.

Earlier, Fujita et al. [2] demonstrated for the pig brain enzyme a relatively high phosphorylation level at pH 5 in Tris-acetate buffer in the absence of added Na^+ (5 mM Mg^{2+}), which is not stimulated by Na^+. This Na^+-free phosphorylation sharply decreases above and below pH 5. They interpreted their results as a proton-activated

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Abbreviation: CDTA, *trans*-1,2-diaminocyclohexane tetra-acetic acid.

phosphorylation with protons substituting for Na^+. Their data have recently been confirmed for a highly purified kidney enzyme [3].

In the present study we have investigated whether the phosphorylation is buffer- or proton-activated or both. Our results are discussed in the light of recent evidence for H^+-transport by the enzyme in the absence of Na^+ [4,5]. If not otherwise specified, the term 'protons' in this paper refers to free protons in solution.

Materials and Methods

$(Na^+ + K^+)$-ATPase is purified from rabbit kidney outer medulla as described by Jørgensen [6], followed by removal of contaminating ATP, washing and storing of the preparation according to the procedure of Schoot et al. [7]. The storage medium contains 5 mM imidazole-HCl (pH 7.0), 0.25 M sucrose and no CDTA. Protein is determined by the method of Lowry et al., following trichloroacetic acid precipitation and using bovine serum albumin as standard [6].

Phosphorylation by ATP (20 μM), in the presence or absence of Na^+ in imidazole-acetate or Tris-acetate buffer at concentrations and pH values specified in the Results, is carried out at 22°C as previously described by us [8]. In order to avoid inactivation at low pH the enzyme and $MgCl_2$ (at specified final concentrations) are added last within a time span of about 12 s to start the 3-s phosphorylation period. The enzyme protein concentration is 0.03 mg/ml in the determination of the K_m for ATP (final concentrations 0.1-20 μM) and 0.1 mg/ml in all other assays with 20 μM ATP. ATP is converted to its imidazolyl- or Tris-salt by passage over a Dowex 50W-X4 exchange resin in the appropriate cation form [9].

Although steady-state phosphorylation levels are determined, which are the result of phosphorylation and dephosphorylation as well as of conformational transition steps in the reaction cycle, we define K_m or $K_{0.5}$ values as the activator concentration giving the half-maximal increase in the 3-s phosphorylation level, I_{50} as the inhibitor concentration reducing the level by 50% and K_i as the inhibitor concentration increasing the apparent K_m of the activator to double the intrinsic K_m.

The $E_2 \rightarrow E_1$ conformational transition is assayed at 22°C with the eosin fluorescence method developed by Skou and Esmann [10]. We monitor the fluorescence increase after addition of 1 vol. aqueous NaCl solution (2.5–100 mM) to 4 vols. basal assay medium (83 μg protein/ml, 2.5 μM eosin, 0.085 mM imidazole-acetate, pH 5.5–8). Alternatively, imidazole-acetate (25–500 mM, pH 5.5–8) is added to the same medium without Na^+. Fluorescence in the Na^+-free basal medium (with water instead of effector added) is taken as the basal E_2 level. The fluorescence increase induced by Na^+ and buffer (E_1 level) is corrected for the fluorescence level in the presence of a saturating (20 mM) K^+ concentration. Since 20 mM K^+ increases, probably by an ionic strength effect, the basal E_2 level by about 4–10%, we subtract this from the fluorescence levels obtained in the presence of 20 mM K^+ and increasing Na^+ or buffer concentrations.

Results

Activation and inhibition by Mg^{2+}

The acid pH optimum for phosphorylation in the absence of added Na^+ led Fujita et al. [2] and also Hara et al. [3] to the assumption of proton activation. We have repeated their experiments using the same high (5 mM) Mg^{2+} concentration. In order to exclude an effect of pH on the buffer cation concentration, which by itself influences the phosphorylation level, we have kept the imidazole-H^+ concentration in the imidazole-acetate medium (pH 4–8) constant (25 mM). The pH optimum under these conditions is 5 (Fig. 1), in agreement with Fujita et al. [2], while Hara et al. [3] reported a value of 5.6. However, when the Mg^{2+} concentration is reduced to 0.1 mM, which is saturating at pH 7 [1], the pH optimum shifts to 6.5 in agreement with the value previously reported by us [1]. The phosphorylation level at this optimum is higher than that at pH 5 at the high Mg^{2+} concentration, which argues against proton activation. In the presence of a saturating Na^+ concentration (100 mM) the phosphorylation levels are still higher (Fig. 1) and the optimum of Na^+ stimulation at pH 7.5 is not influenced by the Mg^{2+} concentration. On the other hand, high Mg^{2+} enhances Na^+-stimulation in the pH range of 4–6 and Na^+-free stimulation in the pH range

