THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 40, Issue of October 5, pp. 36909–36916, 2001

Printed in U.S.A.

K^+-independent Gastric H^+,K^+-ATPase Activity

DISSOCIATION OF K^+-INDEPENDENT DEPHOSPHORYLATION AND PREFERENCE FOR THE E_1 CONFORMATION BY COMBINED MUTAGENESIS OF TRANSMEMBRANE GLUTAMATE RESIDUES*

Received for publication, May 2, 2001, and in revised form, June 22, 2001
Published, JBC Papers in Press, July 24, 2001, DOI 10.1074/jbc.M103945200

Herman G. P. Swarts, Jan B. Koenderink, Harm P. H. Hermsen, Peter H. G. M. Willems, and Jan Joep H. H. M. De Pont‡

From the Department of Biochemistry, Institute of Cellular Signalling, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

Several mutations of residues Glu^{795} and Glu^{820} present in M5 and M6 of the catalytic subunit of gastric H^+,K^+-ATPase have resulted in a K^+-independent, SCH 28080-sensitive ATPase activity, caused by a high spontaneous dephosphorylation rate. The mutants with this property also have a preference for the E_1 conformation. This paper investigates the question of whether these two phenomena are coupled. This possibility was studied by combining mutations in residue Glu^{343}, present in M4, with those in residues 795 and 820. When in combined mutants Glu and/or Gln residues were present at positions 343, 795, and 820, the residue at position 820 dominated the behavior: a Glu giving K^+-activated ATPase activity and an E_2 preference and a Gln giving K^+-independent ATPase activity and an E_1 preference. With an Asp at position 343, the enzyme could be phosphorylated, but the dephosphorylation was blocked, independent of the presence of either a Glu or a Gln at positions 795 and 820. However, in these mutants, the direction of the $E_2 \leftrightarrow E_1$ equilibrium was still dominated by the 820 residue: a Glu giving E_2 and a Gln giving E_1. This indicates that the preference for the E_1 conformation of the E820Q mutation is independent of an active dephosphorylation process.

Negatively charged residues within transmembrane segments of P-type ATPases are assumed to be involved in cation binding and transport (1–3). This is also the case for the gastric H^+,K^+-ATPase. Site-directed mutagenesis of residue Glu^{343} in transmembrane segment four[1] (4, 5), Glu^{795} in transmembrane segment five (6, 7), and Glu^{820} in transmembrane segment six (6, 8) provided strong evidence that these three residues are involved in K^+-stimulation of the ATPase activity. The specificity of these residues is different. Glu^{820} can only be replaced by Asp, indicating that the negative charge of this residue is essential for K^+ activation (8). At position 795, Gln, but not Asp or Asn, can replace Glu resulting in a small increase in K^+ affinity, suggesting that a side chain with a carbonyl residue and with the right length is necessary and sufficient (7). At position 343, only Gln can replace Glu, but this results in a reduction in K^+ affinity, suggesting that the Glu on this position is the preferable amino acid residue (4, 5). Interestingly, several other mutants of Glu^{343}, like E343D, could still be phosphorylated, but no K^+-dependent stimulation of the dephosphorylation of the phosphorylated intermediate was found (5).

In previous studies, we observed that some mutants of Glu^{820} (E820Q, E820A, E820N) (9) and Glu^{795} (E795L, E795A) (7) possessed a relatively high ATPase activity in the absence of K^+. This K^+-independent ATPase activity (also named constitutive ATPase activity) was caused by an enhanced spontaneous dephosphorylation rate of the phosphorylated intermediate (Fig. 1, *step 4*). We postulated that the altered cation-binding site mimics the stimulatory effect of the K^+-filled binding pocket on the hydrolysis of the aspartyl phosphate in the phosphorylated enzyme.

The ATPase activity of these mutants was, like that of the wild type enzyme, very sensitive to inhibition by SCH 28080.[2] In addition, the steady-state ATP-dependent phosphorylation level of the wild-type enzyme as well as of K^+-sensitive mutants was decreased by SCH 28080 (10). In contrast, the steady-state phosphorylation level of the constitutively active mutants was only partly decreased by SCH 28080, and that of the mutant E820Q was even enhanced (10). The latter increase was also found in five double mutants of Glu^{795} and Glu^{820} that displayed K^+-independent ATPase activity (11).

This anomalous behavior of constitutively active mutants was explained by differences in the $E_2 \leftrightarrow E_1$ equilibrium between the enzymes with K^+-dependent and K^+-independent ATPase activity (10). K^+-dependent enzymes are assumed to be present in the E_2 form. Upon preincubation of these enzymes with SCH 28080, a complex is formed (see Fig. 1, *step 7*), and ATP-dependent phosphorylation (*steps 1* and *2*) can no longer occur. Mutants with K^+-independent ATPase activity are assumed to be mainly present in the E_1 form. Preincubation with SCH 28080 does not result in an E_2·SCH complex, and upon addition of ATP, phosphorylation occurs, finally resulting in formation of the phosphorylated intermediate E_2-P (Fig. 1, *steps 2* and *3*). Next, E_2-P forms a stable complex with SCH 28080 (*step 5*) and is accumulated, since its dephosphorylation rate (*step 6*) is slower than that of the phosphorylated intermediate formed in the absence of SCH 28080 (*step 4*) (10).

The preference of mutants with constitutive ATPase activity for the E_1 form was confirmed by recent studies with vanadate (11). Vanadate reacts with the E_2 form of the enzyme (Fig. 1, *step 8*). We found that the ATPase activity of mutants with K^+-independent ATPase activity was much less sensitive toward vanadate than that of enzymes with K^+-dependent

* This work was supported by The Netherlands Foundation for Scientific Research Grant 805-05-041. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 31-24-36-14260; Fax: 31-24-36-16413; E-mail: J.depont@bioch.kun.nl.

[1] Glu^{345} in the rabbit enzyme.

[2] The abbreviation used is: SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2α]-pyridine.

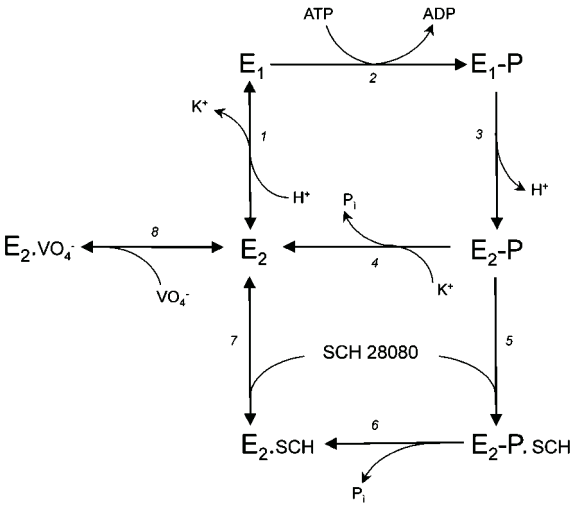

FIG. 1. **Albers-Post scheme for H^+,K^+-ATPase, including inhibition mechanisms for SCH 28080 and vanadate.**

ATPase activity. The K^+-independent mutants have thus a relatively high preference for the E_1 form.

