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Effects of Free Radicals on Partial Reactions of the Na,K-ATPase

M. Mense*, G. Stark, H.-J. Apell
Department of Biology, University of Konstanz, Postfach 5560 M635, D-78434 Konstanz, Germany

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Abstract. The function of the Na,K-ATPase is known to be considerably impaired in the presence of free radicals such as $OH^{\bullet}$. While previous experiments were largely based on the loss of enzymatic activity of the protein, this is the first communication dealing with partial reactions of the pump cycle in the presence of free radicals produced by water radiolysis. Three different system states, which are directly involved in ion transfer catalyzed by the enzyme, showed similar sensitivity to free radical action. This is indicated by largely identical D_{37}-doses of the decay of the reaction amplitudes investigated. The decrease in the efficiency of the enzyme functions was largely due to a lethal damage of pump molecules. A kinetic analysis of the ATP-induced conformational transition $E_1 \rightarrow E_2$ revealed, however, that a minor component of the inactivation is due to a reduction of the transition rate constant. The decrease of the enzymatic activity could be simulated by the decay of the rate-limiting conformational transition. This finding indicates the conservation of a close coupling between ATP-hydrolysis and sodium translocation process throughout free-radical induced inactivation. As a result of the tight coupling, enzyme modification at different system states leads to similar functional consequences for the protein.

Key words: Na,K-ATPase — Partial reactions — Free radicals — Ionizing radiation — Ion transport — Enzymatic activity

Introduction

The Na,K-ATPase is an electrogenic ion pump which utilizes the energy of ATP hydrolysis to translocate Na^+ and K^+ ions against their electrochemical gradient across the plasma membrane (Glynn, 1985). The movement of 3 Na^+ ions out of the cell and the import of 2 K^+ ions accounts for the generation of the membrane potential in animal cells. The enzyme is found in almost all animal cells. Its function is crucial for the cellular metabolism, since it provides for the energy which is necessary to drive a variety of secondary active transport processes across the membrane. The mechanism of action of this ion pump is of great complexity. Cyclic reaction schemes, based on the well-known Post-Albers cycle (Fig. 1), and comprising a great number of intermediate states, are usually applied to explain an increasing number of sophisticated experimental approaches (Läuger, 1991).

The present communication deals with the inactivation of the Na,K-ATPase by free radicals. Previous studies have shown that the activity of the enzyme is strongly and irreversibly reduced in the presence of hydroxyl radicals $OH^{\bullet}$, of oxygen radicals $O_2^{\bullet -}/HO_2^{\bullet}$, or of singlet oxygen, $^1O_2$, (Kim & Akera, 1987; Mishra et al., 1989; Thomas & Reed, 1990; Chen et al., 1992; Vinnikova, Kukreja & Hess, 1992; Rohn, Hinds & Vincenzi, 1993; Elmoselhi et al., 1994; Huang et al., 1994; Shao et al., 1995). Since the Na,K-ATPase is an essential enzyme of the plasma membrane of animals cells, it has been suggested to represent an important target of free radical-induced membrane damage. This has been considered to be of great practical consequence for certain kinds of cardiovascular injuries, e.g., the reperfusion damage of the myocardium after ischaemia (Kukreja & Hess, 1992).

Inactivation of the Na,K-ATPase is usually studied using chemically induced radicals, e.g., by application of Fe^{2+}/H_2O_2, Fe^{2+}/ascorbate or by irradiation of the system with visible light in the presence of appropriate photosensitizers. Water radiolysis is a further convenient method to generate radicals. Absorption of ionizing radiation in oxygen containing aqueous solutions yields definite concentrations of $OH^{\bullet}$ and of $O_2^{\bullet -}/HO_2^{\bullet}$ radicals.

* *Present address:* Department of Cellular and Molecular Physiology, Yale University, New Haven, CT 06510

Correspondence to: H.-J. Apell

Using the latter method, we have recently shown that the enzyme is inactivated via a radical-chain mechanism (Hitschke et al., 1994).

The extent of enzyme inactivation by free radicals was so far characterized mostly via the decrease of ATP hydrolysis. The correlation between the decrease of ATP hydrolysis and the ion pumping activity has not been studied in detail so far. This is the topic of the present communication, in which the consequences of radical action on several partial reactions of the pump cycle are investigated.

Partial reactions of the Na,K-ATPase which are involved in ion transport may be studied by a special fluorescence technique developed in recent years (Klodos & Forbush, 1988; Bühler et al., 1991; Stürmer et al., 1991; Pratap, Robinson & Steinberg, 1991; Heyse et al., 1994; Klodos, 1994). A styryl dye, RH421, which inserts into the lipid domains of Na,K-ATPase membrane fragments, is used to detect changes of local electric fields inside the membrane (Bühler et al., 1991). Such changes are caused by binding or release of ions and/or by movement of charges inside the membrane-bound protein domains. The fluorescence levels have been found to allow discrimination between differently charged states of the enzyme (Grinwald et al., 1982; Bühler et al., 1991). Although the molecular mechanism of the dye is not solved completely so far, the occurrence of at least three partial reactions have been identified in this way by characteristic changes of the fluorescence intensity of RH421 (Visser et al., 1995; Fedosova, Cornelius & Klodos, 1995; Frank et al., 1996): cytoplasmic Na^+ binding (E_1 + 3 $Na^+_{cyt} \rightarrow Na_3E_1$), conformational change and extracellular release of Na^+ ($Na_3E_1ATP \rightarrow P\text{-}E_2$ + ADP + 3 Na^+_{ext}), and extracellular K^+ binding ($P\text{-}E_2$ + 2 $K^+_{ext} \rightarrow E_2(K_2)$ + P_i).