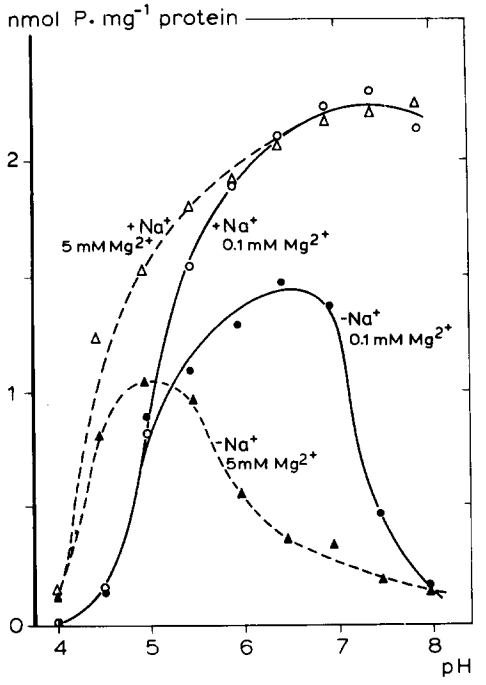

Fig. 1. Phosphorylation as a function of pH. Shown are 3-s phosphorylation levels in imidazole-acetate, containing a constant (25 mM) imidazole-H^+ concentration at 0.1 mM Mg^{2+} (●) and 5 mM Mg^{2+} (▲) in the absence of added Na^+ and in the presence of 100 mM Na^+ (○,△).

of 4–5. At pH values above 5 high Mg^{2+} inhibits Na^+-free stimulation (Fig. 1).

From the latter data it can be concluded that the pH optimum of 5 with 5 mM Mg^{2+} in the absence of Na^+ is due to an inhibition artifact at higher pH and is no indication of proton activation. We had already found this antagonism between millimolar Mg^{2+} and imidazole in the phosphorylation process at neutral pH [1]. This antagonism is reduced at low pH. On the other hand, the stimulation by 5 mM Mg^{2+} below pH 5 indicates that more Mg^{2+} is required for the activation process at low than at high pH. The latter effect of Mg^{2+} cannot be due to stabilization of the enzyme against inactivation [11], since inactivation at pH 5 is negligible during the 15-s assay. The peak value with 5 mM Mg^{2+} at pH 5 is lower than that with 0.1 mM Mg^{2+} at pH 6.5, because 25 mM imidazole-H^+ is not saturating at lower pH values. Actually, at saturating imidazole concentrations the same phosphorylation level is reached as in the case of Na^+ (see next section).

The lack of an effect of the Mg^{2+} concentration on the optimal Na^+-activated phosphorylation at pH 7.5 (Fig. 1) is due to the relatively low Mg^{2+}-Na^+ antagonism. The fact that the Mg^{2+}-Na^+ antagonism is less than that between imidazole-H^+ and Mg^{2+} explains why stimulation by 5 mM Mg^{2+} in the presence of Na^+ extends to pH 6 and in the absence of Na^+ only to pH 5. In the absence of Na^+ the antagonism overrules the activating effect of Mg^{2+} above pH 5.

Our conclusion that activation and inhibition by Mg^{2+} at pH 5 are weaker than at pH 7 is confirmed by Fig. 2, which shows activation and inhibition at pH 5 as a function of the Mg^{2+} concentration. In the absence of Na^+ the maximal phosphorylation level is at 1 mM Mg^{2+} (Fig. 2A), while the K_m value for Mg^{2+} is 40 μM (Fig. 2B). This stimulation is followed by a slight inhibition at higher Mg^{2+} concentrations (about 10% at 5 mM Mg^{2+}). At pH 7 maximal phosphorylation occurs at 0.03–0.1 mM Mg^{2+} (K_m for Mg^{2+} = 1 μM, Ref. 1), while at 5 mM Mg^{2+} phosphorylation is inhibited by 80% (Fig. 1).

In the presence of Na^+ maximal phosphorylation at pH 5 requires higher Mg^{2+} concentrations (2.5 mM, Fig. 2A) and the K_m value for Mg^{2+} (90 μM, Fig. 2B) is more than twice as high as in the absence of Na^+. This is the reverse of the situation at pH 7 [1]. Inhibition by Mg^{2+} appears to be absent at pH 5, which is reflected in the sharp increase in K_i for Mg^{2+} in Na^+-activated phosphorylation (from 2 to 16 mM) upon raising the proton concentration (Fig. 3). The increase in K_i for Mg^{2+} in imidazole-activated phosphorylation without Na^+ is even more pronounced, from 0.34 mM at pH 7 [1] to 7 mM at pH 5 (Fig. 5B). However, the latter values confirm that the greater sensitivity to Mg^{2+} inhibition of the Na^+-free phosphorylation as compared to Na^+-activated phosphorylation remains present in this pH range. Thus the affinity for Mg^{2+} in its activating and inhibitory effects at pH 5 is one to two orders of magnitude less than at pH 7. This is also true, but to a lesser degree, for activation by Na^+, imidazole-H^+ and ATP and for inhibition by K^+ (see below).