The present study aims to investigate whether this preference of the K^+-independent mutants for the E_1 conformation and the high spontaneous dephosphorylation rate are two coupled processes. This was performed by combining mutations of residue Glu^{343}, which block the dephosphorylation step (4, 5), with the E820Q mutation, which gives constitutive ATPase activity (9). The present study shows that the preference for the E_1 conformation in the E820Q mutant also occurs when the dephosphorylation process is blocked, indicating that these two phenomena can be uncoupled.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The cDNAs of the rat gastric H^+,K^+-ATPase α- and β-subunits were cloned in the pFastbacdual vector (Life Technologies, Inc.) behind the polyhedrin and the p10 promotor, respectively (12). Site-directed mutagenesis on Glu^{343} was performed using the Altered Sites II *in vitro* mutagenesis system (Promega, Madison, WI). The mutagenic primer (Biolegio, Malden, The Netherlands) introduced the desired mutation (E343A, E343D, E343L, and E343Q) in the α-subunit together with a silent mutation, thereby creating or deleting a specific restriction site, useful for detection of the mutants. The *Sgr*AI/*Mlu*I fragment (870 bp) was subcloned in the pFastbacdual vector containing either the wild type cDNA or the previously constructed E820Q, E795Q, or E795Q/E820Q cDNAs (7, 11). After selection, the mutants were checked by sequence analysis. All DNA manipulations were carried out according to standard molecular biology techniques described by Sambrook *et al.* (13).

Generation of Recombinant Viruses—The pFastbacdual transfer vector containing the different (mutant) cDNAs was transformed to competent DH10bac *Escherichia coli* cells (Life Technologies) harboring the baculovirus genome (bacmid) and a transposition helper vector. Upon transposition between the Tn7 sites in the transfer vector and the bacmid, recombinant bacmids were selected and isolated (14). Subsequently, insect *Spodoptera frugiperda* Sf9 cells were transfected with recombinant bacmids using Cellfectin reagent (Life Technologies, Breda, The Netherlands). After a 3-day incubation period, recombinant baculoviruses were isolated and used to infect Sf9 cells at a multiplicity of infection of 0.1. Four days after infection, the amplified viruses were harvested.

Preparation of Sf9 Membranes—Sf9 cells were grown at 27 °C in 100-ml spinner flask cultures as described by Klaassen *et al.* (12). For production of H^+,K^+-ATPase, $1.5{\cdot}10^6$ cells·ml^{-1} were infected at a multiplicity of infection of 1–3 in the presence of 1% (v/v) ethanol (15), and 0.1% (w/v) pluronic F-68 (Sigma) in Xpress medium (BioWhittaker, Walkersville, MD) as described before (9). After 3 days, the Sf9 cells were harvested by centrifugation at 2000 × *g* for 5 min. Membranes were resuspended at 0 °C in 0.25 M sucrose, 2 mM EDTA, and 25 mM Hepes/Tris (pH 7.0) and sonicated twice for 30 s at 60 watts (Branson Power Company, Denbury, CT). After centrifugation for 30 min at 10,000 × *g*, the supernatant was collected and recentrifuged for 60 min at 100,000 × *g* at 4 °C. The pelleted membranes were resuspended in the above mentioned buffer and stored at −20 °C.

Protein Determination—The protein concentrations were quantified with the modified Lowry method according to the method of Peterson (16) using bovine serum albumin as a standard.

Western Blotting—Protein samples from the membrane fraction were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer and separated on SDS gels containing 10% acrylamide according to Laemmli (17). For immunoblotting, the separated proteins were transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The α-subunit of the gastric H^+,K^+-ATPase was detected with the polyclonal antibody HKB (18).

ATPase Assay—The SCH 28080-sensitive ATPase activity was determined using a radiochemical method (19). For this purpose, 0.6–5 μg of Sf9 membranes were added to 100 μl of medium, which contained 10 μM [γ-^{32}P]ATP (specific activity 20–100 mCi·$mmol^{-1}$), 1.2 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM EDTA, 0.1 mM ouabain, 1 mM NaN_3, 25 mM Tris-HCl (pH 7.0), and various concentrations of KCl in the presence and absence of 0.1 mM SCH 28080. After incubation for 30 min at 37 °C, the reaction was stopped by adding 500 μl of 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid, and after 10 min at 0 °C the mixture was centrifuged for 10 s (10,000 × *g*). To 0.2 ml of the clear supernatant, containing the liberated inorganic phosphate ($^{32}P_i$), 3 ml of OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added, and the mixture was analyzed by liquid scintillation analysis. In general, blanks were prepared by incubating in the absence of membranes. The H^+,K^+-ATPase activity is presented as the difference of the activity in the absence and presence of SCH 28080.

ATP-dependent Phosphorylation Capacity—ATP-dependent phosphorylation was determined as described before (8, 9, 15). Sf9 membranes were incubated at 21 °C (1–6 μg) in 50 mM Tris-acetic acid (pH 6.0) and 1.2 mM $MgCl_2$ in a volume of 50 μl. After 30–60 min of preincubation, 10 μl of 0.6 μM [γ-^{32}P]ATP (specific activity 50–200 Ci·$mmol^{-1}$) was added, and the mixture was incubated for 10 s at 21 °C. The reaction was stopped by adding ice-cold 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid, and the phosphorylated protein was collected by filtration over a 0.8-μm membrane filter (Schleicher and Schuell). After washing twice with 5 ml of stop-solution, the filters were analyzed by liquid scintillation analysis. Data are corrected for the levels of phosphorylated protein obtained with mock-infected membranes.

Dephosphorylation Studies—After ATP-dependent phosphorylation, the reaction mixture was diluted from 60 to 500 μl with nonradioactive ATP (final concentration 10 μM; in order to prevent rephosphorylation with radioactive ATP) in 50 mM Tris-acetic acid (pH 6.0) and 0–100 mM KCl. The mixture was further incubated for 3 s at 21 °C. Thereafter, the reaction was stopped as described above, and the residual protein phosphorylation levels were determined.

Chemicals—Cellfectin, competent DH10bac *Escherichia coli* cells, and all enzymes used for DNA cloning were purchased from Life Technologies. [γ-^{32}P]ATP (3000 Ci·$mmol^{-1}$ was obtained from Amersham Pharmacia Biotech. SCH 28080 was kindly provided by Dr. C.D. Strader (Schering-Plough, Kenilworth, NJ). The antibodies HKB and 2G11 were gifts from Dr. M. Caplan (Yale) and Dr. J. Forte (Berkeley), respectively.

