Biochimica et Biophysica Acta, 982 (1989) 103–114
Elsevier

BBAMEM 74463

Ethylenediamine as active site probe for Na^+/K^+-ATPase

F M A H Schuurmans Stekhoven, Y S. Zou, H G P Swarts, J Leunissen and J J H.H M De Pont

Department of Biochemistry, University of Nijmegen Nijmegen (The Netherlands)

(Received 19 October 1988)
(Revised manuscript received 9 March 1989)

Key words ATPase, Na^+/K^+- Ethylenediamine Active site probe

(1) Ethylenediamine is an inhibitor of Na^+- and K^+-activated processes of Na^+/K^+-ATPase, i e the overall Na^+/K^+-ATPase activity, Na^+-activated ATPase and K^+-activated phosphatase activity, the Na^+-activated phosphorylation and the Na^+-free (amino-buffer associated) phosphorylation (2) The I_{50} values (I_{50} is the concentration of inhibitor that half-maximally inhibits) increase with the concentration of the activating cations and the half-maximally activating cation concentrations (K_m values) increase with the inhibitor concentration. (3) Ethylenediamine is competitive with Na^+ in Na^+-activated phosphorylation and with the amino-buffer (triallylamine) in Na^+-free phosphorylation Significant, though probably indirect, effects can also be noted on the affinity for Mg^{2+} and ATP, but these cannot account for the inhibition. (4) Inhibition parallels the dual protonated or positively charged ethylenediamine concentration (charge distance 3.7 Å). (5) Direct investigation of interaction with activating cations (Na^+, K^+, Mg^+, triallylamine) has been made via binding studies. All these cations drive ethylenediamine from the enzyme, but K^+ and Mg^+ with the highest efficiency and specificity. Ethylenediamine binding is ouabain-insensitive, however (6) Ethylenediamine neither inhibits the transition to the phosphorylating enzyme conformation, nor does it affect the rate of dephosphorylation Hence, we provisionally conclude that ethylenediamine inhibits the phosphoryl transfer between the ATP binding and phosphorylation site through occupation of cation activation sites, which are 3–4 Å apart

Introduction

Some amines, viz the triple-substituted triallylamine and tripropylamine, effectively substitute for Na^+ in the ATP-dependent phosphorylation of Na^+/K^+-ATPase, probably due to their lipophilic interactions in the centre of phosphoenzyme hydrolysis, thereby inhibiting water entry and antagonizing the opposite K^+-effect [1]

Diamines, however, particularly those displaying a $N \rightarrow N$ distance of 3–4 Å, inhibit steady-state phosphorylation Inhibition by ethylenediamine ($N \rightarrow N$ = 3 7 Å) could be overcome competitively by increasing the Na^+ concentration Inhibition by ethylenediamine of triallylamine activation, on the other hand, not only increased the half-maximally activating concentration of buffer, but also seemed to lower the maximal phosphorylation level attained [2] This would indicate different mechanisms or sites for Na^+ and triallylamine activation Moreover, it hints at the possibility that ethylenediamine may bridge Na^+-activation sites of 3–4 Å distance In addition the mixed-type inhibition of triallylamine activation could indicate different sites of binding for ethylenediamine and activating amine, with the enzyme-inhibitor-activator complex operating at a submaximal level

In view of the prospect that ethylenediamine binding might be used as a tool to localize Na^+-activation sites, we have analysed inhibition by the diamine on overall and partial reactions of the title enzyme in more detail in comparison with its binding characteristics

Materials and Methods

Enzyme preparation and overall hydrolytic activities

Preparation and purification of Na^+/K^+-ATPase from rabbit kidney outer medulla [3], including removal of contaminating ATP, washing and storing of the preparation (Ref 4, omitting CDTA from the storage medium), and determination of protein [3] is carried out according to the references given

Abbreviations $S_{0.5}$, $K_{0.5}$ or K_m are the concentrations of substrate or ligand giving a half-maximal effect I_{50} is the concentration of inhibitor that half-maximally inhibits and K_i is the same but in the absence of a competitive antagonist

Correspondence F M A H Schuurmans Stekhoven, Department of Biochemistry University of Nijmegen, P O Box 9101, 6500HB Nijmegen, The Netherlands

The Na^+/K^+-ATPase and 4-nitrophenylphosphatase activities are determined according to Schoot et al [4] at 37°C and pH 7.4 with minor modifications. The assay medium for Na^+/K^+-ATPase activity contains an additional 0.1 mM EDTA, whereas the 4-nitrophenylphosphatase assay medium contains 7.5 mM $MgCl_2$ in addition to the 1 mM CDTA. The 4-nitrophenyl phosphate is freed from Na^+ by conversion to the imidazole salt by passage over Dowex 50 WX4 (H^+-form), followed by neutralization with solid imidazole and spectrophotometrical determination of its concentration ($\epsilon_{315} = 10.8\ mM^{-1}\ cm^{-1}$). In the control 1 mM ouabain substitutes for K^+.

Determination of Na^+-stimulated ATPase activity is carried out under assay conditions (22°C, pH 7.0) approaching those of the phosphorylation in a medium containing 20 mM triethanolamine-HCl, 1 mM NaCl, 0.1 mM $MgCl_2$ and 20 μM ATP. In the control an additional 1 mM ouabain is present. Inorganic phosphate liberated is determined as phosphomolybdate complex, stained with Malachite green [5].

Phosphorylation and dephosphorylation

Phosphorylation of the enzyme at 23°C and pH 7 follows the procedure outlined in our previous publication [1], using either triallylamine-HCl as only buffer component for optimal buffer-activated phosphorylation, or triethanolamine-HCl as low background buffer support for Na^+-activated phosphorylation. Neutralization and quantification of the buffer components in the stock solutions, including determination of their Na^+ and K^+ contents have been carried out as described [1].

Phosphorylation is started by addition of [γ-^{32}P]ATP (20 μl) or $MgCl_2$ + [γ-^{32}P]ATP (40 μl) to the assay medium, containing the other components, including buffer (+ or −Na^+, + or −Mg^{2+}, and + or − ethylenediamine) and enzyme, making a total volume of 150 μl. Final concentrations in the standard assay medium are 50 mM for buffer, 0.1 mM for Mg^{2+}, 20 μM for ATP and 0.1 mg protein/ml for the enzyme. Concentrations of other components, like Na^+ and ethylenediamine are variable and hence are specified for each experiment in the figure legends. ATP is converted to its imidazole salt by passage of an aqueous solution of the disodium salt over a Dowex 50 WX4 cation-exchange resin in the protonated form, followed by neutralization of the eluent with imidazole. The non-radioactive ATP is mixed with [γ-^{32}P]ATP (Amersham International plc, Amersham, U.K., code PB.170, 333 μM, 3000 Ci/mol [6]) in order to yield a specific radioactivity of 300–500 cpm/pmol (1300–1500 cpm/pmol in the determination of K_m for ATP).

Following phosphorylation for specified times the reaction is quenched by addition of 4–5 ml 5% trichloroacetic acid, containing 0.1 M P_i. The denatured protein is washed on 25 mm diameter, 1.2 μm pore size membrane filters (Schleicher & Schuell, Dassel, F.R.G.) three times with the quenching solution and counted for ^{32}P in a liquid-scintillation analyser after addition of 4 ml Aqualuma Plus counting solution (Lumac b.v., Landgraaf, The Netherlands). Data are corrected for blanks in which the quenching medium is added prior to the MgATP.