In conclusion, the pH optimum in Na^+-free buffer is controlled by the Mg^{2+} concentration as a result of the pH dependence of the K_m and K_i

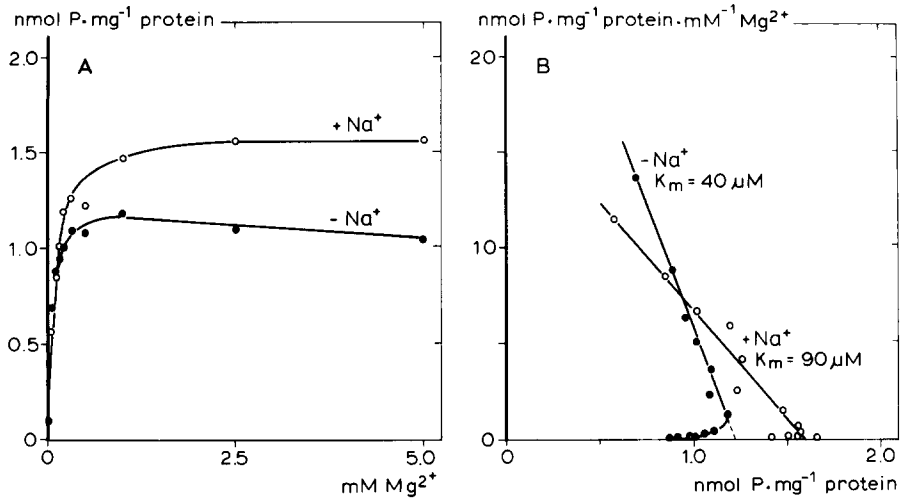

Fig. 2. Activation and inhibition by Mg^{2+}. Phosphorylation is carried out in 25 mM imidazole-acetate (pH 5) at 0–20 mM Mg^{2+} in the absence of added Na^+ (●) and the presence of 100 mM Na^+ (○). (A) Michaelis-Menten plots of the data for Mg^{2+} concentrations up to 5 mM, showing optimal phosphorylation in the absence of added Na^+ is at 1 mM Mg^{2+} and in the presence of Na^+ at 2.5 mM Mg^{2+}. (B) Eadie-Scatchard plots for the full range of Mg^{2+} concentrations, facilitating the determination of the K_m for Mg^{2+} activation from the linear segments.

values for Mg^{2+}, and it can be anywhere between pH 5 and 6.5. It is even likely that the actual pH optimum would coincide with that of Na^+-stimulated phosphorylation, were it not that Na^+-free phosphorylation is inhibited by the buffer itself in above optimal concentrations in the neutral pH range [1]. Hence, the pH optimum of 5 at 5 mM Mg^{2+} is not to be taken as an indication of proton activation. In order to investigate further the alleged involvement of protons in activation of phosphorylation, we have examined the effect of low pH on the kinetic parameters of the activation of phosphorylation by Na^+ and protonated buffers.

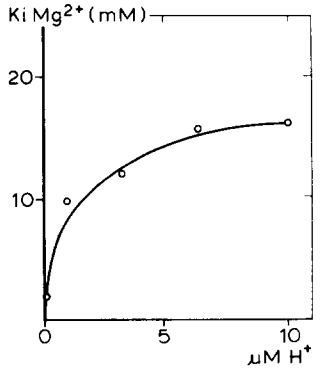

Fig. 3. K_i for Mg^{2+} inhibition of Na^+-activated phosphorylation as a function of the proton concentration. Phosphorylation is carried out in 50 mM Tris-acetate, pH 5.0, 5.2, 5.5, 6.0 and 7.0 ($[H^+] = 0.1$–10 μM) in the presence of 0.1 to 100 mM Na^+ and 2.5–10 mM Mg^{2+}. Na^+ activation is corrected for buffer-stimulated phosphorylation by subtracting the zero Na^+ phosphorylation levels. The K_i values for Mg^{2+} are determined by extrapolation of the linear Dixon plots of $Na^+_{0.5}$ vs. $[Mg^{2+}]$ from the abscissa intercepts.

Activation by Na^+ and protonated buffers

Another argument for believing in proton activation was that Fujita et al. [2] found no stimulation of phosphorylation by Na^+ at pH 5. We cannot confirm this finding when using an imidazole-acetate medium (Fig. 1). Since Fujita et al. used a Tris-acetate medium, we have investigated Na^+ stimulation as a function of pH in this medium. Tris buffer, in contrast to imidazole, gives low background phosphorylation levels down to pH 6, but below this pH the phosphorylation in the absence of Na^+ reaches high levels, up to 50% of the Na^+-activated phosphorylation at pH 5. This indicates that buffer-mediated phosphorylation does occur in Tris at low pH. From straight-line Dixon plots of $Na^+_{0.5}$ vs. the Mg^{2+} concentration (2.5–10 mM) at different pH values (pH 5-7), we have determined the $Na^+_{0.5}$ values at zero Mg^{2+}