Analysis of Data—All data are presented as mean values for 2–5 individual preparations with S.E. Differences were tested for significance by means of the Student's *t* test. IC_{50} and $K_{0.5}$ values were determined by analyzing the plots using the Non-Linear Curve Fitting program (Hill equation function) of Origin 6.1 (Microcal, Northampton, MA).

RESULTS

In this study, we produced a series of mutants of gastric H^+,K^+-ATPase in which Glu^{343}, Glu^{795}, and Glu^{820} were replaced, yielding single, double, and triple mutations in the putative K^+-binding domain. For clarity, these mutants are indicated with their single letter code. The first letter indicates the amino acid at position 343, the second at position 795, and the third at position 820 (*e.g.* DQQ is mutant E343D/E795Q/E820Q). The α-subunit of all mutants expressed in the Sf9 membrane fractions could be detected by the HKB antibody (18) (Fig. 2). The β-subunit was present both in a carbohydrate-free and a core-glycosylated form, similarly as previously reported (12) (data not shown).

Although quantitative analysis of the blots is ambiguous, Fig. 2 suggests that the level of expression of the mutants is rather variable. It is known, however, that part of the protein expressed using the baculovirus expression system is inactive (6). Importantly, the maximal phosphorylation level (expressed per mg of protein) of the various mutants is rather constant. This suggests that in this system, relatively more active protein is produced when the expression level is low. It might be that at very high protein expression levels the amount of chaperones is not sufficient for correct protein folding. We therefore express below the ATPase activity and phosphorylation levels per mg of protein and not per unit of expressed H^+,K^+-ATPase.

Mutagenesis of Glu^{343}

First, four single mutants of Glu^{343} were produced and characterized. These mutants were phosphorylated by ATP, to a similar steady-state level (AEE, 5.5 ± 0.6; DEE, 5.5 ± 1.0; LEE, 7.5 ± 2.0; QEE, 5.5 ± 1.3; values in pmol per mg of protein) as the wild type enzyme (EEE, 5.7 ± 0.5 pmol per mg of protein; Fig. 3*A*). ATP-dependent phosphorylation was completely prevented by preincubation with 0.1 mM SCH 28080. Moreover, the ATPase activities of these mutants measured in the absence of K^+ were comparable with the activities of membranes from mock-infected cells and were not sensitive to SCH 28080. This indicates that they did not possess K^+-independent ATPase activity, as found with some Glu^{795} (7) and Glu^{820} (9) mutants (see also below). The AEE, DEE, and LEE mutants showed, in contrast to the QEE enzyme, no K^+-activated ATPase activity (Fig. 3*B*). The apparent K^+ sensitivity of the latter mutant ($K_{0.5}$ = 0.43 ± 0.04 mM) was about 5 times reduced, compared with that of the wild type enzyme ($K_{0.5}$ = 0.08 ± 0.01 mM). The ATPase activity of both the wild type enzyme and the QEE mutant was inhibited by high K^+ concentrations due to K^+/ATP antagonism that is relatively large at the low (10 μM) ATP concentration used (19). The maximal K^+-stimulated ATPase activity of the QEE mutant (0.19 ± 0.04 μmol of P_i·mg^{-1} protein·h^{-1}) tended to be lower than that of the wild type enzyme (0.30 ± 0.04 μmol of P_i·mg^{-1} protein·h^{-1}). The experiments depicted in Fig. 3*C* show that the dephosphorylation of the AEE, DEE, and LEE phosphointermediates was not stimulated by K^+, which is in line with the lack of K^+-stimulated ATPase activity. The QEE mutant was dephosphorylated by K^+, but the apparent K^+ affinity (0.16 ± 0.02 mM) was 8 times lower than that of the wild type enzyme (0.02 ± 0.01 mM). These results confirm those of Asano *et al.* (4, 5), who expressed these mutants in HEK293 cells. This indicates that expression of these mutants in HEK293 and Sf9 cells yields similar results.

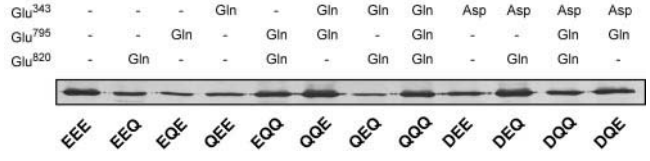

FIG. 2. **Western blot of the expressed H^+,K^+-ATPase mutants.** Membrane proteins (5 μg) isolated from Sf9 cells expressing the recombinant H^+,K^+-ATPase were separated using SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedures." The presence of the α-subunit was detected using the polyclonal antibody HKB (18). The mutations are given with their single letter code (*e.g.* DQQ represents E343D/E795Q/E820Q) and are shown *above* the gel.

Double and Triple Mutants of Glu^{343}, Glu^{795}, and Glu^{820}

Next, we produced H^+,K^+-ATPase mutants of Glu^{343}, Glu^{795}, and Glu^{820} in different combinations. At position 343, either an Asp (no K^+-stimulated activity) or a Gln (reduced K^+ affinity) was used, whereas at positions 795 and 820, Glu was replaced by a Gln. All 12 possible combinations were expressed as shown by Western blotting (Fig. 2). In these 12 combinations, the wild type enzyme (EEE), the previously studied mutants EQE (7), EEQ (9), and EQQ (11), and the Glu^{343} single mutants QEE and DEE (Fig. 3) were included for comparison.

All mutants were functionally active as indicated by ATP-dependent phosphorylation. After determining (i) the steady-state ATP-dependent phosphorylation level, (ii) the K^+ dependence of the ATPase reaction, and (iii) the effect of K^+ on the dephosphorylation process, the mutants and the wild type enzyme were divided in three groups: (i) K^+-sensitive mutants (QEE, EQE, QQE) including the wild type enzyme (EEE), (ii) constitutively active mutants (EEQ, QEQ, EQQ, QQQ), and (iii) nondephosphorylating mutants (DEE, DQE, DEQ, DQQ).