All three reactions are part of the ion transfer catalyzed by the electroenzyme. Therefore, application of the fluorescence technique allows the study of the effect of free radicals on the function of the Na,K-ATPase in considerable more detail than has been possible so far. As a final result of the present analysis, a correlation between the decrease of the rate of ATP hydrolysis and of the ion pumping activity of the Na,K-ATPase is obtained.

Materials and Methods

Sodium dodecylsulfate (SDS) was obtained from Pierce Chemical. Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH, and ATP [disodium salt, Sonderqualität) were from Boehringer, Mannheim, Apyrase (type VI) from Sigma, München. The electric-field sensitive fluorescence dye N-(4-sulfobutyl)-4-[4-(*p*-dipentylaminophenyl)butadienyl]-pyridinium, inner salt (RH 421) and P^3-1-(2-nitro)phenylethyladenosine-5′-triphosphate (caged ATP) were from Molecular Probes, Eugene, OR. Dye purity was checked by thin-layer chromatography. All other reagents were the highest grade commercially available.

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys in form of open membrane fragments using procedure C of Jørgensen (1974). Protein concentration was assessed by the Lowry method, using bovine serum albumin as a standard. Specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971). The specific activity was in the range between 1900 and 2000 μM P_i per mg protein and h at 37°C. Preparations of membranes containing the 19-kDa fragment were prepared according to a recently published procedure (Capasso et al., 1992; Schwappach et al., 1994).

To prepare membrane fragments for the irradiation procedure, they were suspended twice in solutions of 20 mM NaCl, pH 5–6, and centrifuged in a Beckman airfuge at 160,000 × *g*. Under standard conditions the final suspension of fragments was diluted in aqueous solutions of 20 mM NaCl or 10 mM $MgCl_2$, pH 5–6, to a concentration of 0.25 μg/μl. Samples of 40 μl were pipetted into tiny polyethylene vessels, of which 9 could be placed in appropriate holes drilled into a sample holder made from Plexiglas (Hitschke et al., 1994). The holder was placed 6 cm from the focus of an X-ray tube (Philips-Müller RT 100). Attenuation of the 80 kV X-rays was obtained by aluminum filters of various thickness. Underneath the samples an ionization chamber (PTW DL 4, Pychlau, Freiburg) was mounted to monitor the applied dose. Calibration of the chamber was performed by Fricke dosimetry (Spinks & Woods, 1976). For each series of experiments one control sample was stored at room temperature (T = 21 ± 1°C), while the irradiation procedure started with 9 samples. After appropriate times corresponding to defined doses, samples were successively removed from under the X-ray tube. At the end of the irradiation period, all samples were stored on ice until the specific ATPase activity was determined or fluorescence measurements were performed. For each series of experiments, the change of the activity was calculated relative to the activity of the control sample to compensate for the differences between various protein preparations.

Steady-state fluorescence measurements were carried out in a Perkin-Elmer LS 50B fluorescence spectrophotometer as described previously to detect partial reactions of the ion-transport process (Bühler et al., 1991; Stürmer et al., 1991). The thermostatically regulated cell holder was equipped with a magnetic stirrer. For experiments with RH 421, the excitation wavelength was set to 580 nm and the emission wavelength to 650 nm (slit width 15 nm in both cases). The experiments were performed at 20°C. Specific fluorescence levels could be assigned to defined states in the pump cycle of the Na,K-ATPase (Heyse et al., 1994).

Time-resolved fluorescence experiments were performed by application of an ATP-concentration jump in a fluorimeter adapted for fast kinetics experiments (Stürmer et al., 1989). Upon a 10 ns laser flash ATP was released from an inactive precursor, caged ATP, to trigger the reaction sequence $Na_3E_1ATP \rightarrow (Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3) \rightarrow P\text{-}E_2$. To maintain the initial ATP concentration at virtually zero, 3 U/ml apyrase (type VI) were added to the sample. This type of experiments allowed determination of the rate-limiting step in the Na-transport sequence (Fig. 1) which was found previously to be the conformational transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3)$ (Heyse et al., 1994; Wuddel & Apell, 1996).