Phosphorylation is determined as steady-state level (unless stated otherwise), taking 3 s in the absence of ethylenediamine (10–15 s in the determination of the K_m for Mg^{2+} or ATP at suboptimal concentrations of these ligands) and 1–2 min in the presence of ethylenediamine (absence of Na^+). Phosphorylation in triallylamine is preceded by 45 min preincubation (23°C) of enzyme in the buffer medium in order to induce a maximally phosphorylating conformation. This conformation is stable for 30 min, followed by a period of declining phosphorylation capacity, possibly by interference of the lipophilic triallylamine with the protein-lipid interaction. The phosphorylation experiments are carried out in this 30 min period of optimal capacity. ATP is added 2 min after ethylenediamine in order to allow for enzyme-inhibitor equilibrium binding.

In the determination of the K_m for Mg^{2+} an additional 0.2 mM EDTA (neutralized with triethanolamine) is present as Mg^{2+} buffer. Total Mg^{2+} to be added in order to achieve the desired uncomplexed Mg^{2+} concentrations is calculated by means of stability constants reported for the protonated forms of EDTA and ATP and their protonated and unprotonated Mg-complexes [7].

Dephosphorylation is assayed by 10-fold dilution of the phosphorylation medium, following 10 s phosphorylation with 5 μM [γ-^{32}P]ATP, with medium of the same composition, but without enzyme and containing either 5 (method B) or 555 μM (method A) non-radioactive ATP [8]. The phosphoenzyme levels are traced for 10–20 s after dilution. As blanks serve phosphoenzyme determinations in which either acid denaturation of the enzyme takes place before addition of MgATP (method A) or after phosphorylation in the diluted medium for 10 s plus the dephosphorylation time (method B). Rate constants of dephosphorylation are determined from slopes of semilogarithmic plots of phosphoenzyme level vs. time of dephosphorylation.

Binding of ethylenediamine

Binding of ethylenediamine is determined according to a filtration method, previously published by us [9]. The enzyme (1 mg/ml) is incubated at 22°C and pH 7.0 in a medium (100 μl), containing 50 mM triethanolamine-HCl, [^{14}C]ethylenediamine (up to 100 μM, 22 mCi/mmol), [^{3}H]sucrose (5 mCi/mmol, initial $^{14}C/^3H$ counting ratio in the assay medium is between 0.113 and 0.142), 25–50 mM non-radioactive sucrose (contributed by the storage medium of the enzyme

preparation) and ligands in concentrations given in the results section. As blanks serve assays to which non-radioactive ethylenediamine (50 mM, pH 7.0) instead of the effector ligands has been added.

Binding is initiated by adding enzyme last and filtration (40 μl in duplicate) is started after ample (15–30 min) equilibration. Filters are extracted for 30 min in 1 ml 10% (w/v) SDS and counted for ^{14}C and ^{3}H following mixing with 10 ml Insta-Gel (Canberra Packard Benelux bv, Tilburg, The Netherlands), in a liquid scintillation analyser, programmed for double label analysis. Counts of the non-binding [^{3}H]sucrose serve via the initial $^{14}C/^{3}H$ counting ratio for calculation of the amount of free ethylenediamine (F_o) on the filter, uncorrected for a change due to binding of ^{14}C to the enzyme. ^{14}C counts serve for the calculation of the amount of total (T) free plus enzyme-bound ethylenediamine on the filter. Let the total amount of ethylenediamine in the aliquot of medium taken for filtration (40 μl) be M. After n iterative corrections of the free $^{14}C/^{3}H$ ratio for ^{14}C binding and vice versa, the final amount of enzyme-bound ethylenediamine can be described [9] as

$$B_n = (T - F_o)\sum_{0}^{n}\left(\frac{F_o}{M}\right)^n$$

Blanks, that have undergone the same mathematical treatment ($n = 3$), are read from a blank calibration graph of B_n vs $(M - B_n)$. Blank values for B_n are subtracted from assay values at equivalent $(M - B_n)$ values.

Computation of interatomic distances

Calculation of the number of neutral amino acid residues to be inserted between two negatively charged aspartic and/or glutamic acid residues in a helix or pleated sheet structure, allowing the relaxed ethylenediamine anti-conformation (N → N span of 3.7 Å) to fit, has been carried out by the MacroModel programme (Columbia University) via the facilities of the Dutch CAOS/CAMM Center (Science Faculty, Nijmegen).

Radioactive and non-radioactive chemicals

[γ-^{32}P]ATP (3000 mCi/mmol, radiochemical purity ≥ 95%), [^{14}C]ethylenediamine (44 mCi/mmol radiochemical purity 98–99%) and [^{3}H]sucrose (5–9.8 Ci/mmol, radiochemical purity 98–99%) are from Amersham International (Amersham, U.K.). ATP (disodium salt, 98% pure) is from Boehringer Mannheim (Mannheim, F.R.G.). 4-Nitrophenyl phosphate (enzymatic purity 98%), ethylenediamine (99% pure), triallylamine (98% pure), triethanolamine (98% pure) are all from Merck (Darmstadt, F.R.G.) or Merck-Schuchardt (Hohenbrunn, F.R.G.). All other chemicals are of analytical grade.

Results

Inhibition of overall and partial reactions

Na^+/K^+-ATPase activity is inhibited by ethylenediamine but at concentrations of the inhibitor ($I_{50} = 35$ mM, Table I) far beyond those involved in buffer-activated and Na^+-activated phosphorylation ($I_{50} = 0.025$–0.56 mM, Table I). Part of the discrepancy could be due to a difference in temperature (37 vs 22°C) and in the concentration of ligands: $Na^+ = 100$ vs 0–1 mM, $K^+ = 10$ vs 0 mM, $Mg^{2+} = 5$ vs 0.1 mM, ATP = 5 vs 0.02 mM. Comparison with the inhibition of 4-nitrophenylphosphatase activity ($I_{50} = 20$ mM) assayed under comparable conditions of pH, temperature and activating ligand concentrations as the overall Na^+/K^+-ATPase activity (Table I) shows that the presence of high Na^+ in the latter assay probably antagonizes inhibition. The same holds true for K^+, an increase (1 → 15 mM) of which in the phosphatase assay medium increases the I_{50} value for ethylenediamine from 4.2 to 26 mM ($K_i = 2.8$ mM, Fig. 1). On the other hand, the Na^+-activated ATPase activity, which is assayed under

TABLE I

Inhibition by ethylenediamine of overall and partial reactions of Na/K^+ ATPase

Abbreviations: Im, imidazole, TEA, triethanolamine, TAA, triallylamine, S, substrate, E, enzyme, T, temperature, t, time. All buffer components are brought to pH with HCl.