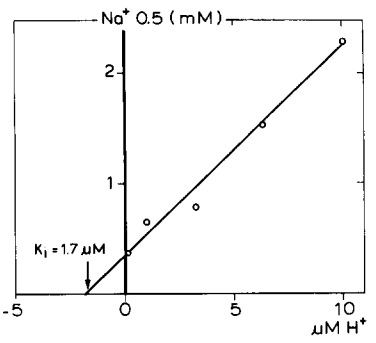

Fig. 4. K_m for Na^+ activation as a function of the proton concentration. Na^+ activation is determined as indicated in the legend of Fig. 3 and $Na^+_{0.5}$ values ($[Mg^{2+}] \rightarrow 0$) are determined by extrapolation from the ordinate intercepts of the linear Dixon plots of $Na^+_{0.5}$ vs. $[Mg^{2+}]$.

concentration (Fig. 4). In contrast to Fujita et al. [2], we find that in Tris the activation by Na^+ also continues down to pH 5. Protons appear to act as a competitive inhibitor towards Na^+ with a K_i value for protons of 1.7 μM.

Previously we have found a decrease in the K_m value for Na^+ at increasing imidazole concentration [1]. This effect, which can be called a synergism between buffer and Na^+ in the phosphorylation process, seems to decrease upon lowering the pH. This appears from Fig. 4, where we obtain a 6.6-fold increase in $Na^+_{0.5}$ (0.35 to 2.3 mM) upon lowering the pH from 7 to 5, in contrast to the 2.3-fold increase (1.9 to 4.3 mM) after extrapolation of the buffer and Mg^{2+} concentrations to zero (not shown).

The relatively high phosphorylation levels at pH 5 in the absence of added Na^+ suggest that both imidazole-H^+ and Tris-H^+ can replace Na^+ at this pH, whereas only imidazole can do this at neutral pH [1]. Eadie-Scatchard plots as in Fig. 5A of the steady-state phosphoenzyme level in relation to the imidazole concentration confirm this. They demonstrate that imidazole-activated phosphorylation at pH 5 (1–10 mM Mg^{2+}) reaches the same maximal level as the Na^+-activated phosphorylation. At pH 7 the maximal level is 30% less than that of the Na^+-activated phosphorylation, possibly due to inhibition by protonated buffer via binding at the Na^+-activation site. At pH 5 the affinity for this site is greatly reduced due to the decreased synergism between the buffer activation and the Na^+-activation site noted in the preceding paragraph. The Dixon plot of imidazole$_{0.5}$ values vs. the Mg^{2+} concentration reveals a K_m of 13 mM (12.8 mM for imidazole-H^+) and a K_i for Mg^{2+} of 7.2 mM (Fig. 5B), while at pH 7 the K_m for imidazole-H^+ is 5.9 mM and the K_i for Mg^{2+} 0.34 mM [1]. Thus the activation by imidazole-H^+ is about equally susceptible to a decrease in pH from 7 to 5 as the activation by Na^+ (ratios of

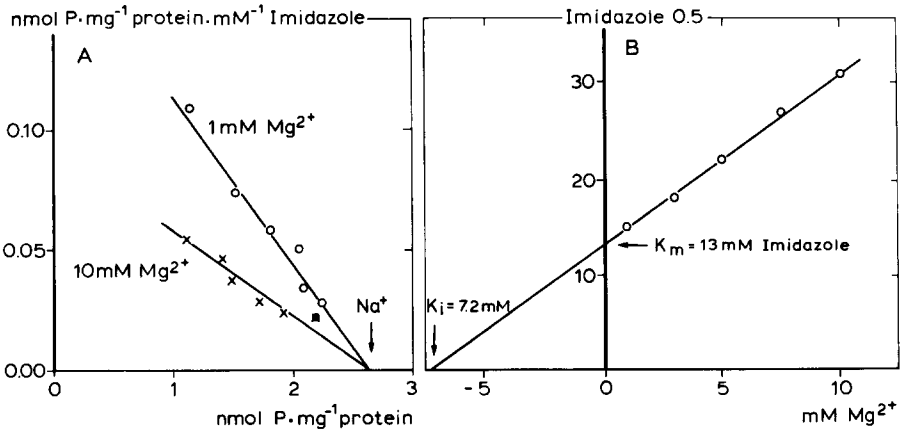

Fig. 5. Determination of the K_m value for imidazole-H^+. Phosphorylation is carried out in 5-100 mM imidazole-acetate (pH 5) in the presence of 1–10 mM Mg^{2+}. (A) Eadie-Scatchard plots of the data at the two extreme Mg^{2+} concentrations. The Na^+ reference is the phosphorylation level in 10 mM imidazole in the presence of 100 mM Na^+. (B) Dixon plot of the half-maximally activating imidazole concentration as a function of the Mg^{2+} concentration, yielding a K_m ($[Mg^{2+}] \rightarrow 0$) for imidazole of 13 mM and a K_i for Mg^{2+} of 7.2 mM.