K^+-sensitive Mutants—Fig. 4*A* shows that the wild type enzyme (EEE) and the three mutants QEE, EQE, and QQE were phosphorylated by ATP. The phosphorylation levels varied somewhat but were not significantly different from the wild type enzyme (EEE, 5.7 ± 0.5; EQE, 3.8 ± 0.5; QEE, 5.5 ± 1.3; QQE, 8.4 ± 1.8; values in pmol/mg of protein). SCH 28080 (0.1 mM) completely prevented ATP-dependent phosphorylation of these preparations. The EQE mutant had a slightly higher K^+ sensitivity (0.03 ± 0.01 mM) in the ATPase reaction than the wild type enzyme (0.08 ± 0.01 mM). The apparent K^+ affinity of the QQE mutant (0.56 ± 0.08 mM) was, like that of the QEE mutant (0.43 ± 0.04 mM), reduced by approximately 1 log unit (Fig. 4*B*). The maximal activity of the QQE mutant (0.30 ± 0.11 μmol of P_i·mg^{-1} protein·h^{-1}) was similar to that of the wild type enzyme (0.30 ± 0.04 μmol of P_i·mg^{-1} protein·h^{-1}) and somewhat, although not significantly, higher than that of the QEE (0.19 ± 0.04 μmol of P_i·mg^{-1} protein·h^{-1}) and EQE (0.19 ± 0.02 μmol of P_i·mg^{-1} protein·h^{-1}) mutants. The data

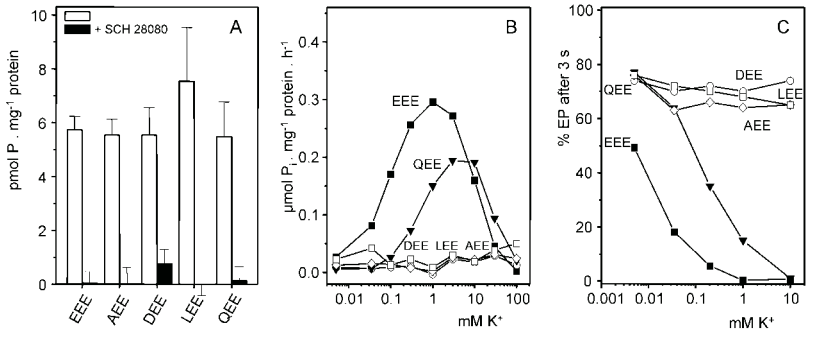

FIG. 3. **Properties of the Glu^{343} mutants.** The ATP phosphorylation capacity (*A*), the ATPase activity (*B*), and the dephosphorylation properties (*C*) of the Glu^{343} mutants are presented. After preparing the Sf9 membranes, the ATP phosphorylation level, the dephosphorylation rate and the ATPase activity in the presence of the indicated K^+ concentration were determined as described under "Experimental Procedures." ATP-dependent phosphorylation (*A*) capacity was determined both in the absence and presence of 0.1 mM SCH 28080. Mean values are given for 2–5 independent membrane preparations; *bars* in *A* represent S.E. values.

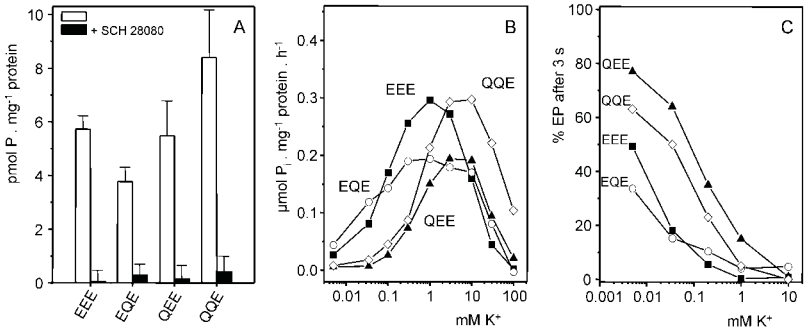

FIG. 4. **Properties of the K⁺-sensitive H⁺,K⁺-ATPase mutants.** The ATP phosphorylation capacity (*A*), the ATPase activity (*B*), and the dephosphorylation properties (*C*) of the K^+-sensitive mutants are presented. After preparing the Sf9 membranes, the ATP phosphorylation level, the dephosphorylation rate, and the ATPase activity in the presence of the indicated K^+ concentration were determined as described under "Experimental Procedures." ATP-dependent phosphorylation (*A*) capacity was determined both in the absence and presence of 0.1 mM SCH 28080. Mean values are given for 2–5 independent membrane preparations; *bars* in *A* represent S.E. values.

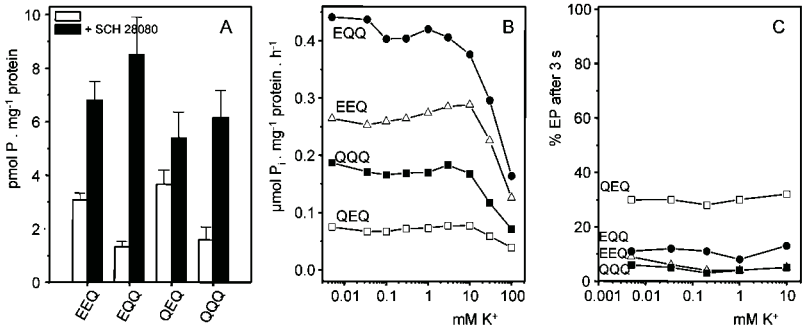

FIG. 5. **Properties of the constitutively active H⁺,K⁺-ATPase mutants.** The ATP phosphorylation capacity (*A*), the ATPase activity (*B*), and the dephosphorylation properties (*C*) of the constitutively active mutants are presented. After preparing the Sf9 membranes, the ATP-dependent phosphorylation level, the dephosphorylation rate, and the ATPase activity in the presence of the indicated K^+ concentration were determined as described under "Experimental Procedures." ATP-dependent phosphorylation (*A*) capacity was determined both in the absence and presence of 0.1 mM SCH 28080. Mean values are given for 2–4 independent membrane preparations; *bars* in *A* represent S.E. values.

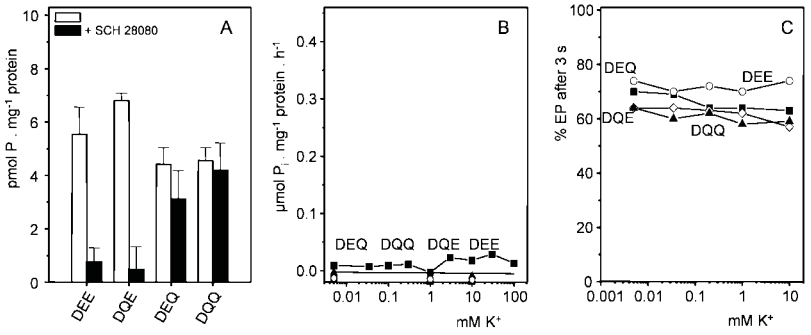

FIG. 6. **Properties of nondephosphorylating H⁺,K⁺-ATPase mutants.** The ATP phosphorylation capacity (*A*), the ATPase activity (*B*), and the dephosphorylation properties (*C*) of the K^+-insensitive mutants are presented. After preparing the Sf9 membranes, the ATP-dependent phosphorylation level, the dephosphorylation rate, and the ATPase activity in the presence of the indicated K^+ concentration were determined as described under "Experimental Procedures." ATP-dependent phosphorylation (*A*) capacity was determined both in the absence and presence of 0.1 mM SCH 28080. Mean values are given for two or three independent membrane preparations; *bars* in *A* represent S.E. values.