Results

The enzymatic activity of Na,K-ATPase-containing membrane fragments shows an exponential decrease to virtually zero as the applied radiation dose is increased (Hitschke et al., 1995). Evidence has been presented that the inactivation is due to an interaction between free

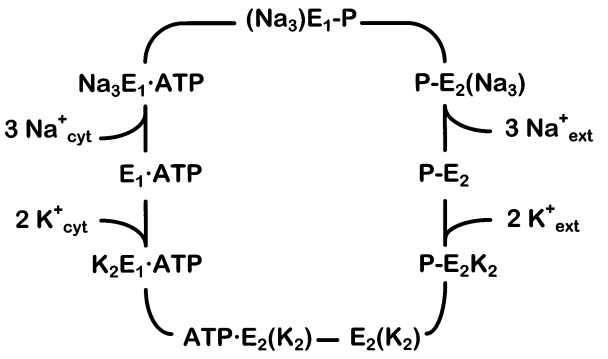

Fig. 1. Post-Albers reaction scheme of the Na,K-Pump under physiological conditions. E_1 and E_2 are conformations of the enzyme with the ion binding sites exposed to the cytoplasm and to the extracellular side of the membrane, respectively. In the occluded states, $(Na_3)E_1$-P, $E_2(K_2)$, and ATP · $E_2(K_2)$, the bound ions are unable to exchange with ions in either aqueous phase.

radicals of water radiolysis and the ion pump. The following experiments were intended to provide a more detailed picture of the inactivation mechanism by studying the effect of radicals on several partial reactions of the pump cycle. This was achieved by application of optical methods which has been developed in recent years to study ion binding and ion release of the Na,K-ATPase (Stürmer et al., 1991; Stürmer & Apell, 1992; Schwappach et al., 1994; Heyse et al., 1994) as well as ion translocation over the membrane interior (Klodos & Forbush, 1988; Pratap, Robinson & Steinberg, 1991; Klodos, 1994; Schulz & Apell, 1995).

Dose Dependence of Steady-State Populations

The distribution of the Na,K-ATPase between the different states of the pump cycle (Fig. 1) can be affected by using specific substrate conditions. When protein (4.5 μg/ml) was incubated in standard buffer (25 mM histidine, 0.5 mM EDTA, 10 mM $MgCl_2$, pH 7.1, but no Na^+ or K^+) with 200 nM of the fluorescence dye RH 421, the pump was stabilized in state E_1 (fluorescence level F_o, *c.f.* Fig. 2*A*). Addition of NaCl (20 mM) in the absence of ATP promoted a transition of the protein into state Na_3E_1 which was accompanied by a fluorescence decrease. Subsequent additions of 0.5 mM ATP and of 20 mM KCl caused transitions to states P-E_2 and $E_2(K_2)$, respectively. These were detected by a fluorescence increase (addition of ATP) and by a fluorescence decrease (addition of KCl).

The amplitudes of the relative intensity changes, $\Delta F/F_o$, are proportional to the percentage of pump molecules participating in the cycle. This holds for intact pump molecules at least. Inactivation of the protein molecules by free radicals should cause a corresponding decrease of all fluorescence amplitudes. Free radicals

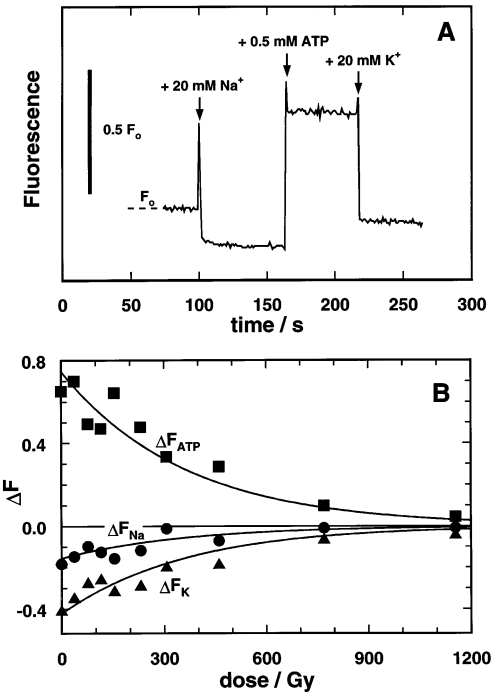

Fig. 2. Dependence of substrate-induced partial reactions of the Na,K-ATPase (detected by the electric field sensitive fluorescence dye RH 421) on the radiation dose. Membrane fragments containing high concentrations of the ion pump were incubated in an aqueous solution containing 10 mM $MgCl_2$, pH 5, and were irradiated by 80 kV X-rays with a dose rate of 75 Gy/min. Subsequently, part of the irradiated solution was added to the buffer (25 mM histidine, 0.5 mM EDTA, 10 mM $MgCl_2$, and 200 nM RH 421, pH 7.1) in which fluorescence experiments were performed. (*A*) Time dependence of the fluorescence intensity, while 20 mM NaCl, 0.5 mM ATP and 20 mM KCl were added successively to membranes which had received a dose of 307 Gy. (*B*) The dependence of the amplitude of fluorescence changes, ΔF, (upon addition of Na^+, ATP and K^+) on the applied radiation dose. ΔF-values were calculated according to $\Delta F_{Na} = (F_{Na} - F_o)/F_o$, $\Delta F_{ATP} = (F_{ATP} - F_{Na})/F_{Na}$ and $\Delta F_K = (F_K - F_{ATP})/F_{ATP}$. The lines represent the function, $\Delta F(D) = \Delta F(0) \cdot \exp(-D/D_{37})$ (D = dose) with the same D_{37} = 360 Gy for all three curves and appropriate values of $\Delta F(0)$.

might, however, affect only specific transitions of the cycle. A selective change of the corresponding amplitudes should be observed upon irradiation in this case. Discrimination between both possibilities was achieved by studying the amplitudes of the normalized intensity changes as a function of the radiation dose (Fig. 2*B*). A normalization procedure was applied to correct for slightly varying protein concentrations in individual measurements and for the dose-dependent decrease of the initial fluorescence, F_o, before the addition of any substrate.