Activity	Buffer (mM)	pH	Na^+ (mM)	K^+ (mM)	Mg^{2+} (mM)	S (mM)	E (μg/ml)	T (°C)	t (min)	I_{50} (mM)
Na^+/K-ATPase	Im 30	7.4	100	10	5 [a]	5	1.25	37	60	35
4-Nitrophenylphosphatase	Im 30	7.4	0.0	10	7.5 [b]	5	1.25	37	60	20
Na^+-activated ATPase	TEA 20	7.0	1	0.0	0.1	0.02	10	22	15	4.2
Na^+-activated phosphorylation	TEA 40	7.0	1	0.0	0.1	0.02	100	23	0.05	0.56
Buffer-activated phosphorylation	Im 50	7.0	0.0	0.0	0.1	0.02	100	23	0.05	0.025
	TAA 40	7.0	0.0	0.0	0.1	0.02	100	23	0.05	0.067

[a] 0.1 mM EDTA present.
[b] 1 mM CDTA present.

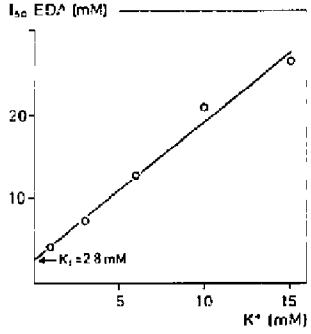


Fig. 1 Competition between K^+ and ethylenediamine (EDA) in the 4-nitrophenylphosphatase activity Shown is I_{50} EDA as a function of the K^+ concentration Assay conditions as indicated in Table I

conditions (22°C, pH 7 0, 1 mM Na^+, 0 1 mM Mg^{2+}, 0 02 mM ATP) comparable to those of the Na^+-activated phosphorylation, also displays reduced sensitivity to the inhibitor ethylenediamine (I_{50} = 4 2 vs 0 56 mM, Table I) The difference may be due to the difference in rate limiting step, being dephosphorylation in Na^+-activated ATPase and the phosphorylation step per se in Na^+-activated phosphorylation

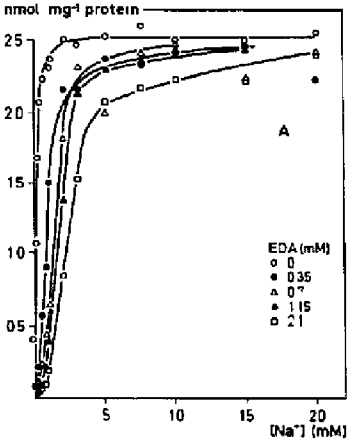
Finally, comparison of Na^+- and buffer-activated phosphorylation confirms the antagonistic action of Na^+ on ethylenediamine inhibition where 1 mM Na^+ is able to increase the I_{50} for ethylenediamine at least 8-fold under comparable assay conditions (Table I) The antagonism will be analysed in further detail in the next section

Inhibition of Na^+-activated phosphorylation

Since inhibition by ethylenediamine on the Na^+-activated phosphorylation is more apparent in the rate than in the eventual steady state, we have confined here ourselves to 3-s phosphorylation levels (presteady-state in presence of the inhibitor at suboptimal Na^+ concentrations) This inhibition by ethylenediamine (0–2 1 mM) can be fully overcome during the 3 s of phosphorylation by increasing the Na^+ concentration (Fig 2A), concomitant with an increase (from 0 12 to 2 68 mM) of the half-maximally activating Na^+ concentration (Fig 2B, inset) This phenomenon represents competitive inhibition of Na^+ activation by ethylenediamine

The upward curvature in the double reciprocal plot of phosphorylation level vs Na^+ concentration indicates positive cooperativity (Fig 2B) Hill-plot analysis of the data demonstrates that the Hill coefficient (n_H) also increases (from 1 34 to 2 52) with the inhibitor concentration (Fig. 2B, inset) This response is in agreement with ligand exclusion in which the binding of an inhibitor to a single site prevents the activating ligand from binding to a multiplicity of sites [10], possibly three, judging from the Hill coefficient approaching a value of 3

Because of the low background phosphorylation level (10–16% in the control without Na^+ and ethylenediamine) triethanolamine-HCl has been chosen as buffer component The $S_{0.5}$ of 0 12 mM for Na^+ in this buffer in the absence of ethylenediamine is similar to the half-maximally activating Na^+ concentration (0 2 mM) in imidazole-HCl at the same concentration, pH and temperature [11], indicating that triethanolamine is equivalent to imidazole in inducing the phosphorylating



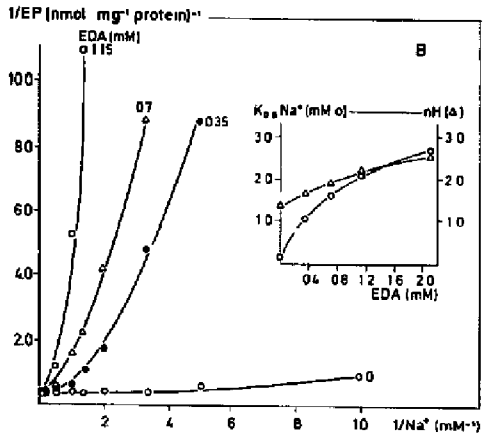


Fig. 2 Inhibition of Na^+-activated phosphorylation by ethylenediamine Phosphorylation takes place under the conditions of Table I (3rd line from bottom) in the presence of the indicated Na^+ and ethylenediamine (EDA) concentrations (A) Michaelis-Menten plots (B) Lineweaver-Burk plots (Inset in B) $K_{0.5}$ (○) and Hill-coefficient (n_H △) for Na^+ as determined from Hill plots of the data

E_1 conformation This contention is supported by the kinetics of the $E_2 \rightarrow E_1$ transition which in triethanolamine and imidazole are of similar magnitude (Van Uem et al, unpublished data) The I_{50} for ethylenediamine as a function of the Na^+ concentration in triethanolamine extrapolates at 0 Na^+ to 0 075 mM (not shown), which is virtually equivalent to the I_{50} value for triallylamine activated phosphorylation (0 067 mM, Table I) This may mean that triethanolamine and triallylamine have the same affinity for a common site but that the latter, due to stronger inhibition of dephosphorylation, leads to appreciably higher steady-state phosphorylation levels (90 vs 10–16% of the maximal Na^+ activation level)

Inhibition of buffer-activated phosphorylation

Where Na^+ can fully overcome in 3 s the inhibition of phosphorylation by ethylenediamine, this is not the case for triallylamine as representative of buffer-activated phosphorylation This raises the question as to whether steady-state phosphorylation in buffer-activated phosphorylation is reached within 3 s or not, and whether this level is stable in time Besides a slow (30–45 min) rise in the (3-s) phosphorylation level during preincubation in triallylamine (maybe due to insertion of the lipophylic molecule into the lipid bilayer) addition of ethylenediamine disturbs the reaching of an equilibrium by giving a downward overshoot of about 2 min duration (Fig. 3) Under conditions of Na^+-activated phosphorylation (Table I) the overshoot phenomenon is absent and the new equilibrium is attained within 3 s upon addition of the inhibitor Apparently Na^+ is a more potent cation to control the equilibrium shift caused by ethylenediamine. Time courses of ethylenediamine inhibition at different triallylamine concentrations display patterns similar to those shown in Fig. 3 Therefore, in subsequent experiments on ethylenediamine inhibition of buffer-associated phosphorylation 2–3 min of equilibrium setting for the enzyme-inhibitor complex is allowed to take place before phosphorylation is started by addition of ATP Though 3 s is sufficient to reach the steady state in buffer-activated phosphorylation in the absence of ethylenediamine (10–15 s at suboptimal concentrations of Mg^{2+} or ATP Fig. 6) prolonged phosphorylation times (0 25–1 5 min) are required in the presence of the inhibitor (0 033–0 6 mM) The steady-state lasts for another 1 5–2 75 min (Fig 4), except at suboptimal ATP concentration where the decline, due to substrate exhaustion, sets in after 1 min Hence, routinely triallylamine generated steady-state phosphoenzyme levels are determined following 2 min of phosphorylation (Figs 5 and 6B)