from the dephosphorylation experiments were in line with those of the ATPase assays, showing, a slightly higher apparent K^+ affinity for the EQE mutant (0.01 ± 0.01 mM) and a reduced apparent affinity for the QEE (0.16 ± 0.02 mM) and QQE (0.08 ± 0.02 mM) mutants, compared with the wild type enzyme (0.02 ± 0.01 mM) (Fig. 4*C*). If the values for the ATPase activities were divided by those of the maximal steady state phosphorylation levels, "turnover numbers" that were not significantly different (varying between 576 and 877 min^{-1}) were obtained. These turnover numbers are an underestimation of the actual turnover numbers because of the low ATP concentration used in the ATPase assay. Thus, with a Gln at position 343 and/or position 795, the ATPase activity was K^+-sensitive if the native Glu was present at position 820. In the case of a Gln at position 343, the K^+ affinity was 1 log unit lower than with a Glu at this position.

Constitutively Active Mutants—The phosphorylation levels of the four mutants with a Gln at position 820 and either a Glu or a Gln at positions 343 and 795 were lower than those of the K^+-sensitive preparations (values in pmol/mg of protein; EEQ, 3.1 ± 0.3; EQQ, 1.3 ± 0.2; QEQ, 3.7 ± 0.5; QQQ, 1.6 ± 0.5; *cf.* Fig. 5*A* with Fig. 4*A*). The EQQ and the QQQ mutants had especially low phosphorylated intermediate levels. Preincubation of the membranes with SCH 28080 did not prevent ATP-dependent phosphorylation of these mutants. Conversely, it enhanced the steady-state phosphorylation levels (values in pmol per mg of protein; EEQ, 6.8 ± 0.7; EQQ, 8.5 ± 1.4; QEQ, 5.4 ± 1.0; QQQ, 6.2 ± 1.0) as previously shown for the EEQ and EQQ mutants (10, 11). Of note, the phosphorylation level measured in the presence of SCH 28080 was of the same order of magnitude as that of K^+-sensitive mutants measured in the absence of SCH 28080, suggesting that they have similar in-

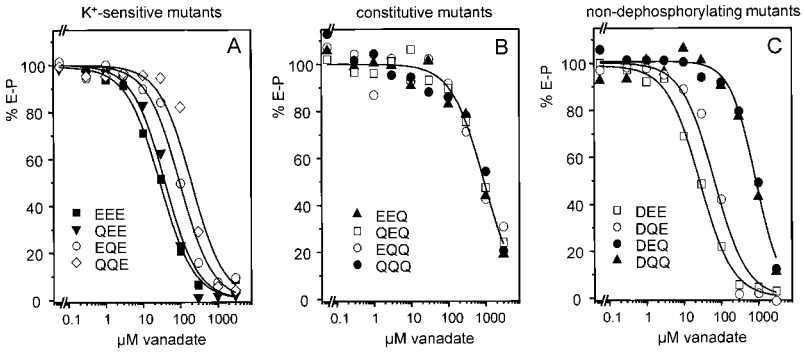

FIG. 7. **Vanadate sensitivity of the steady-state phosphorylation level of H^+,K^+-ATPase mutants.** The 12 preparations were divided into three groups, each containing four preparations: the K^+-sensitive mutants (*A*), the constitutively active mutants (*B*), and the nondephosphorylating mutants (*C*). After preparing the Sf9 membranes, the ATP-dependent phosphorylation level was determined in the presence of the indicated vanadate concentrations as described under "Experimental Procedures." The phosphorylation levels obtained in the absence of vanadate were set at 100%, and the results are expressed as function of the used vanadate concentrations. Mean values are given for two or three independent membrane preparations. The following IC_{50} values were measured: EEE, 0.03 ± 0.01 mM; QEE, 0.04 ± 0.01 mM; EQE, 0.10 ± 0.02 mM; QQE, 0.20 ± 0.05 mM; EEQ, 0.81 ± 0.12 mM; EQQ, 0.88 ± 0.18 mM; QEQ, 0.91 ± 0.10 mM; QQQ, 0.98 ± 0.20 mM; DEE, 0.03 ± 0.01 mM; DEQ 0.86 ± 0.19 mM; DQE 0.07 ± 0.01 mM; DQQ, 0.76 ± 0.14 mM.

trinsic capacities. The constitutively active mutants showed an SCH 28080-sensitive ATPase activity in the absence of K^+ (Fig. 5*B*). This activity was not affected by K^+ concentrations up to 10 mM and decreased at higher concentrations. The highest constitutive ATPase activity (0.44 ± 0.09 μmol of $P_i \cdot mg^{-1}$ protein$\cdot h^{-1}$) was obtained with the EQQ double mutant (11). Further neutralization of the putative K^+ binding pocket by an additional introduction of a Gln at position 343 (QQQ) did not enhance the constitutive ATPase activity but lowered it to 0.19 ± 0.01 μmol of $P_i \cdot mg^{-1}$ protein$\cdot h^{-1}$. Similarly, the constitutive ATPase activity of the QEQ mutant (0.08 ± 0.01 μmol of $P_i \cdot mg^{-1}$ protein$\cdot h^{-1}$) was less compared with that of the single mutant EEQ (0.26 ± 0.02 μmol of $P_i \cdot mg^{-1}$ protein$\cdot h^{-1}$). If the values for the ATPase activities were divided by those of the steady state phosphorylation levels (obtained in the presence of SCH 28080), the apparent turnover numbers decreased in the same rank order as shown in Fig. 5*B*, from 863 min^{-1} for the EQQ mutant to 247 min^{-1} for the QEQ mutant. These apparent turnover numbers also underestimate the actual turnover numbers because of the low ATP concentration used in the ATPase assay. Fig. 5*C* demonstrates that in 3 s at 21 °C, the phosphorylated intermediates of the EEQ, EQQ, and QQQ mutants were hydrolyzed to 10% or less of the original level. The phosphorylated intermediate of the QEQ mutant was reduced to 30% of the original level in this time period. The residual phosphorylation levels were not affected by K^+. Thus, constitutive ATPase activity was obtained with a Gln at position 820, independent of the presence of either a Gln or a Glu at positions 343 and 795.