Experiments were performed with native and irradiated enzyme in the dose range up to 1160 Gy (1 Gy = 1 J/kg). The general shape of the fluorescence changes was found to be independent of the radiation dose. The fluorescence trace shown in Fig. 2*A* was obtained after

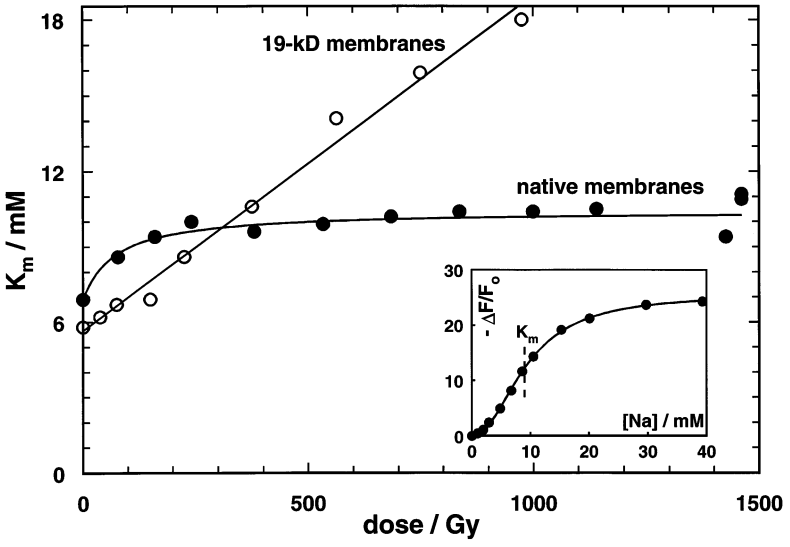

Fig. 3. Dependence of the apparent cytoplasmic sodium binding affinity on the applied radiation dose for native enzyme and 19-kD membranes. The half-saturating concentration, K_m, was obtained from titration of the Na^+-induced RH 421 fluorescence change, $\Delta F/F_o$, as shown in the inset. The K_m-values were determined from fits of the titration data by the Hill function, $\Delta F/F_o([Na]) = F_\infty/(1 + (K_m/[Na])^p)$. The analyses were carried out for native enzyme (''native membranes'') and for trypsinolyzed enzyme (''19-kD membranes'') which were irradiated in the dose range up to 1500 Gy. Experimental conditions as given in Fig. 2.

application of 307 Gy and is identical (apart from the amplitudes) to that found for the native enzyme (Stürmer et al., 1989). In Fig. 2*B* the relative fluorescence changes, $\Delta F \equiv (F_1 - F_o)/F_o$ (where F_o and F_1 represent the fluorescence levels before and after substrate addition, respectively), are presented as function of the applied dose. Each data point is an average of 4–6 experiments. The data indicate an exponential decrease of the relative fluorescence changes according to

$$\Delta F = \Delta F(0) \cdot \exp(-D/D_{37}) \quad (1)$$

where $\Delta F(0)$ represents the relative fluorescence change of the native ion pump, D is the radiation dose, and D_{37} is the dose which reduces the initial signal to 1/e (≈37%). The fluorescence changes decreased to virtually zero for all three substrate additions with comparable D_{37} values of about 360 Gy (Fig. 2*B*) under standard irradiation conditions (i.e., 0.25 μg/μl protein in buffer containing 10 mM $MgCl_2$, pH 5 and a dose rate of 75 Gy/min). The identical dose dependence of the three partial reactions is indicative of a lethal damage of pump molecules, i.e., a complete inactivation comprising all important states of the reaction cycle (*see above*). The sensitivity of the fluorescence method applied does not exclude, however, the additional presence of partially inactivated pump molecules. This will be shown by specific functional tests of partial reactions presented in the following sections.