We have furthermore tested the reversibility of the inhibition either by incubating the enzyme for 2 min with the inhibitor in 2 7-times concentrated form prior to dilution with triallylamine, or by adding the inhibitor

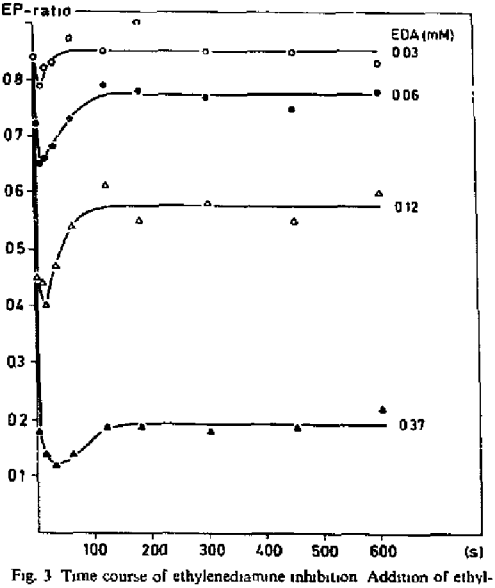


Fig. 3 Time course of ethylenediamine inhibition Addition of ethylenediamine (EDA, final concentration upon addition of ATP as indicated) follows 45 min preincubation of enzyme with triallylamine (204 5 mM) Inhibition is pursued by addition of ATP at the indicated times after ethylenediamine and traced by the indicated 3-s presteady-state phosphorylation levels as ratio of the control without EDA The phosphorylation conditions are those of Table I (bottom line, but at 150 mM triallylamine) following a 15% reduction in ligand concentrations upon addition of EDA and a further 12% reduction upon the subsequent addition of ATP Conditions of temperature and pH during preincubation and phosphorylation are the same

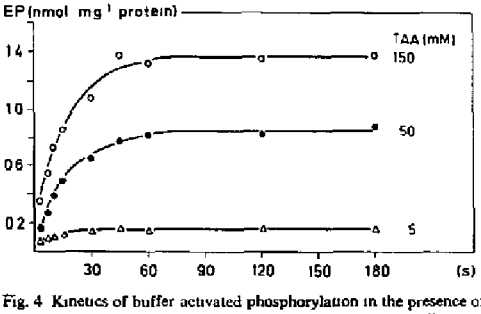


Fig. 4 Kinetics of buffer activated phosphorylation in the presence of ethylenediamine. Phosphorylation for the indicated times follows 45 min of preincubation in triallylamine (TAA final concentrations indicated) and a subsequent 2–3 min of preincubation in the presence of ethylenediamine (final concentration 0 37 mM) Conditions of preincubation and phosphorylation, including the dilutions caused by addition of ethylenediamine and ATP are essentially those given in Fig. 3

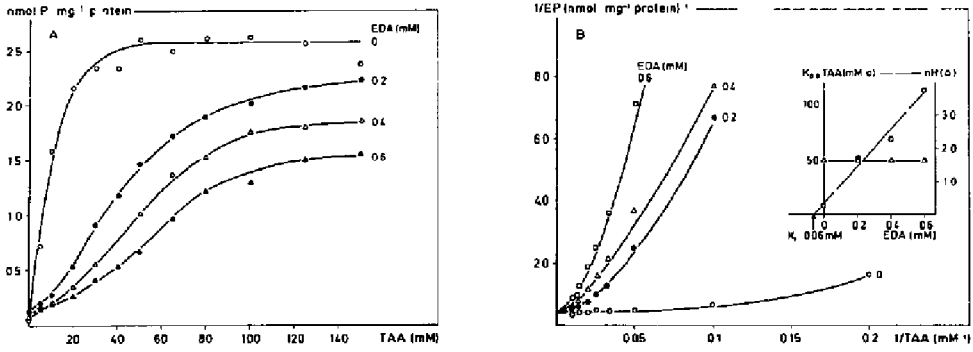


Fig 5 Inhibition of buffer-activated phosphorylation by ethylenediamine The conditions of Fig 4 are applied in preincubation and phosphorylation (3 s without ethylenediamine 2 min in the presence of ethylenediamine) Final concentrations of triallylamine (TAA) and of ethylenediamine (EDA) are presented (A) Michaelis-Menten plots (B) Lineweaver-Burk plots, corrected for back-ground levels in (A), due to imidazole (0 54 mM) accompanying the enzyme (Inset in B) $K_{0.5}$ (○) and Hill-coefficient (n_H, △) determined from Hill plots of the data from (B)

46 min after the activator In either case final concentrations of inhibitor (0 6 mM) and activator (150 mM) upon addition of ATP were the same as were the final steady-state phosphorylation levels (within the experimental error of 10%) In addition, the inhibitor can be washed out in binding experiments to be presented below Hence, we consider inhibition of ethylenediamine to be reversible

Following these preliminaries we have determined the type of inhibition by ethylenediamine on buffer-associated phosphorylation (Fig 5) In contrast to an earlier conclusion [2], based on Fig 5A, which seems to demonstrate mixed-type inhibition, we now interpret the inhibition as being merely competitive (Fig. 5B) The half-maximally activating triallylamine concentration increases in a virtually linear fashion, whereas the

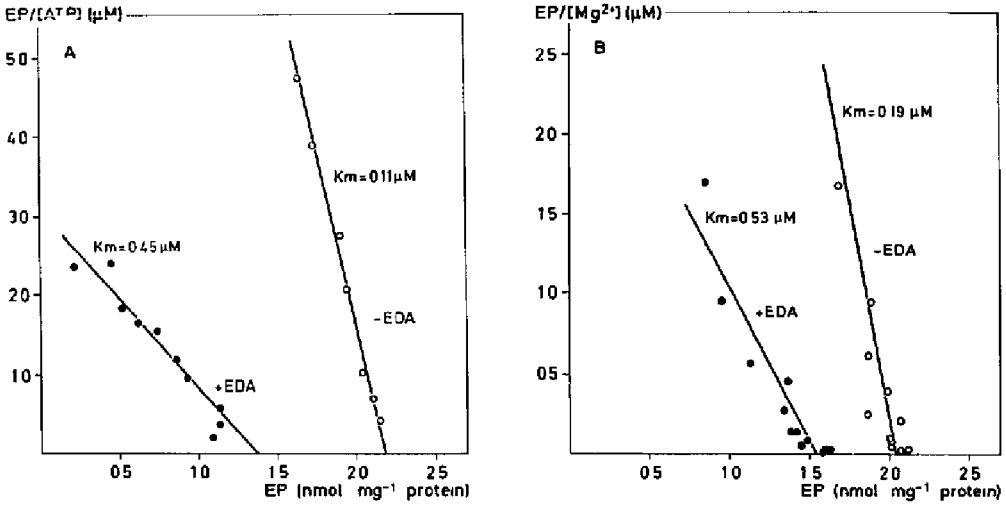


Fig 6 Effects of ethylenediamine on the K_m for ATP (A) and for uncomplexed Mg^{2+} (B) Preincubation and phosphorylation are essentially according to Fig 5 with minor modifications Concentrations during preincubation are 15–28% higher than during phosphorylation, following the addition of EDA and ATP Final concentrations in (A) ATP = 0 1–5 μM Mg^{2+} = 100 μM, and enzyme = 0 03 mg/ml, (B) ATP = 20 μM total Mg^{2+} = 26 8–302 8 μM (free 0 5–100 μM), and enzyme = 0 1 mg/ml Triallylamine in (A) and (B) is 150 mM and ethylenediamine (EDA) is 0 6 mM Phosphorylation to steady state without EDA takes 15 s and with EDA 1 min in (A) 10 s and 2 min respectively in (B) K_m values derived from the slopes, as calculated by linear regression analysis are presented The ATP concentrations are corrected for consumption during phosphorylation