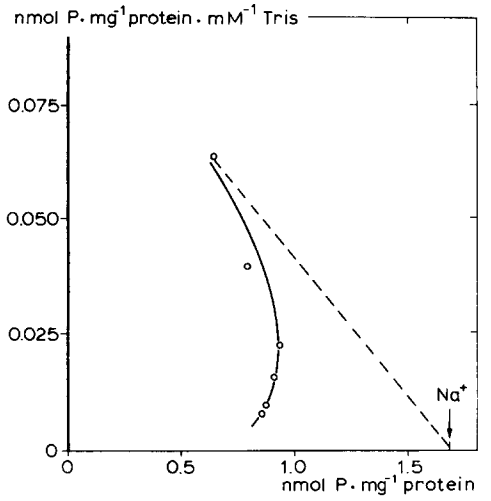

Fig. 6. Activation of phosphorylation by Tris buffer at pH 5. Phosphorylation is carried out in 10–100 mM Tris-acetate (pH 5) in the presence of 2.5 mM Mg^{2+}. Shown is an Eadie-Scatchard plot of the data with reference to the phosphorylation level given by 100 mM Na^+ in 40 mM Tris. The dashed line is based on the hypothesis of no inhibition by Tris > 10 mM. Its slope indicates a K_m value of 17 mM, equal to the K_m for imidazole at 2.5 mM Mg^{2+} (Fig. 5B).

intrinsic K_m values at pH 5 over pH 7 are 12.8/5.9 = 2.2 vs. 4.3/1.9 = 2.3). However, under those circumstances the imidazole-H^+ stimulated process becomes more than twice as sensitive to Mg^{2+} activation (90/40 = 2.3; Fig. 2B) and is less sensitive to Mg^{2+} inhibition than the Na^+-activated process (ratio of K_i values for Mg^{2+} at pH 5 over pH 7 is 21/8 = 2.6). In other words, lowering the pH narrows the specificity gap between the activators of phosphorylation.

This conclusion also extends to Tris. At pH 7 Tris predominantly inhibits phosphorylation [1]. At pH 5 it exerts a concentration-dependent activation as well as an inhibition, as demonstrated by the non-linear Eadie-Scatchard plot (Fig. 6). In the absence of inhibition (dashed line in Fig. 6), Tris would be equally effective as imidazole in the stimulation of phosphorylation (K_m values for both Tris and imidazole 17 mM at 2.5 mM Mg^{2+}, Fig. 5B). The inhibitory effect of Tris is unlikely to be due to a contaminant in this buffer, since after recrystallization from methanol the same effect is seen.

Thus, protons appear to inhibit activation (and inhibition) by the effectors of phosphorylation (Na^+, Mg^{2+}, buffer cations) in a competitive fashion, leading to increased K_m and K_i values. However, they do not appear to substitute for these effectors, since the phosphorylation levels are primarily dependent on the effector concentration.

The effect of pH on substrate affinity and K^+ sensitivity

It is essential that under the assay conditions at pH 5 the applied ATP concentration (20 μM) is saturating for the phosphorylation. Hence, we have determined from Eadie-Scatchard plots the K_m for ATP at low (25 mM) and high (100 mM) im-

TABLE I

KINETIC PARAMETERS FOR ACTIVATORS AND INHIBITORS OF ATP-DEPENDENT PHOSPHORYLATION

Data at pH 5 summarize the results from the present publication, those at pH 7 are from Ref. 1 and have been determined in similar fashion. The parameters for Na^+ activation ($K_m(Na^+)$) and inhibition of this activation by mmolar Mg^{2+} ($K_i(Mg^{2+})$, $+Na^+$ column) are given for assays in 50 mM Tris because of low background phosphorylation in this medium at pH 7. At this pH the $K_m(Na^+)$ ($[Mg^{2+}] \rightarrow 0$) is only slightly higher than that in 50 mM imidazole (0.35 vs. 0.24 mM) and $K_i(Mg^{2+})$ is the same (2 mM, Ref. 1). All other parameters are for assays in imidazole medium. Values between parentheses indicate intrinsic $K_m(Na^+)$ values, determined by extrapolation to zero buffer concentration and correction for competitive inhibition by Mg^{2+}.

Parameter		pH 7		pH 5	
		$-Na^+$	$+Na^+$	$-Na^+$	$+Na^+$
K_m(imidazole-H^+)	(mM)	5.9	–	12.8	–
K_m (Na^+)	(mM)	–	0.35 (1.9)	–	2.3 (4.3)
K_m (Mg^{2+})	(μM)	1	<1	40	90
K_i (Mg^{2+})	(mM)	0.34	2	7.2	16.2
I_{50} (K^+)	(μM)	12.5	180	45	151
K_m (ATP)	(μM)	0.09	0.06	0.35	0.15

idazole concentrations as well as at low (0.2 mM) and high (2 mM) Mg^{2+} concentrations in the absence and presence of Na^+ (100 mM). Since we find in all cases a K_m value below 0.4 μM at pH 5, the 20 μM ATP concentration must be saturating at this low pH. Only Na^+ has a significant effect at pH 5: it decreases the K_m for ATP from 0.3–0.4 μM to 0.1–0.2 μM, whereas at pH 7 we obtain values of 0.09 μM (Na^+ absent) and 0.06 μM (Na^+ present; Table I). This is another example of the synergism, which we have previously noted to exist between the actions of Na^+ and the substrate effects on phosphorylation [12].