Effects of Free Radicals on Cytoplasmic Sodium Binding

Since binding of Na^+ to the Na,K-ATPase is an electrogenic process, it can be detected via fluorescence changes of the dye RH 421 and the binding affinity of the protein can be determined by titration experiments (Heyse et al., 1994; Schulz & Apell, 1995). The experiments were similar to the first addition shown in Fig. 2*A*. Aliquots of NaCl, which corresponded to concentration steps of 1–10 mM, were added to native or irradiated protein preparations incubated in standard buffer and 200 nM RH 421. The fluorescence changes were plotted against Na^+ concentration (*see inset* of Fig. 3) and fitted by the Hill function, $\Delta F/F_o([Na]) = \Delta F_\infty/(1 + (K_m/[Na])^p)$. The dose dependence of the maximum fluorescence change, ΔF_∞, of the Na,K-ATPase (*data not shown*) agreed with the findings presented in Fig. 2*B*. The Na^+ affinity, K_m, showed an initial increase in the low dose range (<150 Gy) from 7 mM to 10 mM and remained constant with a value of 10.2 ± 0.2 mM (mean ± SEM) in the dose range up to 1500 Gy. The Hill coefficient p varied between 1.5 and 2.5 in a dose-independent manner (*data not shown*).

Similar experiments were performed with 19-kD membranes. Recent investigations showed that the Na^+ binding sites remain intact with respect to their binding affinity and electrogenicity (Schwappach et al., 1994) when the membrane-bound enzyme is selectively digested by trypsin (Capasso et al., 1992). By this treatment approximately 50% of the protein mass is removed and the remaining structure consists of four aggregated fragments of the α-subunit and an intact β-subunit. The trypsinolyzed Na,K-ATPase has lost almost completely the cytoplasmic protrusions of its α-subunit (Goldshleger et al., 1994). 19-kD membranes were irradiated with doses up to 1,000 Gy (at a protein concentration of 0.125 μg/μl in 10 mM $MgCl_2$). In spite of their nearly identical Na-binding behavior before irradiation, clear differences were found between native and trypsinolyzed enzyme in their response to radiation-induced free radicals. The half-saturating concentration, K_m, of the trypsinolyzed

protein showed a linear increase with the applied dose from 6 mM (nonirradiated) to 18 mM (980 Gy) (open circles in Fig. 3). This indicates an enhanced susceptibility of 19-kD membranes towards free radicals.

Dose Dependence of the Conformational Change $E_1 \rightarrow E_2$

The transition $E_1 \rightarrow E_2$ can be studied as part of the reaction sequence $Na_3E_1 \rightarrow (Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3) \rightarrow P\text{-}E_2$. The experiments were performed as follows: After irradiation in 20 mM NaCl (pH 5–6) membrane fragments were incubated in standard buffer with 20 mM NaCl, 200 nM RH 421 and 300 μM caged ATP. At time $t = 0$, about 15–25 μM ATP were released by laser-flash photolysis and were used to trigger the reaction sequence. Since Na_3E_1 has a high affinity binding site for ATP with $K_{ATP} < 0.2$ μM (Heyse et al., 1994), the concentration of released ATP was always high enough to ensure that the phosphorylation step was not rate-limiting with respect to the subsequent conformational transition, $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3)$. The extracellular ion release is the main electrogenic event detected by RH 421 (Stürmer & Apell, 1992). It is, however, fast compared to the preceding steps (Wuddel & Apell, 1995). Therefore, the kinetics of this partial reaction (starting at state Na_3E_1 and ending at $P\text{-}E_2$) is limited by the rate of the conformational transition.

The RH 421-fluorescence change induced by the laser flash was detected with a time resolution of 1 or 2 msec. A typical experiment is shown in Fig. 4*A* from a membrane preparation irradiated with 380 Gy. The fluorescence intensity before the ATP-concentration jump was defined as F_o. The UV-laser flash (at $t = 0$) induced a pronounced fluorescence increase. (The initial artifact of less than 1 msec duration was generated in the photomultiplier by incomplete shielding against the UV flash.) The ATP-induced fluorescence increase could be satisfactorily approximated by a single exponential function (*cf.* Fig. 4*A*), which is characterized by the rate constant of the process, k, and by the normalized change of fluorescence, $\Delta F_\infty \equiv (F_\infty - F_o)/F_o$. Experiments of this kind were performed with membranes irradiated with different radiation doses.

The rate constant, k, shows a small but significant decrease with increasing dose (Fig. 4*B*). The data were found to be in fair agreement with the function

$$k(D) = k_\infty + (k_o - k_\infty) \exp(-D/D_{37}) \qquad (2)$$

where $k_o = 11.3\ \text{sec}^{-1}$ is the rate constant of the native enzyme and $k_\infty = 3.9\ \text{sec}^{-1}$ is the value approached at high doses. The D_{37} dose was determined as 720 Gy. The decrease of k indicates that an increasing part of the pump molecules experiences a partial inactivation, i.e., a