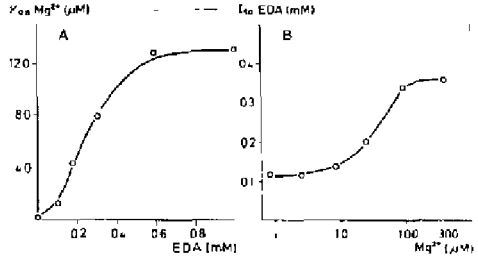


Fig. 7 Ethylenediamine-Mg^{2+} antagonism in Na^+-activated phosphorylation. Conditions of phosphorylation are essentially those given in Table I (3rd line from bottom) at 1–300 μM uncomplexed Mg^{2+} (EDTA = 0.2 mM) and 0–3 mM ethylenediamine (EDA). (A) $K_{0.5}$ Mg^{2+} as a function of EDA. (B) I_{50} EDA as a function of Mg^{2+}.

Hill coefficient ($n_H = 1.64$) remains horizontal, i.e. independent of the inhibitor concentration (Fig. 5B, inset). According to the ligand exclusion theory [10] this is possible when n_H is close to the number of ligand binding sites. This may mean that 2 sites (the next integer of 1.64), rather than 3 (the next integer of 2.52 in Fig. 2B) are involved in amino-buffer activation.

Effects on the activation by substrate and Mg^{2+}

The effect of ethylenediamine on activation by ATP and Mg^{2+} in buffer-associated phosphorylation appears to be of secondary importance. The K_m values for ATP and Mg^{2+} only increase 3–4-fold to values (0.45 and 0.53 μM, respectively, Fig. 6), which cannot account for the inhibition observed with 44-times the K_m for ATP and 189-times the K_m for Mg^{2+} present. The same holds for ATP in Na^+-activated phosphorylation (conditions of Table I) where the increase in K_m for ATP (0.8 mM ethylenediamine) is only 2-fold (from 0.02 to 0.04 μM). The effect on the $K_{0.5}$ for Mg^{2+} in Na^+-activated phosphorylation is more severe and leads to a value that levels off at about 13 μM (Fig. 7A). The increase may be due (in part) to the increase in $K_{0.5}$ for Na^+ (Fig. 2B) since increasing the Na^+ concentration decreases the $K_{0.5}$ for Mg^{2+} in the μmolar range [11]. In addition, the saturating inhibition indicates separate binding sites for ethylenediamine and Mg^{2+}, reminiscent of a similar response of the K_d for ATP upon titration with K^+ [12]. Again, the K_m value of 13 μM, though being relatively high, cannot have been a cause of inhibition at the saturating Mg^{2+} concentration of 100 μM in the phosphorylation experiments.

Conversely, Mg^{2+} also antagonizes the inhibition by ethylenediamine of the Na^+-activated phosphorylation (Fig. 7B). This antagonism comes into full effect beyond the maximal $K_{0.5}$ value for Mg^{2+} shown in Fig. 7A. The half-maximal effect is at 36 μM. This probably means that we are dealing here with a separate Mg^{2+} site of lower affinity.

The effect of ethylenediamine on dephosphorylation

The reduced phosphorylation levels, seen in the presence of ethylenediamine (Figs. 2, 3, 5, 6), might occur when the inhibitor would enhance the dephosphorylation rate. For that reason the effect of ethylenediamine on the dephosphorylation kinetics was investigated. Meanwhile the kinetics of dephosphorylation in triallylamine as optimal activator of buffer-associated phosphorylation were compared with those of imidazole as second-best activator [1,11] in order to check the concept that the activation of phosphorylation, seen as an increase of the phosphorylation level, is due to inhibition of dephosphorylation. In that case the kinetics of dephosphorylation in triallylamine should be lower than in imidazole. Since millimolar (non-radioactive) ATP, used to quench the incorporation of ^{32}P, inhibits dephosphorylation [8] the reaction is also quenched by 10-fold dilution of the radioactive ATP with non-radioactive ATP of the same concentration (5 μM).

TABLE II

Effects of imidazole, triallylamine and ethylenediamine on the kinetics of dephosphorylation at 23°C and pH 7

Imidazole or triallylamine is present throughout the experiment; ethylenediamine (EDA, 0.5 mM) only during dephosphorylation (added together with the non-radioactive ATP used to quench the incorporation of ^{32}P). The data on imidazole (3rd and 4th column) are calculated from results already published by us [8,13].

Medium (phosphorylation) / (dephosphorylation)		a Imidazole (50 mM) id (10 mM)	b Imidazole (50 mM) id (50 mM)	c Triallylamine (40 mM) id (40 mM) − EDA	d Triallylamine (40 mM) id (40 mM) + EDA
ATP during dephosphorylation (μM)	Method	Rate constant of dephosphorylation (k (s^{-1}))			
		a	b	c	d
5	B	0.78	0.21	0.074	0.085
500	A	0.33	0.04	0.026	0.025

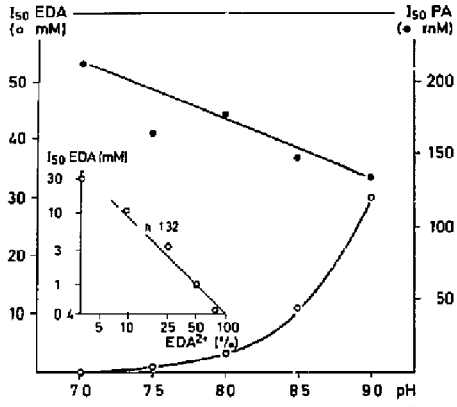


Fig. 8 Effect of molecular charge on ethylenediamine inhibition Shown is I_{50} for ethylenediamine (EDA) in the Na^+-activated phosphorylation as a function of pH and as a function of the mol fraction of double-charged EDA (double logarithmic plot inset) For comparison the effect of pH on the I_{50} for the single-charged propylamine (PA) is shown Assay conditions as in Table I, 3rd line from bottom, at 5-times the K_m for Na^+ at each pH 1 mM (pH 7 and 7 5), 1 5 mM (pH 8), 2 25 mM (pH 8 5), 2 75 mM (pH 9)