Nondephosphorylating Mutants—Fig. 6*A* shows that the steady-state phosphorylation level of the four mutants with an Asp at position 343 and either a Glu or a Gln at positions 795 and 820 was rather similar to that of the wild type enzyme (values in pmol/mg of protein; DEE, 5.5 ± 1.0; DQE, 6.8 ± 0.3; DEQ, 4.4 ± 0.6; DQQ, 4.6 ± 0.5; *cf*. Fig. 6*A* with Fig. 4*A*). SCH 28080 prevented ATP-dependent phosphorylation of the DEE (residual activity 0.8 ± 0.5 pmol/mg of protein) and DQE (0.5 ± 0.9 pmol/mg of protein) mutants. This inhibition, however, did not occur when, in addition to an Asp at position 343, a Gln was present at position 820 (DEQ, 3.1 ± 1.0 pmol/mg of protein; DQQ, 4.2 ± 1.0 pmol/mg of protein). No K^+-activated or constitutive ATPase activity could be demonstrated in any of these mutants (Fig. 6*B*). The dephosphorylation measurements (Fig. 6*C*) showed a rather low spontaneous dephosphorylation and no K^+ activation of the dephosphorylation process. The nondephosphorylating H^+,K^+-ATPase mutants all had an Asp at position 343, indicating that this mutation completely blocked the dephosphorylation step and overruled the effect of all other mutations on this step.

Vanadate Effect on Phosphorylation Level

The effects of SCH 28080 on the steady-state phosphorylation level suggest that the K^+-sensitive mutants are present in the E_2 form and the constitutively active mutants in the E_1 form. Of the four mutants without ATPase activity (Fig. 6), the two with a Glu at position 820 (DEE, DQE) were SCH 28080-sensitive, whereas the two with a Gln on this position (DEQ, DQQ) were insensitive toward this compound. This suggests that the residue at position 820 determined the conformational preference.

In a previous study (11), vanadate was used to determine the $E_2 \leftrightarrow E_1$ preference by measuring the sensitivity of the ATPase reaction. This cannot be performed with the mutants that do not possess ATPase activity. However, the effect of vanadate on the steady-state phosphorylation level is also an indication for the $E_2 \leftrightarrow E_1$ equilibrium (see Scheme 1).

$$\begin{array}{ccc} E_2 + VO_4^- & \rightleftharpoons & E_2 \cdot VO_4^- \\ \Updownarrow & & \\ E_1 + ATP^* & \longrightarrow & E_2P^* \end{array}$$

SCHEME 1

If the enzyme is in the E_2 form, it forms a complex with vanadate. For ATP-dependent phosphorylation, the enzyme has to be in the E_1 form. The sensitivity of the ATP-dependent phosphorylation step for vanadate is thus a measure for the direction of the $E_2 \leftrightarrow E_1$ equilibrium. All preparations were therefore preincubated with increasing vanadate concentrations, and the phosphorylation level obtained with 0.1 μM ATP in 10 s was determined and expressed as a function of the level in the absence of vanadate. Fig. 7*A* shows that the K^+-sensitive mutants had IC_{50} values between 0.03 and 0.2 mM vanadate. The IC_{50} values of the mutants with a Gln at position 795 were 5 times higher than those with a Glu at this position. The four mutants with constitutive ATPase activity all showed IC_{50} values around 0.9 mM (Fig. 7*B*). Fig. 7*C* shows the vanadate sensitivity of the four mutants without ATPase activity with an Asp at position 343. The two mutants with a Glu at position 820 had similar IC_{50} values (0.03–0.07 mM) as the K^+-sensitive mutants. The two mutants with a Gln on residue 820 had

similar IC_{50} values (IC_{50} = 0.76–0.86 mM) as the mutants with constitutive ATPase activity. This confirms the experiments with SCH 28080 and indicates that the presence of a Gln at position 820 resulted in a preference for the E_1 conformation, whereas a Glu on this position favored the E_2 conformation.

DISCUSSION

The present paper shows that two properties of constitutively active mutants of gastric H^+,K^+-ATPase, a high K^+-independent dephosphorylation rate and preference for the E_1 conformation, can be uncoupled. This was performed by combining mutations of Glu^{343} and Glu^{820}. The E343D mutant alone had no ATPase activity but could be phosphorylated by ATP and had an E_2 preference. On the other hand, the E820Q mutant possessed K^+-independent ATPase activity, a high spontaneous dephosphorylation rate, and an E_1 preference. Combination of these two mutations no longer resulted in an enhanced K^+-independent dephosphorylation rate. However, the E_1 preference, the other property of constitutively active mutants, was kept in this double mutant.

Our recent finding that some mutants of Glu^{820} and Glu^{795} in the catalytic subunit showed a constitutive ATPase activity is a puzzling phenomenon. It is clear that this ATPase activity was primarily the result of a high spontaneous dephosphorylation rate. When in the wild type enzyme K^+ is absent from the cation-binding site, dephosphorylation is very slow. Enhanced dephosphorylation only occurs when K^+ binds to the cation-binding pocket. In constitutively active mutants, a minor modification in the transmembrane domain resulted in a similar effect as found with K^+ in the wild type enzyme, an enhanced dephosphorylation rate.

All mutations that displayed constitutive ATPase activity neutralize the negative charge of at least one of the amino acids that may be involved in cation binding. Neutralization, however, was not sufficient for constitutive ATPase activity. In previous studies, we found that mutant E795Q still possesses a K^+-stimulated activity (7). In addition, the mutants E820L (8) and E795N (7) could be phosphorylated, although to a relatively low level, but showed no ATPase activity. In the present study, we demonstrate that none of the single Glu^{343} mutants, although being phosphorylated to a level similar to the wild type enzyme, possessed K^+-independent ATPase activity. Thus, there have to be other factors, in addition to charge neutralization, that determine if a mutant exhibits constitutive ATPase activity.

The preference of constitutively active mutants for the E_1 conformation was already concluded from the increasing rather than decreasing effect of SCH 28080 on the steady-state phosphorylation level of mutant E820Q (10). The wild type enzyme was present in the E_2 form and formed a complex with SCH 28080 (see Scheme 2).

$$E_2 + \mathrm{SCH} \rightleftharpoons E_2 \cdot \mathrm{SCH}$$
$$\Updownarrow$$
$$E_1 + \mathrm{ATP}^* \longrightarrow E_2\mathrm{P}^* \xrightarrow{\mathrm{SCH}} E_2\mathrm{P}^* \cdot \mathrm{SCH}$$

SCHEME 2

Upon the addition of ATP, no phosphorylation occurred. The E820Q mutant did not form such an E_2·SCH 28080 complex because of its preference for the E_1 conformation. After ATP-dependent phosphorylation, however, the E_2P intermediate of this mutant formed a complex with SCH 28080. The dephosphorylation rate of the latter complex was smaller than that of E_2P alone, resulting in an increase in the steady-state phosphorylation level.