In view of the extreme sensitivity of the imidazole-activated phosphorylation to K^+ at pH 7, we have also studied the effect of K^+ at pH 5. At pH 7 (25 mM imidazole-H^+, 0.1 mM Mg^{2+}, 20 μM ATP) half-maximal inhibition occurs in the absence of Na^+ at 12.5 μM K^+ and in the presence of 100 mM Na^+ at 180 μM K^+ (Table I). Lowering the pH to 5 under otherwise identical conditions decreases the sensitivity to K^+ in the absence of Na^+ ($I_{50} = 45$ μM). However, in the presence of Na^+, it increases the K^+ sensitivity ($I_{50} = 150$ μM, Fig. 7). This may be related to the greater reduction in the affinity for Na^+ in buffered medium at pH 5 than in the affinity for the buffer cation (Table I). Using the relation $I_{50} = (1 + (S/K_m))K_i$, we arrive at a K_i value for K^+ of 15 μM at pH 5 (no Na^+) that is 20-times higher than at pH 7 ($K_i = 0.7$ μM, Ref. 1).

The $E_2 \rightarrow E_1$ transition

Our earlier studies of the effect of imidazole on phosphorylation suggest that the $E_2 \rightarrow E_1$ transition has a regulating effect on the phosphorylation process, in which buffer and Na^+ act synergistically [1]. Therefore we have investigated whether buffer, Na^+ and possibly protons might primarily act on this transition. It was already known that protonated amines have a Na^+-like effect in facilitating the transition of the dephosphorylated enzyme from the E_2-conformation to the phosphorylating E_1-conformation [10,13]. We have determined the effects on the $E_2 \rightarrow E_1$ transition per se by using eosin as a fluorescent label for the high-affinity nucleotide binding site in the E_1 conformation [10]. The method gives reliable results down to pH 5.5, but at pH 5 it leads to spurious data due to a 50% inactivation of the enzyme after

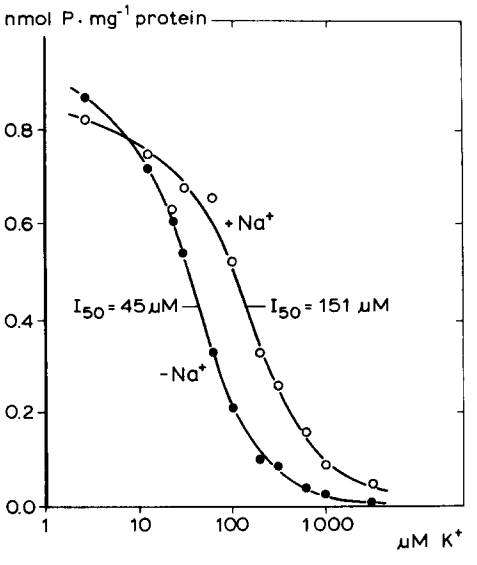

Fig. 7. Inhibition of phosphorylation by K^+. Phosphorylation is carried out in 25 mM imidazole-acetate (pH 5) and 0.1 mM Mg^{2+} in the absence (●) and presence (○) of Na^+ (100 mM) and in the presence of the indicated K^+ concentrations. Half-maximally inhibitory concentrations of K^+ under the two conditions are indicated in the figure.

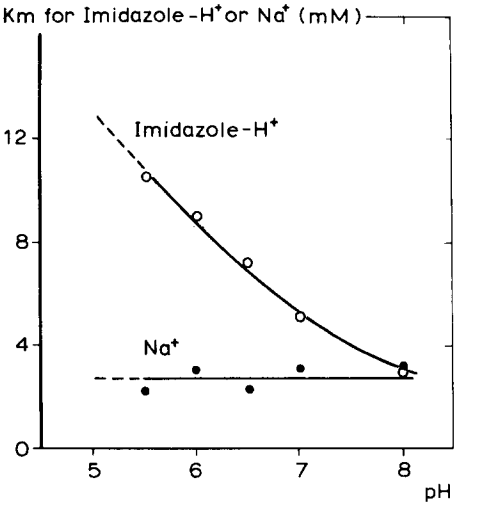

Fig. 8. K_m values for Na^+ and protonated imidazole in the $E_2 \rightarrow E_1$ transition as a function of pH. The transition is determined with eosin as fluorescent probe as described under Materials and Methods. The K_m values at pH 5 are estimated by extrapolation as indicated (dashed lines).

prolonged standing (60 min at 22°C) at that pH. Hence, we have determined the $E_2 \rightarrow E_1$ transition over a range of pH values between 5.5 and 8.0 by allowing the samples to come to equilibrium in several minutes and estimating the effect of the ligands at pH 5 by extrapolation. Fig. 8 shows the results of this strategy.