The data (Table II) show that increasing the imidazole concentration from 10 to 50 mM decreases the rate constant of dephosphorylation 4–8-fold, followed by a further 1 5–3-times by changing the medium to triallylamine (40 mM) Ethylenediamine, present during dephosphorylation, does not alter the rate constant significantly, indicating that enhancement of dephosphorylation is not involved in the ethylenediamine inhibition Since ethylenediamine also does not inhibit the conformational change to the phosphorylating E_1-form of the enzyme (Van Uem et al , unpublished data) the phosphorylation step per se is the actual target of ethylenediamine inhibition

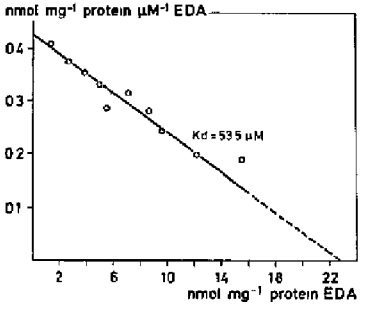


Fig 9 Ethylenediamine binding to Na^+/K^+-ATPase Binding to one of the preparations, also represented in Table III, is shown here in a Scatchard plot, following incubation in the presence of 5–100 µM (initial concentration) of ethylenediamine (EDA) These concentrations are corrected for the binding

Effect of molecular charge on ethylenediamine inhibition

In our previous article [1] we postulated that the strong inhibition by ethylenediamine is caused by stapling of the double-charged diamine to neighbouring carboxyl groups With pKa values for the amino groups of 7 52 and 9 98 one should be able to shift the molar fraction of double-charged diamine from 77 back to 3% by increasing the pH from 7 to 9 Since buffer-activated phosphorylation sharply drops above pH 7 0 [11] the effect of elevated pH on ethylenediamine was only tested in Na^+-activated phosphorylation. The maximal level of Na^+-activated phosphorylation at pH 9 is still 86% of that at pH 7 0. Occupation of Na^+-activation sites was tried to be standardized by taking 5-times the half-maximally activating concentration of Na^+ (0 2–0 55 mM) Since inhibition may also depend on the degree of protonation of the enzyme [14] the inhibition by ethylenediamine is compared with that of propylamine (pK_a = 10 53), which is single-charged at all pH values (7–9) investigated The I_{50} value for ethylene-

TABLE III

Parameters of ethylenediamine binding and phosphorylation

Maximal binding (EDA_{max}) of ethylenediamine (5–100 µM) and half-maximally saturating ethylenediamine concentrations ($S_{0.5}$) are determined from Scatchard plots like that given in Fig 9 The interaction coefficient (n) is determined from the binding in the maximum of the plot, relative to that of the abscissa intercept = $(n-1)/n$ [15] Maximal phosphorylation is determined in the absence of ethylenediamine, but in the presence of 100 mM Na^+ Enzyme preparations (E) used with specific Na^+/K^+-ATPase activity (spec act) and phosphoenzyme level (EP_{max}) are given in the left columns

E (No)	Spec act (µmol/mg protein per h)	EP_{max} (nmol/mg protein)	EDA_{max} (nmol/mg protein)	$S_{0.5}$ (µM)	n	$\frac{EDA_{max}}{EP_{max}}$ ratio
1	1213	2 5	23	54	1 0	9 2
2	1253	2 2	18	41	1 3	8 2
3	1395	2 5	18	35	1 0	7 2
Av	1290±55	2 4±0 1	20±1 7	43±5 6	1 1±0 1	8 2±0 6

diamine exponentially increases from 0 45 mM at pH 7 to 30 mM at pH 9 (Fig 8) The dual logarithmic plot of I_{50} vs the molar fraction of double-charged molecules is straight with a slope of 1 3 This indicates that the double-charged ethylenediamine is the strongly inhibitory molecule and that the binding sites are weakly cooperative Note that the interaction coefficient of 1 3 is similar to that for ethylenediamine binding (next section) The single-charged *n*-propylamine displays an opposite trend, namely that an increased pH and concomitant deprotonation of the enzyme leads to an increased affinity for the inhibitor (I_{50} = 214 mM at pH 7, 134 mM at pH 9, Fig 8)

Cation-ethylenediamine binding interplay

Since inhibition by ethylenediamine and its release by activating cations, as studied in the previous sections, should be preceded by binding of the inhibitor to the enzyme, we have tried to find a relation between kinetic and binding studies involving the antagonism between inhibitor and activating cations

Ethylenediamine binding in the apparent absence of antagonists is not or only weakly cooperative with Hill coefficients between 1 0 and 1 3 and half-maximally saturating concentrations averaging at 43 ± 6 μM The binding capacity is 7–9-times the phosphorylation capacity (Fig 9 , Table III), suggesting that not all of the binding may be directed to activating cation binding sites as only 3 Na^+ sites, 2 K^+ sites and 1 Mg^{2+} site have been reported [16–18] Some kind of aspecificity is also revealed by the uniformity of high K_i and I_{50} values (17–23 mM, Table IV, Fig 10) as displayed in the antagonism exerted on ethylenediamine binding by such various cations, like Na^+, K^+ (> 1 mM), Li^+ and triallylamine The Dixon plots of reciprocal binding versus the concentration of antagonist intersect on the abscissa (not shown), indicating that high ionic strength increases the binding capacity aspecifically

Apparently more specific is the antagonism as exerted by submillimolar K^+ (K_i = 0 2 mM) and Mg^{2+} (K_i = 0 4 mM) Here Dixon plots intersect above the abscissa at the reciprocal maximal binding capacity in the absence of the antagonist Ethylenediamine binding is ouabain-insensitive in the sense that up to 1 mM ouabain in the presence of 1 mM Mg^{2+} (≈ I_{50}, Fig. 10C) at 0 1 mM ethylenediamine does not decrease the binding level below that given by Mg^{2+} alone

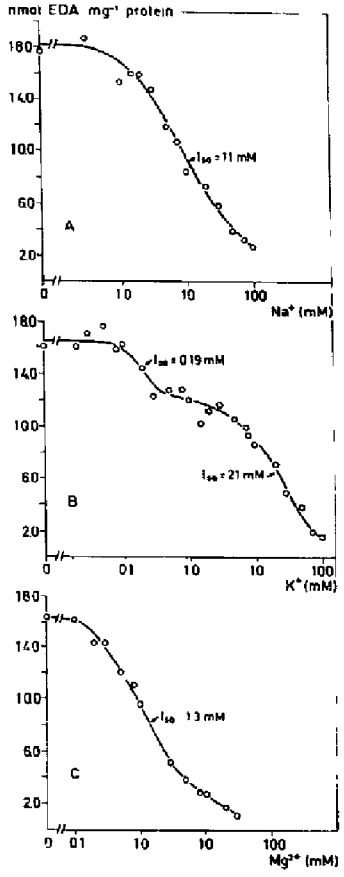


Fig 10 Mono- and divalent cation antagonism on ethylenediamine binding Binding occurs at 100 μM initial ethylenediamine (EDA) in the presence of the indicated Na^+ (A), K^+ (B) and Mg^{2+} (C) concentrations Also indicated are the I_{50} values for the cations under these conditions The K_i value for the Na^+ antagonism in (A) as determined by linear regression analysis of the Dixon plot (reciprocal binding vs the concentration of Na^+) is 17 mM (Table IV)