In the wild type enzyme, inorganic phosphate inhibits ATP-dependent phosphorylation, since it forms a complex with the E_2 form (Fig. 1, *reaction 4* in opposite direction). Inorganic phosphate did not inhibit ATP-dependent phosphorylation of the E820Q mutant (10). This confirms that the $E_2 \leftrightarrow E_1$ equilibrium in this mutant is directed to the right. The present study confirms the preference of mutants with K^+-independent ATPase activity for the E_1 form by showing that higher vanadate concentrations were necessary to decrease the steady-state ATP-dependent phosphorylation level of K^+-independent mutants as compared with K^+-sensitive mutants. The ATPase activity of the former mutants was also rather insensitive toward this compound (11). In contrast, the K^+-sensitive mutants, all characterized by the presence of the original Glu residue at position 820, had a preference for the E_2 form, as is the case for the wild type enzyme.

The first atomic structure for a P-type ATPase, the Ca^{2+}-ATPase from rabbit sarcoplasmic reticulum, has recently been published (20). The crystallization was carried out in the presence of 10 mM Ca^{2+}, indicating that the obtained structure is a model for the $E_1(Ca^{2+})$ form of this enzyme. In this model, two Ca^{2+} binding sites were found. The residue homologous to Glu^{795} participates in Ca^{2+} binding site I, whereas the residues homologous to Glu^{343} and Glu^{820} are involved in Ca^{2+} binding site II. Using this model, it is not yet possible to understand why constitutive ATPase activity in H^+,K^+-ATPase is found with some mutants of Glu^{820} and Glu^{795} but not with mutants of Glu^{343}. Furthermore, Ca^{2+} ions stimulate phosphorylation, and the constitutive ATPase activity primarily reflects an increase in the spontaneous dephosphorylation rate. Even if the structure of the catalytic subunit of H^+,K^+-ATPase would be rather similar to that of Ca^{2+}-ATPase, one has to realize that the published structure is of the enzyme in the $E_1(Ca^{2+})$ form, whereas K^+-stimulated or spontaneous dephosphorylation takes place with the enzyme present in the E_2-P form. There is some evidence that there are large structural changes in P-type ATPases during the catalytic cycle (3). As long as we do not know how the amino acid residues in the membrane are positioned in the E_2 conformation, the mechanism of spontaneous activation of the dephosphorylation process is unclear.

Although a direct mimicking of the K^+-filled binding pocket by mutagenesis is possible, it might also be that some of these mutations simulate the effect of K^+ binding without a direct effect on the K^+ binding site. It could for instance be that they change the mobility of the transmembrane domains five and six (21, 22) and so mimic the K^+ activation. Mutation of Glu^{820} may also have unexpected effects elsewhere in the protein. In T4 lysozyme, it has been reported that insertion of three alanine residues at position 73 results in the conversion of two elsewhere located α-helices, which form a bend of 105°, into a single straight α-helix (23). Another possibility is that the binding pocket is modified in such a way that H^+ can replace K^+ in the dephosphorylation reaction. We recently observed a similar change in cation selectivity (Na^+ *versus* K^+) in the D804A mutant of Na^+,K^+-ATPase (24).

All single, double, and triple mutants used in the present study could be phosphorylated by ATP. It is generally assumed that binding of H^+ ions is necessary for phosphorylation and that most of the amino acid residues involved in H^+ binding have a similar function in K^+ binding. Nevertheless, the specific orientation of these residues must be different in the E_1 form that binds protons compared with the E_2 form that binds K^+ ions. Glu^{309} in SERCA Ca^{2+}-ATPase, which is equivalent to Glu^{343} in H^+,K^+-ATPase, has been studied in detail, but

mainly regarding its role in Ca^{2+} binding and transport that occurs in the E_1P to E_2P step (25–28). It is clear that the degree of freedom for Ca^{2+} binding in Ca^{2+}-ATPase is much smaller than for H^+-binding in H^+,K^+-ATPase, since with most of the mutants of this residue in Ca^{2+}-ATPase, no ATP-dependent phosphorylation or Ca^{2+} binding was found. Only in the E309Q mutant half of the amount of Ca^{2+} was still bound, but this was only measurable when the enzyme was in the E_2 conformation and at pH 7.0 (27).

Mutation of Glu^{343} in gastric H^+,K^+-ATPase resulted in the loss of K^+ activation unless Glu was replaced by a Gln. In the latter case, however, the apparent affinity was 5 times reduced. All other single Glu^{343} mutants (E343D, E343A, and E343L) could not be dephosphorylated. Interestingly, Vilsen and Andersen (28) found that in the Ca^{2+}-ATPase mutant E309D, the dephosphorylation of the phosphoenzyme intermediate formed with P_i (E_2P) was blocked, a finding comparable with the result with the E343D mutant in the present study.

Why is the dephosphorylation in the E343D mutant blocked? One explanation is that Glu^{343} is indeed directly involved in K^+ binding, in which interaction both oxygens of the carboxyl group are involved. Asp cannot replace Glu in K^+ binding, apparently because of its shorter length. Gln has the right length but can only partially replace Glu, probably because it has only one of the two binding oxygens left, explaining the decrease in affinity by 1 order of magnitude. The alternative explanation for the lack of dephosphorylation of the E343D mutant is that all mutations other than Gln block the conformational change needed for communication with the phosphorylation domain. In that sense, it is interesting that in the Toyoshima model of Ca^{2+}-ATPase, transmembrane segment 4 is unwound around Glu^{309}. Mutation of the latter residue might affect the unwinding of the α-helix, thus disrupting the coupling to the phosphorylation site.

The Na^+,K^+-ATPase residue Glu^{327} is analogous to Glu^{343} in gastric H^+,K^+-ATPase. Mutation of this residue to a Gln residue results in a complete loss in high affinity K^+ occlusion (29). Despite this fact, the residual Na^+,K^+-ATPase activity was still 37% of that of the wild-type enzyme, suggesting that high affinity occlusion is not absolutely essential for ATPase activity. The E327Q mutant[3] shows in several systems Na^+,K^+-ATPase activity, although with a lower apparent K^+ affinity (28, 30, 31). In this respect, it behaves similarly to the E343Q mutant in H^+,K^+-ATPase. Mutation of the Glu^{327} residue in transmembrane domain 4 of Na^+,K^+-ATPase into an Asp also results in a (nearly) complete loss in ATPase activity (29, 31).

We previously showed that mutation of Glu^{795} in transmembrane segment 5 into a Gln residue had hardly any effect on the gastric H^+,K^+-ATPase activity (6, 7). In all double and triple mutants used in the present study, the results were rather similar when a Glu or a Gln was present on this position. Only the K^+-independent ATPase activities were higher with mutant EQQ compared with EEQ (11) and with mutant QQQ compared with QEQ. The constitutive ATPase activity of the QQQ mutant, in which three Glu residues were neutralized, is less than that of the EQQ mutant, indicating that neutralization alone does not determine the ATPase activity.