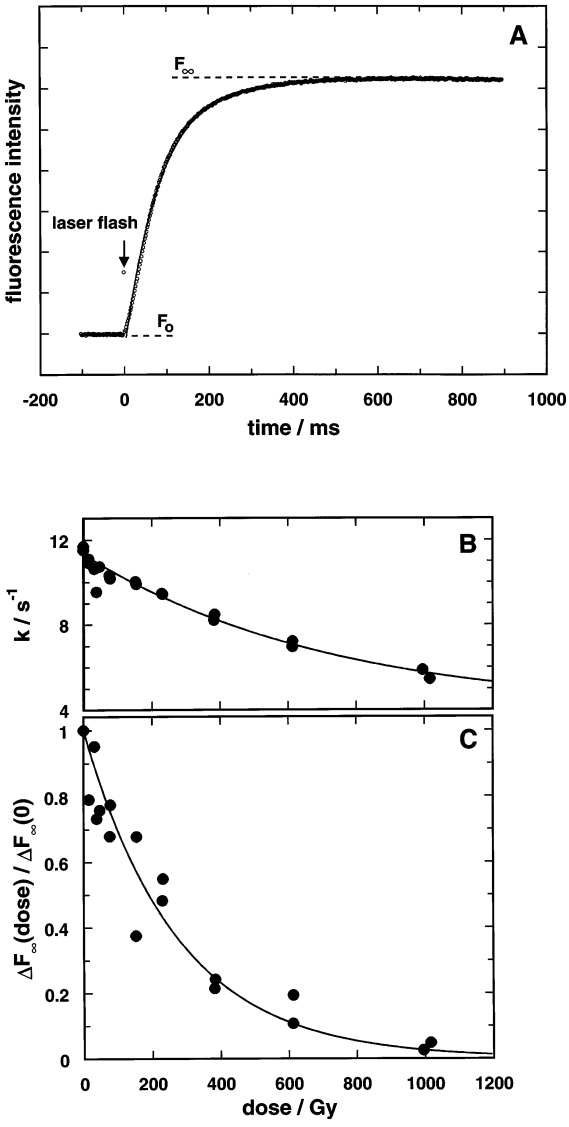

Fig. 4. Analysis of the dose dependence of the rate-limiting step of sodium translocation. (*A*) Time course of the RH 421—fluorescence in an ATP-concentration jump experiment. 20 μg/ml of Na,K-ATPase-containing membrane fragments, which were irradiated with 380 Gy, were added to buffer (25 mM histidine, 20 mM NaCl, 0.5 nM EDTA, 5 mM $MgCl_2$, pH 7.1) including 200 nM RH 421 and 300 μM caged ATP. The concentration of ATP was increased at $t = 0$ from virtually 0 to 20 μM by laser photolysis of inactive caged ATP. The time course of the fluorescence intensity was fitted by the function $F_o + (F_\infty - F_o) \cdot (1 - \exp(-k \cdot t))$, characterized by the rate constant k and by the relative fluorescence change, $\Delta F_\infty \equiv (F_\infty - F_o)/F_o$. These experimental parameters were obtained for membrane preparations irradiated in the dose range up to 1,000 Gy. (*B*) Plot of the rate constant k against the radiation dose. The experimentally obtained dose dependence was fitted by the function $k(D) = k_\infty + (k_o - k_\infty) \cdot \exp(-D/D_{37})$ (D = dose), with $k_o = 11.3\ \text{sec}^{-1}$, $k_\infty = 3.9\ \text{sec}^{-1}$ and $D_{37} = 720$ Gy (solid line). (*C*) Plot of the relative fluorescence change, $\Delta F_\infty(D)/\Delta F_\infty(0)$, against the radiation dose. The ATP-induced fluorescence change decreased to virtually zero for doses higher than 1000 Gy. The data could be approximated by a single exponential of the form $\exp(-D/D_{37})$ with $D_{37} = 240$ Gy (solid line).

reduction of their pumping efficiency, with increasing radiation dose. Nevertheless, the main effect of free radicals is the lethal damage of the ion pump, as will be apparent from the detailed discussion of the results.

In Fig. 4*C* the dose-dependent relative fluorescence changes $\Delta F_\infty(D)/\Delta F_\infty(0)$ are plotted against the applied dose, D, where $\Delta F_\infty(0)$ is the fluorescence change of the nonirradiated preparation. The amplitudes of the relative intensity change are proportional to the percentage of pump molecules participating in the partial reaction. The amplitude declined exponentially according to Eq. (1) with a D_{37} value of 240 Gy. When doses above 1,000 Gy were applied, the ATP-concentration jump experiments could no longer be analyzed due to the vanishing changes of the fluorescence intensities. The D_{37} dose of 240 Gy should be identical to the corresponding value from the dose dependence of the steady state populations (Fig. 1) which was found to be 360 Gy. The latter value was obtained, however, under different experimental conditions (presence of $MgCl_2$ instead of NaCl), which is of consequence for the concentration of radicals generated.

Dose Dependence of the Enzymatic Activity

Experiments as presented by Hitschke et al. (1994) were repeated in which the overall ATPase activity of the Na,K-ATPase-containing membrane fragments was determined by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971). Membrane fragments were irradiated in a solution containing 18.75 mM NaCl, pH 5. The enzyme preparations used in this study showed (*under comparable conditions*) the same dose-dependent increase of the activity with a D_{37} value of 206 Gy (Fig. 5) as our previous data (200 ± 33 Gy; 11).