TABLE IV

Inhibition of ethylenediamine binding by various ligands

The effects of various cations on ethylenediamine binding have been analysed from Dixon plots of reciprocal binding at three different ethylenediamine concentrations (25 30, 50 and 100 μM) as a function of the indicated cation concentrations K_i values are defined by the common intersections of the graphs, lying on the abscissa (Na^+, K^+ > 1mM, triallylamine) or above the abscissa at the level of the reciprocal binding capacity in the absence of the pertinent cation (K^+ <1 mM, Mg^{2+})

Ligand	Concn (mM)	K_i (mM)
Na^+	0–75	17–20
K^+	0–0 5	0 2
K^+	1–50	20
Mg^{2+}	0–5	0 4
Triallylamine	0–75	18–23

Discussion

Inhibition of cation activations

Ethylenediamine with a distance of 3 7 Å between its two positive charges in the relaxed *trans*-configuration (2 7 Å in the opposite *cis*-configuration [1]) appears to be an excellent probe for the interaction of cations with Na^+/K^+-ATPase, e g Na^+, as appears from comparison of its inhibitory potency on the Na^+/K^+-ATPase and 4-nitrophenylphosphatase activity at 100 and 0 0 mM Na^+, respectively Under similar assay conditions of buffer, activating cations (K^+, Mg^{2+}) pH and temperature this Na^+ concentration difference leads to a 43% decrease in I_{50} for the diamine (35 vs 20 mM) In the Na^+-activated phosphorylation the competition with ethylenediamine is even more unambiguous since here the assay conditions are identical except for the Na^+ concentration An increase of this cation from 0 5 to 3 mM increases the I_{50} for ethylenediamine several-fold (from 0 18 to 3 1 mM) The 6-fold increase in I_{50} for ethylenediamine upon increasing the K^+ concentration in the 4-nitrophenylphosphatase assay 15-fold (from 1 to 15 mM) indicates interaction with the K^+ activation sites for this activity as well Interaction with Mg^{2+} activation sites appears to be more indirect, but present All these cation activations take place at the inner membrane side [19–21], i e , at the target side for ethylenediamine inhibition (Van der Hijden et al , unpublished data)

On the other hand, the inhibition of amino-buffer associated phosphorylation by ethylenediamine must be transmembranal as ethylenediamine inhibits at the cytoplasmic side and amino-buffers activate at the extracellular side (Van der Hijden et al , unpublished data) As shown in this paper the amino-buffer associated increase of the phosphorylation level is due to an inhibition of dephosphorylation, which in turn is caused by μmolar K^+ that accompanies the enzyme preparation [13] Since K^+ activates dephosphorylation at the extracellular side [22] amino-buffer cations may enhance the phosphorylation level by direct competition with K^+ This would agree with the stoichiometry of 2 for activation by triallylamine as 2 K^+ ions are involved in the dephosphorylation process [23] assuming that the stoichiometry of subsequent occlusion is the same That triallylamine is a stronger inhibitor of dephosphorylation than imidazole may be due to the more lipophylic nature of the triallyl substituted nitrogen compound In addition to its competition versus K^+ it may also hinder, by virtue of lipophylic interactions, access of water to the phosphorylation site

Comparison of inhibition by, and binding of ethylenediamine

The $K_{0.5}$ (43 ± 6 μM, Table III) for equilibrium binding of ethylenediamine in the presence of triethanolamine as kinetically 'inert' buffer [1] is of the same order of magnitude as the I_{50} value (75 μM) determined for phosphorylation in the same buffer at $Na^+ \rightarrow 0$ mM (Results), indicating that binding and inhibition describe the same phenomenon The competitive action of Na^+, K^+ and triallylamine on the binding of the inhibitor is also in agreement with their competitive nature in the kinetic experiments described above, but their I_{50} values in the binding assays are higher For instance, 20 mM Na^+ abolishes the inhibition of phosphorylation by ethylenediamine (0 35–2 1 mM) almost completely (Fig 2), whereas 20 mM Na^+ abolishes the binding of ethylenediamine (0 025–0 1 mM) only half-maximally (Table IV) A similar discrepancy is found for Mg^{2+} 36 μM increases the I_{50} for ethylenediamine in Na^+ (1mM) activated phosphorylation 2-fold (Fig 7B), whereas a 10-fold higher K_i (0 4 mM, Table IV) for Mg^{2+} is recorded in the binding studies The difference may be reconciled by the data of Pedemonte and Kaplan [24], showing that phosphorylation may reduce the sensitivity to electrophiles Hence, Na^+ may overcome the weaker inhibition, that occurs during phosphorylation, with more ease than it reduces the stronger binding in the absence of phosphorylation As for Mg^{2+}, this cation is known to display high affinity for the phosphoenzyme anyway [25]

Since Mg^{2+} probably inhibits ethylenediamine binding from a different site, as indicated by the saturation curves of inhibition (Fig 7), we expect mixed-type inhibition in the binding experiments The Dixon formulation for this type of inhibition is

$$\frac{[E]_{tot}}{[ES]+[EIS]} = \frac{1+([I]/K_i)}{([S]/K_S)(1+([I]/\alpha K_i))}+1$$

relating the fraction of total enzyme containing the ligand S with the concentration of ligand and inhibitor (I) K_s, K_i and αK_i ($\alpha > 1$) are the dissociation constants of the ES, EI and EIS complexes, respectively Plotting the reciprocal fraction as a function of $[I]/K_i$ ($= 0–5$, $[S]/K_s = 0\,5–2$) yields curved lines, except when $\alpha \geq 5$ The plots then become virtually straight, intersecting one unit above the abscissa (the position of the reciprocal maximal binding capacity in the absence of inhibitor) in agreement with our binding data

The antagonism, exerted by triallylamine on ethylenediamine binding in the absence of phosphorylation ($K_i = 18–23$ mM, Table IV) is more commensurate with the affinity of the enzyme for this amino-buffer in the presence of ethylenediamine during phosphorylation (K_m for triallylamine = 26 mM at ethylenediamine = 0 1 mM, Fig 5B) This could mean that phosphorylation has no profound influence on the affinity for the extracellularly activating amino-buffers, although it causes a strong affinity increase for K^+ at the same membrane side [26] with which the amino-buffers are thought to compete [13]

More direct may be the competition exerted at low concentrations of K^+ (< 1 mM, $K_i = 0.2$ mM, Table IV and Fig 10C) although intersection of the Dixon plots in this concentration range still indicates a stoichiometry of 7 ethylenediamine binding sites per phosphorylation site (enzyme No 3 in Table III) Thus the number of the ethylenediamine binding sites and K^+ occlusion sites (2 per functional unit [23]) do not match Ethylenediamine, however, strongly inhibits ($K_i = 0.06$mM [27]) the occlusion of Rb^+ ($E_1 \xrightarrow{Rb^+} E_2Rb$) or a similar transition ($E_1 \xrightarrow{K^+} E_2K$) as induced by K^+ [28] with a similar K_d for K^+ as the K_i reported above

Another matter of concern is the ouabain insensitivity of the ethylenediamine binding as noticed by us, while binding of Na^+ or K^+ and the occlusion of K^+ has been reported to be ouabain-sensitive [17,29] On the other hand, ouabain-stabilized occlusion of Na^+ as well as K^+ has also been described [30,31], even under non-phosphorylating conditions, which may indicate, like our stoichiometries, a multiplicity of cation binding sites So far, only part of the single-charged cation binding has been reported to be ouabain-sensitive Hence, the reason for resistance to ouabain of ethylenediamine binding may be caused by the dual charge, which is involved in tight binding of the diamine (Fig 8)