The starting point for the present investigation was our finding that mutant E820Q showed constitutive ATPase activity associated with K^+-independent dephosphorylation and a preference for the E_1 conformation. These properties remained when in addition to a Gln at position 820, Gln residues were present at positions 343 and/or 795. We assume that constitutive ATPase activity requires a permanent signaling between the cation-binding site and the phosphorylation domain, so that spontaneous dephosphorylation occurs. This signaling is not negatively influenced by the presence of Gln instead of Glu residues in transmembrane segments 4 and 5. We could, however, block the constitutive ATPase activity of mutant E820Q when, in addition, Glu^{343} was replaced by an Asp residue. This occurred with either a Glu or a Gln at position 795. Strikingly, the tendency of the E820Q mutation toward the E_1 form was kept in these mutants.

This leads to the conclusion that the high spontaneous dephosphorylation rate and the preference for the E_1 conformation are two separate effects of the E820Q mutation and that the preference for the E_1 conformation is not the result of the increased dephosphorylation rate. The K^+-independent dephosphorylation in the E820Q mutant could be blocked when in this mutant Glu^{343} was additionally replaced by an Asp. The latter mutation only reduces the length of this side chain by a single CH_2 group. This additional mutation left the second effect (preference for the E_1 conformation) intact. It is likely that both effects were due to conformational changes caused by the E820Q mutation. The enhanced spontaneous dephosphorylation rate must be due to a better accessibility to water of the aspartyl phosphate bond located in the P-domain. The E343D mutation would then physically block the conformational change between the cation-binding pocket and the phosphorylation domain. According to the Toyoshima model (20), this could be possible, since Glu^{343} is located between Glu^{820} and the P-domain. The change in the $E_2 \leftrightarrow E_1$ equilibrium in the rightward direction by the E820Q mutation might be caused by a change in the cation-binding domain itself, resulting in a change in the cation selectivity of this pocket in favor of H^+. The latter change is probably not affected by the additional E343D mutation. This speculative explanation for the findings of this paper can be tested when detailed atomic structures of gastric H^+,K^+-ATPase in several conformational states are available.

[3] This residue is named Glu^{329} in Refs. 28 and 30.

REFERENCES

1. Arguello, J. M., and Kaplan, J. H. (1991) *J. Biol. Chem.* **266,** 14627–14635
2. Arguello, J. M., and Kaplan, J. H. (1994) *J. Biol. Chem.* **269,** 6892–6899
3. Møller, J. V., Juul, B., and Le Maire, M. (1996) *Biochim. Biophys. Acta* **1286,** 1–51
4. Asano, S., Tega, Y., Konishi, K., Fujioka, M., and Takeguchi, N. (1996) *J. Biol. Chem.* **271,** 2740–2745
5. Asano, S., Furumoto, R., Tega, Y., Matsuda, S., and Takeguchi, N. (2000) *J. Biochem.* (*Tokyo*) **127,** 993–1000
6. Swarts, H. G. P., Klaassen, C. H. W., De Boer, M., Fransen, J. A. M., and De Pont, J. J. H. H. M. (1996) *J. Biol. Chem.* **271,** 29764–29772
7. Hermsen, H. P. H., Koenderink, J. B., Swarts, H. G. P., and De Pont, J. J. H. H. M. (2000) *Biochemistry* **39,** 1330–1337
8. Hermsen, H. P. H., Swarts, H. G. P., Koenderink, J. B., and De Pont, J. J. H. H. M. (1998) *Biochem. J.* **331,** 465–472
9. Swarts, H. G. P., Hermsen, H. P. H., Koenderink, J. B., Schuurmans Stekhoven, F. M. A. H., and De Pont, J. J. H. H. M. (1998) *EMBO J.* **17,** 3029–3035
10. Swarts, H. G. P., Hermsen, H. P. H., Koenderink, J. B., Willems, P. H. G. M., and De Pont, J. J. H. H. M. (1999) *Mol. Pharmacol.* **55,** 541–547
11. Hermsen, H. P. H., Swarts, H. G. P., Wassink, L., Koenderink, J. B., Willems, P. H. G. M., and De Pont, J. J. H. H. M. (2001) *Biochemistry* **40,** 6527–6533
12. Klaassen, C. H. W., Van Uem, T. J. F., De Moel, M. P., De Caluwé, G. L. J., Swarts, H. G. P., and De Pont, J. J. H. H. M. (1993) *FEBS Lett.* **329,** 277–282
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) *J. Virol.* **67,** 4566–4579
15. Klaassen, C. H. W., Swarts, H. G. P., and De Pont, J. J. H. H. M. (1995) *Biochem. Biophys. Res. Commun.* **210,** 907–913
16. Peterson, G. L. (1983) *Methods Enzymol.* **91,** 95–106
17. Laemmli, U. K. (1970) *Nature* **227,** 680–685
18. Gottardi, C. J., and Caplan, M. J. (1993) *J. Biol. Chem.* **268,** 14342–14347
19. Swarts, H. G. P., Klaassen, C. H. W., Schuurmans Stekhoven, F. M. A. H., and De Pont, J. J. H. H. M. (1995) *J. Biol. Chem.* **270,** 7890–7895
20. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) *Nature* **405,** 647–651
21. Lutsenko, S., Anderko, R., and Kaplan, J. H. (1995) *Proc. Natl. Acad. Sci.*

U. S. A. **92,** 7936–7940
22. Gatto, C., Lutsenko, S., Shin, J. M., Sachs, G., and Kaplan, J. H. (1999) *J. Biol. Chem.* **274,** 13737–13740
23. Vetter, I., Baase, W., Heinz, D., Xiong, J., Snow, S., and Matthews, B. (1996) *Protein Sci.* **5,** 2399–2415
24. Koenderink, J. B., Swarts, H. G. P., Hermsen, H. P. H., Willems, P. H. G. M., and De Pont, J. J. H. H. M. (2000) *Biochemistry* **39,** 9959–9966
25. Clarke, D. M., Loo, T. W., and MacLennan, D. H. (1990) *J. Biol. Chem.* **265,** 22223–22227
26. Clarke, D. M., Loo, T. W., Inesi, G., and MacLennan, D. H. (1989) *Nature* **339,** 476–478
27. Skerjanc, I. S., Toyofuku, T., Richardson, C., and MacLennan, D. H. (1993) *J. Biol. Chem.* **268,** 15944–15950
28. Vilsen, B., and Andersen, J. P. (1998) *Biochemistry* **37,** 10961–10971
29. Nielsen, J. M., Pedersen, P. A., Karlish, S. J. D., and Jørgensen, P. L. (1998) *Biochemistry* **37,** 1961–1968
30. Vilsen, B. (1993) *Biochemistry* **32,** 13340–13349
31. Jewell-Motz, E. A., and Lingrel, J. B (1993) *Biochemistry* **32,** 13523–13530