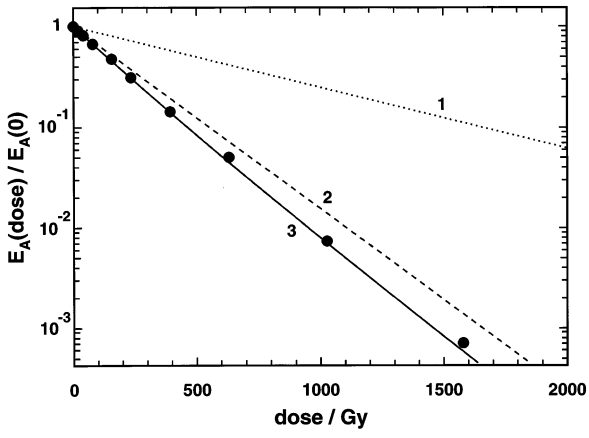

Fig. 5. Inactivation of the enzymatic activity of the Na,K-ATPase in membrane fragments by free radicals. Membrane fragments containing high concentrations of the ion pump were incubated in an aqueous solution containing 18.75 mM NaCl, pH 5, and were irradiated by 80 kV X-rays with a dose rate of 75 Gy/min. The enzymatic activity E_A, normalized to the activity before irradiation, $E_A(0)$, decayed exponentially with dose and showed a D_{37} dose of 206 Gy (data points). Three simulations were calculated on the basis of Eqs. (1) and (2) to reproduce the dose dependence of E_A: 1, $E_A(D) \propto k(D)$, 2, $E_A(D) \propto \Delta F_\infty(D)$, and 3, $E_A(D) \propto \Delta F_\infty(D) \cdot k(D)$ (*see* Discussion).

Discussion

X-ray absorption in water represents a convenient way of free radical generation (v. Sonntag, 1987; Stark, 1991). The primary radicals of water radiolysis are the $OH^\bullet$, the $H^\bullet$ and the $e_{aq}^{\bullet-}$ species. In the presence of oxygen, $H^\bullet$ and $e_{aq}^{\bullet-}$ are converted into the secondary radicals $HO_2^\bullet$ and $O_2^{\bullet-}$, which represent a conjugated acid-base pair. The differential action of the hydroxyl radical and of the oxygen radicals may be estimated by addition of appropriate radical scavengers. The use of scavengers also allows one to distinguish between indirect radiation effects (by free radicals) and the direct radiation effect (e.g., ionization) by absorption of radiation at the macromolecule itself. In the case of Na,K-ATPase, free radicals have been found to represent by far the most important source of enzyme inactivation, i.e., the direct radiation effect can be largely neglected. This was shown by application of radical scavengers and by the finding of a considerable enhancement of the sensitivity of Na,K-ATPase with increasing dilution of this protein (Hitschke, 1994). The experiments presented here further confirm the importance of free radicals. Figure 3 shows an enhanced radiation effect on cytoplasmic sodium binding for 19-kD membranes. If direct absorption of radiation in the protein would account for this phenomenon, the hit theory (Dertinger & Jung, 1970) would predict a reduced sensitivity of 19-kD membranes due to the decrease in target mass, which is in contrast to the experimental results.

The pronounced increase in the dose dependence of K_m for 19-kD membranes may be understood assuming comparatively free access of radicals to the binding sites for Na^+. It has been shown recently that the complete ion-binding sites are part of the transmembrane segments of the Na,K-ATPase (Capasso et al., 1992; Schwappach et al., 1992). For native membranes on the other hand, the small radiation effect seems to indicate a relatively good shielding of the binding sites towards a free radical-induced chemical modification of the protein. This is achieved by the large cytoplasmic domain of the α subunit of the protein which covers the transmembrane segments. The shielding effect by the cytoplasmic domain suggests that the modification of ion binding is produced by free radicals from the aqueous phase rather than by interaction of lipid peroxidation products with the protein. This is, however, in contrast to the suggestions of

different authors according to which products of lipid peroxidation represent the main source of inactivation (Mishra et al., 1989; Thomas & Reed, 1990; Siems, Hapner & van Kuijk, 1996). An alternative explanation of the decrease in Na affinity of 19-kD membranes after irradiation could be a destabilization of the protein complex by radical effects in analogy to the thermally induced lability of the complex (Or et al., 1993; Schwappach et al., 1994). We think, however, that this assumption is less likely, since SDS gel electrophoresis of irradiated membranes showed cross-linking instead of fragmentation (*unpublished results*).

The cyclic reaction scheme, which underlies complex functional properties of the Na,K-ATPase, consists of a considerable number of intermediate states, of which the most important are shown in Fig. 1. The question arises, whether one of the different states shows particular sensitivity towards free radical action and will therefore be of special importance for enzyme inactivation. The experiments illustrated in Fig. 2 indicate that the D_{37} doses (for the three partial reactions investigated) did not vary significantly, since the decay of the corresponding steady states could be approximated by the same value of 360 Gy (lines in Fig. 2*B*). Therefore, all system states seem to show similar sensitivity.