The K_m values for imidazole-H^+ in phosphorylation (5.9 mM, Ref. 1) and in the $E_2 \rightarrow E_1$ transition (5.1 mM, Fig. 8) are nearly equal at pH 7 as well as at pH 5 (12.8 mM, from Fig. 5B, and 13 mM, latter value extrapolated by the dashed line in Fig. 8). This suggests that the $E_2 \rightarrow E_1$ transition can be rate-limiting for the phosphorylation process. On the other hand, for Na^+ the intrinsic K_m value for phosphorylation (at 'zero' buffer and Mg^{2+} concentration, see section on Na^+ activation) increases from 1.9 mM at pH 7 to 4.3 mM at pH 5, whereas the K_m value for the $E_2 \rightarrow E_1$ transition remains unchanged (about 3 mM, Fig. 8). The effect on the K_m for steady-state phosphorylation could be due to an effect of pH on the Na^+-stimulated dephosphorylation.

Discussion

Buffer- and Na^+-activated phosphorylation

In contrast to the earlier observations of Fujita et al. [2], who did not find Na^+-activation of the phosphorylation at pH 5, we present evidence that both Na^+ as well as imidazole and Tris activate phosphorylation at this pH. Even at 0°C, the assay temperature used by Fujita et al., we still find the Na^+ effect, although the phosphorylation level then achieved is only about 4% of that at 22°C.

Other arguments against proton activation of phosphorylation are:

(1) The maximal level of Na^+-free phosphorylation at pH 5 (5 mM Mg^{2+}) is lower than that at pH 6.5 (0.1 mM Mg^{2+}), which is due to a suboptimal imidazole-H^+ concentration at pH 5 and not to a suboptimal proton concentration.

(2) The apparent optimum for Na^+-free phosphorylation at pH 5 in imidazole medium (5 mM Mg^{2+}) is due to a decreasing K_i value for Mg^{2+} above that pH (Table I) and thus it is an inhibition artifact.

(3) The optimal Na^+-free phosphorylation in Tris-medium at pH 5 is probably due to a decreasing K_i value for Tris above pH 5 and thus another example of a pH optimum determined by inhibition, this time by the buffer itself.

(4) Protons induce the non-phosphorylating E_2-conformation and reduce the rate of the $E_2 \rightarrow E_1$ transition [13,14]. It is unlikely that protons exert the dual effect of activating phosphorylation in the Na^+- or buffer-induced E_1 conformation, and also of promoting the $E_1 \rightarrow E_2$ transition.

It cannot be excluded that buffer cations donate a proton to the activating site, as has been suggested by Eigen and Hammes [15] for localized enzymatic acid-base catalysis. This might fit in with the recent report by Hara and Nakao [5] of an ATP-dependent proton uptake by K^+-loaded inside-out vesicles containing ($Na^+ + K^+$)-ATPase. However, the notion that H^+ could replace Na^+ in activating phosphorylation would imply that the affinity of protons for these sites is about 2–3 orders of magnitude higher than that of Na^+, which is the natural activator ion ($K_m < 0.1$ μM vs. 240 μM at pH 7; $K_m < 10$ μM v. 3500 μM at pH 5, each at 25 mM imidazole-H^+ in the absence of Mg^{2+}). On the other hand, the K_m values of 5.9–12.8 mM for imidazole-H^+ in the pH range of 5–7 would imply that very much higher concentrations of protons are available near the active site than are free in solution. We have evidence to believe that protonated buffer inhibits phosphorylation when binding to the Na^+-activation site, but stimulates it when binding to another site. Therefore, protonation should occur at the latter site. Protonation of that site would hamper rather than stimulate phosphorylation because of buffer cation repulsion (next section). Hence, we favour the idea that proton transfer follows the phosphorylation step and that proton extrusion occurs during the subsequent $E_1P \rightarrow E_2P$ transition. In this assumption proton transport is linked to buffer cation-activated phosphorylation. Since the buffer cation- and Na^+-activation sites interact, proton extrusion may actually occur via the latter sites.

Protons as inhibitors rather than activators

Protons more likely inhibit than activate phosphorylation, since all activators (protonated buffer, Na^+, Mg^{2+} and ATP) and inhibitors (K^+, Mg^{2+}) show a lower affinity for this process at pH 5 than at pH 7 (Table I). Effector binding sites may

become protonated, thereby being neutralized or positively charged and thus repelling the effector in addition to being shielded via intramolecular salt bridge formation.

The inhibition by protons may be at least partially related to their inhibition of the $E_2 \rightarrow E_1$ transition and to a reduction of the synergism between the buffer cation and Na^+ in this transition and the subsequent phosphorylation. Arguments for this conclusion are:

(1) The K_m for imidazole-H^+ in phosphorylation increases in line with that for the $E_2 \rightarrow E_1$ transition upon decreasing the pH.