Ethylenediamine as structural probe

The presence of dual positive charge on ethylenediamine as prerequisite for strong inhibition of phosphorylation could inevitably only be tested in the presence of Na^+ as much more potently activating cation than the amino-buffers The latter fail to give significant phosphorylation levels above the pK value (7.5) of the second protonated group of the inhibitor [11] due either to a reduced affinity for the amino-buffer cation or an increased dephosphorylation rate The forementioned dual charge at a distance of 3.7 Å in the relaxed anti-configuration of ethylenediamine could mean that activating cation binding sites, which could be occupied either by Na^+ or K^+, are such a distance apart The effects on ATP and Mg^{2+} binding (the two other effectors of phosphorylation) are more remote, judging from the moderate or limited effects of the inhibitor on the $K_{0.5}$ for ATP and Mg^{2+}, although these effects may equally well reveal a distortion of the enzyme, which could cause the inhibition of the phosphoryl transfer

In order to allow the distance of 3.7 Å by ethylenediamine to be spanned, the negative charges of aspartic or glutamic acid residues in the proteins should be either 2–3 amino acid residues apart in the helix or only 1 in the pleated sheet structure Helix and pleated sheet structures are present in a ratio of 1.3 : 1 in the extramembraneous domains of the enzyme's α-subunit [32] An analogy is found in the Asp-36 to Asp-38 spanning property of ethylenediamine toward bacteriorhodopsin [33]

We arrive now at the general principle that cations can induce conformational changes into the enzyme that may eventually lead to an inhibition of phosphorylation, even though the inhibitor does not or hardly affects the affinity for substrate Ethylenediamine, like other amines, increases the fluorescence of eosin in the presence of enzyme, eosin being a probe for high-affinity ATP binding to the E_1-conformation of the enzyme [34] A similar strong E_1-conformation inducing effect of ethylenediamine has been detected with the covalently bound fluorescent probe fluorescein isothiocyanate [28] Hence, ethylenediamine does not inhibit the transition from the low-affinity substrate binding E_2 to the high-affinity substrate binding conformation E_1, nor does it increase dephosphorylation (Table II) Consequently, it must deteriorate by conformational change the juxtaposition of the ATP-binding Lys-719 residue and the phosphorylation site (Asp-369 [35]) In conclusion, we favour the concept that ethylenediamine by occupation of cation activation sites inhibits the phosphoryl transfer from ATP to the enzyme

References

1 Schuurmans Stekhoven F M A H Swarts H G P., 't Lam G K Zou Y S and De Pont, J J H H M (1987) Biochim Biophys. Acta 937 161–176
2 Schuurmans Stekhoven F M A.H Swarts, H G P 't Lam, G K Zou Y S and De Pont J J H H M (1988) The Na^+ K^+ pump (Proc 5th Int Conf Na,K-ATPase) (Skou, J C., Nørby J G., Maunsbach, A B and Esmann, M., eds.), pp 355–362, Alan R Liss, Inc New York
3 Jørgensen P L (1974) Biochim Biophys. Acta 356 36–52
4 Schoot, B M, Schoots A F M De Pont J J H H M Schuurmans Stekhoven, F M A H and Bonting, S L. (1977) Biochim Biophys Acta 483 181–192
5 Van Veldhoven, P P and Mannaerts, G P (1987) Anal Biochem 161, 45–48
6 Schuurmans Stekhoven, F M A H., Swarts, H G P., Fu, Y F., Kuypers G A J De Pont J J H H M and Bonting, S L (1984) Biochim Biophys. Acta 774, 277–287
7 Sillén L G and Martell A E (1964) Stability Constants of Metal-Ion Complexes, The Chemical Society, London Special Publication No 17
8 Swarts H G P., Zwartjes, H.A H.T., Schuurmans Stekhoven, F M A H and De Pont J J H H M. (1987) Biochim Biophys Acta 903, 525–532
9 Schuurmans Stekhoven, F M A H Swarts H G P De Pont, J J H H M and Bonting, S L. (1981) Biochim Biophys. Acta 649, 533–540
10 Segel I H (1975) Enzyme Kinetics pp 385–387 Wiley-Interscience, New York
11 Schuurmans Stekhoven, F M A.H., Swarts, H G P., De Pont J J H H M and Bonting, S L (1985) Biochim Biophys Acta 815 16–24
12 Nørby, J G and Jensen J (1971) Biochim Biophys Acta 233 104–116
13 Schuurmans Stekhoven F M A.H., Swarts, H G P De Pont

J J H H M and Bonting, S L (1986) Biochim Biophys Acta 855, 375–382
14 Schuurmans Stekhoven, F M A H, Swarts, H G P, Helmich-De Jong M L De Pont, J J H H M and Bonting S L (1986) Biochim Biophys Acta 854, 21–30
15 Segel, I H (1975) Enzyme Kinetics, p 370, Wiley Interscience, New York
16 Yamaguchi, M and Tonomura, Y (1980) J Biochem 88, 1365–1375
17 Matsui H and Homareda H (1982) J Biochem 92, 193–217
18 Grisham, C M and Mildvan, A S (1974) J Biol Chem 249, 3187–3197
19 Post R L Merrit, C R Kinsolving C R and Albright C D (1960) J Biol Chem 235, 1796–1802
20 Glynn, I M (1961) J Physiol 160, 18–19 P
21 Blostein R., Pershadsing, H A, Drapeau, P and Chu, L (1979) Na,K-ATPase Structure and Kinetics (Skou, J C and Nørby, J G, eds), pp 233–245, Academic Press, New York
22 Blostein, R and Chu, L (1977) J Biol Chem 252, 3035–3043
23 Forbush III, B (1987) J Biol Chem 262, 11104–11115
24 Pedemonte, C H and Kaplan J H (1986) J Biol Chem 261 16660–16665
25 Fukushima, Y and Post R L (1978) J Biol Chem 253, 6853–6862
26 Robinson, J D (1973) Arch Biochem Biophys 156, 232–243
27 Forbush III, B (1988) J Biol Chem 263, 7979–7988
28 Mezele M, Lewitzki E., Ruf, H and Grell, E (1988) Ber Bunsenges Phys Chem 92, 998–1004
29 Glynn, I M and Richards, D E (1982) J Physiol 330, 17–43
30 Forbush III B (1983) Curr Top Membr Transp 19, 167–201
31 Forbush III, B (1987) Biophys J 51 179a
32 Ovchinnikov, Y A, Arystarkhova, E A, Arzamazova, N M, Dzhandzhugazyan, K N, Efremov, R G, Nabiev, I R and Modyanov, N N (1988) FEBS Lett 227, 235–239
33 Ovchinnikov, Y A, Abdulaev, N G, Vasilov, R G, Vtiurina, I Y, Kuryatov A B and Kiselev A V (1985) FEBS Lett 179, 343–350
34 Skou, J C and Esmann, M (1981) Biochim Biophys Acta 647, 232–240
35 Ohta, T Nagano, K and Yoshida M (1986) Proc Natl Acad Sci USA 83, 2071–2075