The experiments in Fig. 2 may be explained by a lethal damage of pump molecules due to free radical reactions with the protein. The kinetic experiments shown in Fig. 4, however, which were designed to analyze the rate-limiting step of a series of partial reactions, provided evidence for a reduction of the mean transition rate constant, *k*, of this process (Fig. 4*B*) (in addition to the lethal damage of pump molecules, *c.f.* Fig. 4*C*). In these experiments an ATP concentration jump triggers a reaction sequence of several steps, $Na_3E_1 \rightarrow (Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3) \rightarrow P\text{-}E_2$. Only the last step produces the RH 421 fluorescence change shown in Fig. 4*A*. To test by which of the three steps the kinetics of the overall reaction is controlled, the rate constants have to be compared: The phosphorylation reaction with a rate constant of 200 sec^{-1} (Heyse et al., 1994) and the ion-release with a rate constant of >1400 sec^{-1} (Wuddel & Apell, 1995) are significantly faster than the observed process ($k \leqslant 12$ sec^{-1}, Fig. 4*B*). Therefore the latter has to be attributed to the conformational change, $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3)$. Our data are in fair agreement with previously determined rate constants of this process (Heyse et al., 1994). There is another conclusion which may be derived from Fig. 4*B:* One might argue that the decrease of *k* is influenced by the enzyme phosphorylation, $Na_3E_1 \rightarrow (Na_3)E_1\text{-}P$, preceding the conformational transition. In that case, however, a shoulder curve should be found, i.e., *k* should remain constant at low doses, where the conformational change is rate limiting. The conformational change follows enzyme phosphorylation and which is significantly slower than the phosphorylation reaction in the not irradiated protein. This is in contradiction to the actual behavior observed (Fig. 4*B*).

Though the reduced transition rate constant, *k*, contributes to enzyme inactivation, the lethal damage appears to be the most important modification of the enzyme. This may be concluded from Fig. 5. The figure compares experimental data of the dose-dependent decay of the enzymatic activity of the ion pump with predictions based on the concentration-jump experiments of Fig. 4. The simulations of the dose dependence of the enzyme activity were performed making three different assumptions. The assumption of a dose-dependent rate constant (Fig. 4*B*) as the only reason of the decrease of the enzymatic activity can be excluded (dotted line). The dashed line shows a small (but significant) deviation from the actual experimental values. It was calculated by assuming that the number of active enzyme molecules decays only according to the number of molecules participating at the reaction sequence $Na_3E_1 \rightarrow \ldots \rightarrow P\text{-}E_2$ (Fig. 4*C*), i.e., $E_A(D) \propto \Delta F_\infty(D)$. An almost perfect agreement between data and simulation is obtained, if the reduction of the rate constant of the transition is considered in addition to the lethal damage, i.e., $E_A(D) \propto \Delta F_\infty(D) \cdot k(D)$ (solid line). The comparatively small difference between the latter two simulations indicates that partial enzyme inactivation due to lowering of transition rates is less important (though not negligible) than the complete inactivation of pump molecules.

The data in Fig. 5 show the decrease of the activity of ATP-hydrolysis of the pump molecules. The simulations on the other hand were obtained from a kinetic analysis of the conformational transition $E_1 \rightarrow E_2$, which is part of the ion-translocation pathway. The close correspondence between data and simulation may be considered as evidence for the conservation of a tight coupling between ATP-hydrolysis and the ion pumping activity of the electroenzyme (Glynn, 1985; Läuger, 1991). Interaction with free radicals seems to transform the protein in such a way that both functional properties investigated, ATP hydrolysis and Na^+ translocation, which are part of the same branch of the Post-Albers scheme, are affected simultaneously. This indicates that the loss of the enzymatic activity is not necessarily due to an inhibition of the phosphorylation reaction, $Na_3E_1ATP \rightarrow (Na_3)E_1\text{-}P$, as could be expected from previous studies, which were based mainly on the analysis of ATP-hydrolysis.

In a different approach Elmoselhi et al. (1994) studied the effect of radicals on the sodium pump in pig coronary artery. They observed uncoupling between ouabain-sensitive Rb^+ uptake and ouabain-sensitive p-nitrophenylphosphatase activity (pNPPase) induced by peroxide and superoxide. This seems to be in contrast to our findings in which tight coupling between ATPase

activity and Na^+ transport was found to be preserved throughout inactivation. In the experiments of Elmoselhi et al., however, instead of ATP hydrolysis, which includes the complete reaction cycle, pNPPase activity was studied. The latter comprises only the partial reaction of $E_2(K_2) \leftrightarrow P\text{-}E_2K_2$. This kind of ''low energy'' phosphorylation occurs without subsequent conformational change, E_1/E_2, which is essential for ion transport. On the basis of the experiments presented by Elmoselhi et al., it cannot be excluded that pNPPase activity is possible by inactivated forms of the enzyme (i.e., unable to participate in the pump cycle).

The present study was performed with a well-characterized membrane preparation in which the only protein present is the Na,K-ATPase. Our results cannot exclude inactivation via different pathways. The pathways may start from various states of the pump cycle and will possibly arrest the protein in different (lethal) molecular states. In view of the tight coupling, the functions of the enzyme will be affected, however, in the same way, irrespective of the inactivation pathway. The detailed nature of the lethal state(s) is unknown at present and will have to be analyzed by methods different from those applied throughout the present investigation.

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