(2) The inhibition by protons of the Na^+-dependent phosphorylation at a high buffer concentration is triple that at a low buffer concentration.

The pH independence of the K_m for Na^+ in the $E_2 \rightarrow E_1$ transition at low buffer concentration does not exclude an inhibitory effect of protons on the rate of this transition, which could explain the reduced phosphorylation levels at low pH. The pH dependence of the K_m for Na^+ in the steady-state phosphorylation under identical conditions may be due to an effect of pH on Na^+-stimulated dephosphorylation. The buffer cations probably lack the ability to stimulate dephosphorylation [1], so their K_m values in steady-state phosphorylation are not increased above those for the transition.

The discrepancy in proton-sensitivity of the K_m for Na^+ and buffer cations in the $E_2 \rightarrow E_1$ transition leads us to the conclusion that these effectors bind at different sites. The Na^+ binding sites have a low pK value, probably that of a carboxyl group [16] and are therefore little affected by protons in the pH range of 5.5–8. The buffer cation binding sites have a higher pK value, probably falling in this pH range, and they thus become protonated at low pH. A histidyl-imidazole group [14] might be activated by hydrogen bridge formation with protonated buffer and become inactive in the $E_2 \rightarrow E_1$ transition by protonation and buffer cation repulsion. Interaction between both sites is revealed by the synergism between the buffer cations and Na^+ in transition and phosphorylation as well as by the decrease of this synergism upon protonation of the buffer cation binding sites. This decrease in synergism may also explain the decreased inhibition by high buffer cation concentrations upon lowering the pH, which elicits Tris-activation (next section).

Tris-buffer activation and inhibition

A favourable action of protons that leads to a substantial activation by Tris buffer at pH 5, but much less above pH 6 [1], may actually be due to an increased K_i value for Tris as has also been observed for high imidazole and Mg^{2+} concentrations and for K^+ (Table I). Since the net activation is the sum of an activation and an inhibition, it appears that there may be both an activator and an inhibitor site for Tris on the enzyme and that lowering the pH increases the K_m less than the K_i for this ligand.

Tris-H^+ might inhibit the phosphorylation by occupying the Na^+-activation site, the affinity of which is controlled by occupation of the separate buffer cation binding site (see previous section). Due to a decreased synergism between the two sites at decreasing pH, the reduced affinity of the Na^+-site for Tris-H^+ leads to a decreased inhibition. Competition of Tris-H^+ and Na^+ for the same site is suggested by experiments showing an increase of K_m for Na^+ in phosphorylation at pH 7 at Tris concentrations above 50 mM, following a decrease caused by the synergism at lower Tris concentrations (Schuurmans Stekhoven et al., to be published). Tris does not inhibit, but rather stimulates the $E_2 \rightarrow E_1$ transition [10] with a K_m value similar to the K_m for imidazole-H^+ (5.8 mM at pH 7.4 (Ref. 10) vs. 4.3 mM, Fig. 8). This leads to the conclusion that occupation of the specific buffer cation binding site by Tris-H^+ leads to activation of phosphorylation, i.e. to a phosphorylating E_1-conformation, but additional binding to the Na^+-activation site to inhibition. Na^+, which only activates via its own sites, lacks the inhibitory effect found for buffers. Imidazole-H^+ displays effects similar to those of Tris-H^+, with K_m values of similar magnitude but with K_i values which must be higher than those of Tris-H^+ at the Na^+-activation site. Hence, its activating effect predominates in the pH range of 5–7, while its inhibitory effect overrules activation above pH 7. The difference between Tris-H^+ and imidazole-H^+ in their fit to the Na^+-activation site may be related to Tris being a primary amine and imidazole a tertiary amine.

Conclusions

In conclusion, we find no clear evidence for proton activation of phosphorylation, but rather a competitive inhibition by protons of the activation by Na^+, Mg^{2+} and imidazole-H^+ and also of the $E_2 \rightarrow E_1$ transition. In addition, protons diminish the inhibition by buffer, K^+ and Mg^{2+} of the phosphorylation. The reduced ligand inhibition can be seen as a favourable action of protons, but it cannot be considered as proton activation.

In view of the recent evidence for proton transport by the Na^+-pump in the nominal absence of Na^+ [4,5], we propose that during the $E_1P \rightarrow E_2P$ transition unprotonated buffer is released from the buffer cation binding site to the 'cytoplasmic space'. There the buffer will accept another proton and stimulate the $E_2 \rightarrow E_1$ transition, while the proton left on the enzyme is expelled during the $E_1P \rightarrow E_2P$ transition. Protonation of E_1P may even help drive the $E_1P \rightarrow E_2P$ transition, like protonation of E_1 drives the $E_1 \rightarrow E_2$ transition [13,14]. In this hypothesis protonated buffer is seen as donating protons to the phosphorylated enzyme in the E_1P conformation, rather than to Na^+-sites activating phosphorylation, where it would inhibit rather than promote.

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